APPENDIX F
Silliker, 1969

AN EVALUATION OF THE SALMONELLA PROBLEM

A Report of the
U.S. DEPARTMENT OF AGRICULTURE
and the
FOOD AND DRUG ADMINISTRATION,
U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

prepared by the
COMMITTEE ON SALMONELLA
DIVISION OF BIOLOGY AND AGRICULTURE
NATIONAL RESEARCH COUNCIL

NATIONAL ACADEMY OF SCIENCES
WASHINGTON, D.C.
1969

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Page 1175

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Prefac

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Appendix D

"Wet Compositing" as an Approach to Control Procedures for the Detection of Salmonellae

Silliker (unpublished) has conducted a feasibility study of "wet compositing" as an approach to control procedures for the detection of salmonella. His results are summarized here.

The samples involved in the study were dried foods and feeds that were being analyzed for salmonellae as a part of quality-control programs. All samples were pre-enriched for 18–24 hours at 35°C before subculture. The nature of the pre-enrichment medium varied depending on the nature of the sample. For example, dried milk samples were enriched in water containing brilliant green, dried eggs in lactose broth, rendered animal by-products in lactose broth containing tergitol, and candy in reconstituted nonfat dried milk containing brilliant green.

The pre-enrichment cultures were subcultured in tetrathionate broth containing brilliant green. Approximately half of the samples were subcultured into cystine selenite broth, in addition to tetrathionate broth. The selective broths were incubated at 35°C for 24 hours, at which time the cultures were streaked onto brilliant green, SS, and bismuth sulfite agars.

After incubation, the selective agars were examined for suspect salmonella colonies. These were studied and identified using conventional biochemical and serological procedures.

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To evaluate the "wet compositing" approach, incubated pre-enrichment cultures were randomly segregated in groups of 10. Within each group of 10, then, a variety of different samples were represented. Each of the 10 samples was subsequently analyzed according to routine procedures as described above. In the "wet compositing" technique, 1 ml from each of the 10 enrichment cultures was introduced into 10 ml of double-strength selective medium. This "wet composite," representing 10 randomly selected samples, was then analyzed as a single unit, using the procedures described above. Those samples that would ordinarily be subcultured in tetrathionate broth only were "wet composited" only in tetrathionate broth. On the other hand, from those samples that were analyzed using both tetrathionate and selenite broths, "wet composites" were prepared in both selenite and tetrathionate broths. The "wet composites" were then analyzed using the procedures described above.

Table D1 summarizes results obtained in the analysis of 106 "wet composites" representing a total of 1,060 individual samples. Thirty-nine composites were positive for salmonella, 66 negative. In seven instances (designated false negative), one or more of the individual samples comprising the "wet composite" yielded salmonellae. Interestingly, in one case (designated false positive) the "wet composite" was positive for salmonella, even though the analysis of each of the 10 samples comprising the composite was negative.

TABLE D1 Sensitivity of "Wet Compositing" for *Salmonella* Detection

No composites	105
No. composites positive	39
No. composites negative	66
No. false negative composites*	7
No. false positive composites*	1

* False negative indicates failure to isolate salmonellae from the "wet composite," even though one or more of the 10 components was found positive.

* False positive indicates "wet composite" was found positive for salmonella, even though each of the 10 components was found negative.

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APPENDIX G
Sales Brochure, Idaho Technology



PERFORMANCE TESTED METHODS℠

AOAC RESEARCH INSTITUTE NEWS

Idaho Technology Salmonella LT Food Security System Granted Performance-Tested Method℠ Status

AOAC RESEARCH INSTITUTE
Leading the World in Performance-Based Validation

Idaho Technology, Inc.

Salmonella LT Food Security System
for the R.A.P.I.D.® LT
Cat. No.: ASAY-ASY-0116
PTM Status: March 2008
Certificate No.: 030803

Summary

The *Salmonella* LT Food Security System (FSS; Figure 1) is a rapid detection method for *Salmonella* that combines sensitive, rapid, real-time polymerase chain reaction (PCR) with simple sample preparation. The PCR process takes only 35 min and the entire procedure only 17 h. The method involves a 16 h sample enrichment using traditional media, sample processing by cell lysis to release DNA, rehydration of freeze-dried assay reagents, DNA amplification (PCR) in the Idaho Technology (Salt Lake City, Utah, USA) R.A.P.I.D. LT instrument, and automatic result interpretation by the R.A.P.I.D. LT FSS software.

The AOAC Research Institute certified the Salmonella LT FSS as a *Performance-Tested Method℠* in March 2008 (Certificate No. 030803). Idaho Technology, Inc. (ITI) developed the *Salmonella* LT FSS to enable fast, simple, inexpensive testing for *Salmonella*-contaminated foods. ITI utilized experience with patented rapid thermocycling technology, real-time PCR detection systems, and pathogen detection for the military to design a custom system for the food industry. The FSS is approved for both single and pooled samples. To qualify for AOAC approval, the *Salmonella* FSS was evaluated for sensitivity, specificity, ruggedness, and stability of reagents.

This report describes the evaluation of the *Salmonella* LT FSS compared to reference methods in three foods: cooked ham, raw chicken, and chocolate.

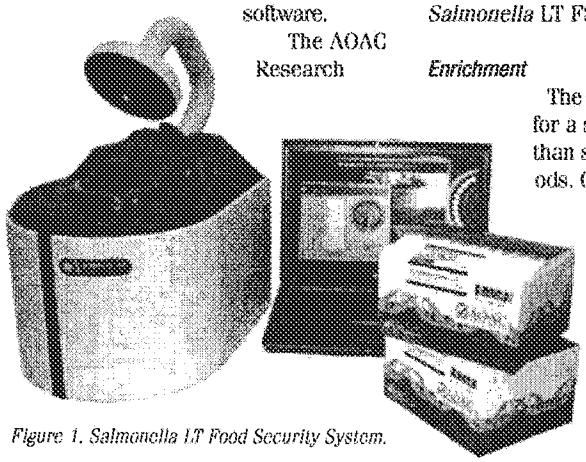


Figure 1. Salmonella LT Food Security System.

Method

See Figure 2 for an outline of the *Salmonella* LT FSS method.

Enrichment

The sensitivity of PCR allows for a shorter enrichment time than standard detection methods. One colony forming unit (CFU) of Salmonella in 25 g of food can be detected after only 16 h of enrichment using traditional media (buffered peptone water or nonfat dry milk with brilliant green) at 37 C. After enrichment, up to 5 samples can be pooled for analysis. *See* the pooling section below.

Sample Processing

DNA is extracted from the *Salmonella* bacteria by mechanical cell lysis. Bead tubes for mechanical lysis are contained in the *Salmonella* LT test kit. The bacteria are lysed either directly after enrichment or after cell concentration with the MATRIX MicroScience PATHATRIX® System. The direct method includes a dilution step to remove PCR inhibitors before amplification (referred to as PCR-only). The PATHATRIX is useful for isolating *Salmonella* from dirty food backgrounds that may interfere with detection.

Freeze-Dried Reagents

PCR is simple with the RAPID LT FSS because all PCR components are included in easy to use freeze-dried reagent vials contained in the *Salmonella* LT test kit. Freeze-dried reagents are stored at room temperature. The processed sample is used to rehydrate freeze-dried assay reagents with a buffer, and the mix is added directly to a PCR vessel.

PCR

Real-time DNA amplification (PCR) occurs in the Idaho Technology R.A.P.I.D. LT instrument. *Salmonella* LT has two levels of specificity. PCR primers specifically amplify *Salmonella* DNA that is then detected by specific fluorescent probes that only detect *Salmonella*. Thus,

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Table 1. Method comparison results: individual samples [a]

Matrix	Inoculating organism	Level	MPN, CFU/25 g[a]	Samples	*Salmonella* LT positive	Confirmed positive	Reference positive (paired samples)
Raw chicken	*Salmonella* typhimurium	Low	<0.8	20	13	13	13
		Control	0	5	0	0	0
Cooked ham	*Salmonella* enteriditis	Low	<0.8	20	7	7	7
		Control	0	5	0	0	0
Cooked ham[b]	*Salmonella* typhimurium	Low	2.3	20	13	13	13
		Control	0	5	0	0	0
Chocolate	*Salmonella* senftenberg	High	10.8	20	17	17	17
		Low	0.9	20	9	9	9
		Low	<0.8	20	1	1	1
		Control	0	15	0	0	0

[a] MPN = Most Probable Number. [b] Independent laboratory results.

fluorescent detection only occurs if a *Salmonella* target is amplified. The freeze-dried reagents also contain an internal amplification control to ensure that amplification is successful. The R.A.P.I.D. LT instrument is faster than traditional PCR instruments (35 min versus 3 h) because it uses air thermocycling to heat and cool the sample.

Final Result

Results are reported by the RAPID LT FSS software as "positive" or "negative." The final result is determined within about 17 h from the beginning of enrichment.

Pooling

Post-enrichment sample pooling is an excellent way to increase throughput and decrease costs. A 5:1 pooling approach enables the cost per test to approach that of traditional methods, reducing PCR assay costs by up to 80%, when most samples are negative. Samples are individually enriched and then pooled into a composite sample, while preserving the individual samples in case of a positive. The composite sample is tested as a single sample. If the composite sample is positive, the individually enriched samples may be retested to determine which of the samples is positive. Retesting takes less than 1 h because of the short 35 min PCR run. In the *Salmonella* LT FSS evaluation, pooled samples yielded the same results as individual samples with no false negatives.

Validation Results

With both individual and pooled samples, the *Salmonella* LT FSS demonstrated 100% sensitivity and 100% specificity for the three different food types (cooked ham, raw chicken, and chocolate). Each sample was grown in standard media and tested with both the *Salmonella* LT FSS and the reference method.

The FSS also proved to be robust and reproducible. The ruggedness study demonstrated that the system produced consistent results with variability in reagent preparation time and sample volumes. Lot-to-lot and shelf-life studies demonstrated that the *Salmonella* LT produced consistent results with several lots of reagents produced at different times.

Method Comparison, Individual Samples

The *Salmonella* LT FSS is as sensitive as the USDA and FDA reference methods at low inoculum levels in raw chicken, cooked ham, and chocolate. Results are summarized in Table 1. The *Salmonella* LT FSS was compared to reference methods from the FDA *Bacteriological Analytical Manual* (BAM; 1) and USDA *Microbiology Laboratory Guidebook* (MLG; 2). A total of 25 samples per food type were prepared for primary enrichment in the

(Continued on page ??.)

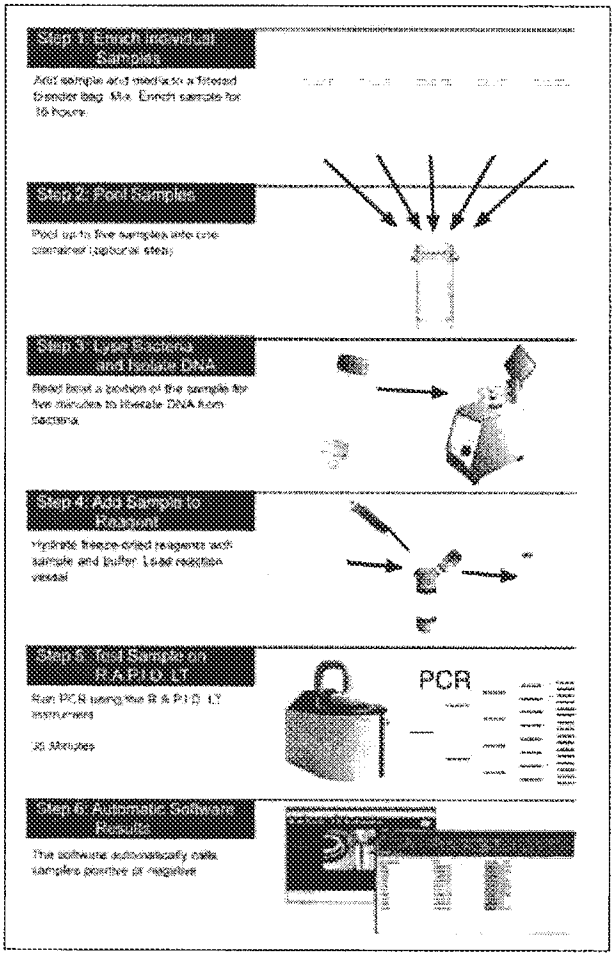


Figure 2. Salmonella LT FSS method workflow.

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Idaho Technology Salmonella LT Food Security System Granted Performance-Tested Method℠ Status

Continued from page ??.

recommended broth according to the reference method. Twenty samples were inoculated with a low level of target organism (approximately 1 CFU per 25 g food sample), while 5 samples were not inoculated. Cooked ham and raw chicken samples were inoculated with liquid culture and allowed to equilibrate at 4°C for 48-72 h. Chocolate samples were melted, inoculated with liquid culture, allowed to harden at room temperature, and equilibrated at room temperature for 2 weeks. Each food matrix was inoculated with a different *Salmonella enterica* serovar (see Table 1). These serovars have been responsible for foodborne illness or associated with recent outbreaks. Each sample was tested with both the reference method and the *Salmonella* LT FSS PCR-only protocol (no cell concentration with the PATHATRIX) since they both use the same primary enrichment protocol. This allowed for a paired sample analysis. All presumptive positive samples were confirmed by standard plating methods.

Method Comparison, Pooled Samples

The system is sensitive even with pooled samples (5 samples combined). Results are summarized in Table 2. Ten samples were inoculated with a low level of target organism (approximately 1-10 CFU per 25 g food sample), while 40 samples were not inoculated. A 50 mL aliquot from each individual positive sample was combined with 50 mL aliquots from 4 negative samples to create a 250 mL pooled sample. A total of 10 pools comprised of the 50 individual samples were constructed. Following a 16 h incubation, the pools were analyzed with one (PCR-only) or both (PCR-only and PATHATRIX with PCR) *Salmonella* FSS protocols.

Inclusivity and Exclusivity

The *Salmonella* LT FSS was able to detect 121 out of 121 strains tested in the inclusivity panel. It did *not* detect 30 out of the 30 bacteria tested in the exclusivity panel consisting of closely related non-*Salmonella* species. Each strain was grown in buffered peptone water and nonfat dry milk with brilliant green.

Conclusions

The *Salmonella* LT FSS is as sensitive as the USDA and FDA methods at low inoculum levels in food, with individual and pooled samples. Out of a total of 230 samples evaluated in the study, 126 samples were positive with both the *Salmonella* LT and the reference method. Sensitivity and specificity were 100% for the *Salmonella* LT FSS with individual or pooled samples, and with PATHATRIX for pooled samples. False-positive and false-negative rates were 0%. *Salmonella* LT detected all 121 Salmonella serotypes evaluated. It detected none of the 30 non-Salmonella species tested.

The *Salmonella* LT FSS is a significant improvement over standard methods. It provides results significantly faster, in 17 h compared to 72 h for the USDA and FDA reference methods. The R.A.P.I.D LT performs real-time PCR and provides automated results in about 30 min (after enrichment and sample processing). Results are easier to interpret than standard methods because the software gives a "positive" or "negative" answer. The *Salmonella* LT FSS is easy to use, with very few steps and minimal sample handling. Pooled samples yield the same results as individual samples. Pooling enables five times the throughput at the same cost.

Based on results of the validation study, the *Salmonella* LT FSS for the detection of *Salmonella* in cooked ham, raw chicken, and chocolate was adopted as AOAC *Performance-Tested Method℠* No. 030803.

—Stephanie Thatcher, Haleigh Millward, and Mike Powers
Contributing Writers
stephaniet@idahotech.com, haleigh@idahotech.com

Mention of trade names or commercial products is for identification only and does not constitute preference over similar ones not mentioned. If you are interested in submitting an article regarding a test kit that has been granted Performance-Tested MethodSM status, contact Deborah McKenzie at dmckenzie@aoac.org.

References

(1) U.S. Food and Drug Administration, Bacteriological Analytical Manual, http://www.cfsan.fda.gov/~ebam/bam-5.html

(2) U.S. Department of Agriculture/Food Safety and Inspection Service, Microbiology Laboratory Guidebook, http://www.fsis.usda.gov/PDF/MLG_4_03.pdf

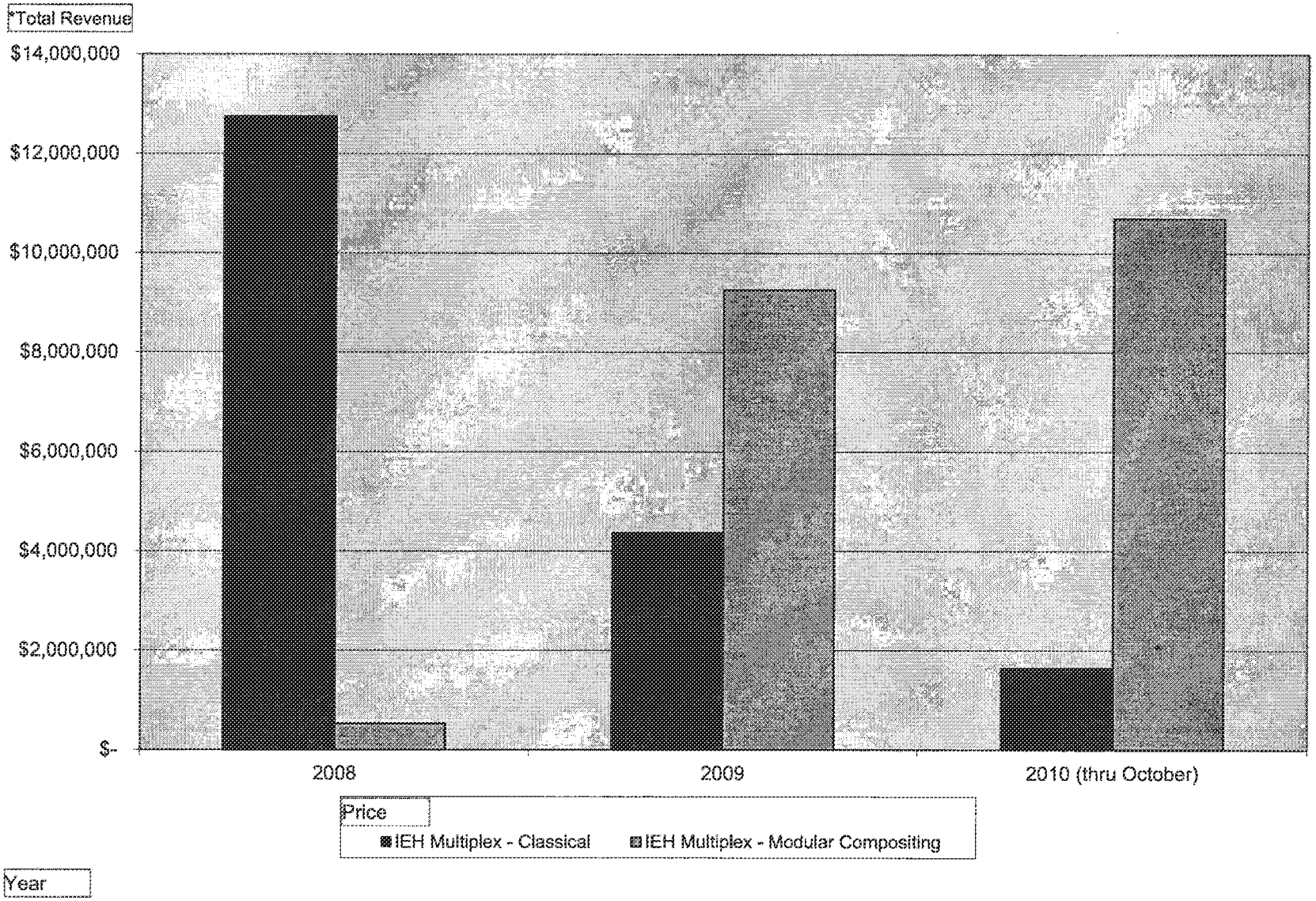
Table 2. Method comparison results: Pooled samples

Matrix	Inoculating organism	Protocol	MPN, CFU/25 g[a]	Samples	*Salmonella* LT positive	Confirmed positive	Reference positive (paired samples)
Raw chicken	*Salmonella* typhimurium		18.8	10	10	10	10
		PATH[b]	18.8	10	10	10	10
Cooked ham	*Salmonella* enteriditis		5.8	10	10	10	10
		PATH	5.8	10	10	10	10
Cooked ham[c]	*Salmonella* typhimurium		0.9	10	4	4	4
		PATH	0.9	10	4	4	4
Chocolate	*Salmonella* senftenberg		10.8	10	9	9	9
		PATH	10.8	10	9	9	9

[a] MPN = Most Probable Number. [b] PATH = Cell concentration with PATHATRIX. [c] Independent laboratory results.

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APPENDIX H
IEH Sales Figures


Total Revenue
$14,000,000
$12,000,000
$10,000,000
$8,000,000
$6,000,000
$4,000,000
$2,000,000
$-
2008
2009
2010 (thru October)
Price
IEH Multiplex - Classical
IEH Multiplex - Modular Compositing
Year

Via Electronic Submission
Attorney Docket No.:66090-008US2

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mansour Samadpour
Serial No.:	95/001,294
Filed:	05 April 2010
For:	MODULAR COMPOSITING-MULTIPLE LOT SCREENING PROTOCOLS FOR DETECTION OF PATHOGENS, MICROBIAL CONTAMINANTS AND/OR CONSTITUENTS
Examiner:	Brenda G. Brumback
Art Unit:	3991
Attorney Docket No.:	66090-008US2
Date:	31 January 2011

Attn: Mail Stop "Inter Partes Reexam"
Central Reexamination Unit
Commissioner for Patents
P. 0. Box 1450
Alexandria VA 22313-1450

DECLARATION OF MANSOUR SAMADPOUR TO REBUT THE DECLARATION OF DR. BAILEY UNDER 37 C.F.R. § 1.132
(IN SUPPORT OF RESPONSE UNDER 37 C.F.R. § 1.111)

Madam:

I, Dr. Mansour Samadpour, being duly sworn, say:

1. "Dr. J. Stan Bailey, a citizen of the United States of America, hereby declare and state: I have over thirty-five years of work and research experience in the area of testing for microorganisms in foods.

2. "I have a Bachelor of' Science in Environmental Health Science, which was conferred upon me by the University of Georgia in Athens, Georgia, in 1974. I have a Master of Science in Food Science, which was conferred upon me by the University of Georgia in Athens,

Georgia, in 1978. I have a Ph.D. in Poultry Science, which was conferred upon me by the University of Georgia in Athens, Georgia, in 1989.

3. "I have been employed by bioMerieux, Inc., the real party in interest under 37 C.F.R. §1.915(b)(8) in the *Inter Partes* Reexamination of the above-identified patent (hereinafter "the real party in interest"), since January 2008 as the Director of Scientific Affairs. Prior to my employment with bioMerieux, Inc., I spent thirty-four years as a research microbiologist employed by the United States Department of Agriculture ("USDA") within the Agricultural Research Service ("ARS") during which time I received seventeen USDA Certificates of Merit and was named "Outstanding Senior Research Scientist of the Year" in 2002. I have taught as an Adjunct Professor in the Poultry Science Department at the University of Georgia and at the Department of Food Science at Kansas State University. I have been a Faculty Member at the "Rapid Methods and Automation in Microbiology Workshop" taught at Kansas State University where over one thousand scientists from fifty countries have received training in the area of microbiological testing.

4. "I have authored or co-authored over five-hundred scientific publications in the area of food microbiology. I am on the editorial boards of *Poultry Science, Journal of Rapid Methods and Automation in Microbiology,* and the *Journal of Applied Poultry Research.*

5. "I have received numerous honors in the area of food microbiology that include the following awards and appointments:

"a) Chairman of the Food Microbiology Division of the American Society for Microbiology (1992);

"b) Secretary of the Microbiological Methods Committee of the Association of Official Analytical Chemists ("AOAC") (1990-95);

"c) Food and Agriculture Organization of the United Nations Expert Consultant on Animal Feeding and Food Safety;

"d) Fellow of the American Academy of Microbiology (1994);

"e) Technical Advisor to the National Advisory Committee on Microbiological Criteria in Foods for poultry production (1995 and 2006);

"f) Scientific Advisor to the International Life Sciences Institute, Food Microbiology Committee (1997 -2008);

"g) Federal Laboratory Consortium Award for Technology Transfer;

"h) Laboratorian of the Year by International Association for Food Protection ("IAFP"), (2003);

"i) Fellow of the International Association for Food Protection (2005); and

"j) President of the International Association for Food Protection (2009).

6. "I am a member of the following Professional Societies: Southern Poultry Science Society, Worlds Poultry Science, A1llerican Society for Microbiology, American Academy of Microbiology, Poultry Science Society, International Association for Food Protection, Georgia Association for Food Protection, AOAC.

7. "I have a professional relationship with the real party in interest. In the course of that professional relationship, I receive compensation directly from the real party in interest for my work relating to food microbiology. I am not being separately compensated for my work in connection with this Declaration and my compensation is not dependent on the outcome of these proceedings.

8. "I have read U.S. Patent 7,534,584 which issued to Mansour Samadpour on May 19,2009 (referred to herein as the "584 patent"). Based on my experience and knowledge of the relevant literature, it is my opinion that the method of sampling, testing and validating test lots (or singleunit production lots) for microbial contamination claimed in the '584 patent is an aggregation of sample-compositing, enrichment, wet-compositing, testing, validation and optional re-testing steps that were known in the art prior to August 2004. It is my opinion that each of the steps comprising the claimed method performs its art-recognized function and that the combination of steps, as defined by the claims, yields predictable results. It is also my opinion that it would have been obvious to a person or ordinary in August 2004 to have combined the steps, as set forth in the claims, to improve efficiencies and reduce costs. The scientific field relevant to '584 patent is the field of microbiology as it relates to the microbiological analysis of food products and industrial food safety. A person of ordinary skill in that field in August 2004 would have been involving in developing methodology for sampling, testing and validating food products for microbial contamination. Such a person would have possessed either an advanced scientific degree in microbiology, environmental health and safety, or food science or would have possessed a Bachelor's degree and bad several years of experience in developing methodology for the sampling and analysis of food products. The person of ordinary skill would have been familiar with the scientific literature relevant to the

microbiological analysis of food also would have been required to possess a working knowledge of rules, regulations, guidelines and directives relating to sample preparation and testing methodology to evaluate food for the presence of absence of pathogenic microorganisms."

RESPONSE BY INVENTOR:

Dr. Bailey defines a person with ordinary skill in the field as a Bachelor's degree in microbiology, environmental health, or food science. Then he decides that the person would have had several years of experience in developing methodologies for the sampling and analysis of food products. Then he throws in comprehensive familiarity with the literature, and a working knowledge of rules, regulations, guidelines, and directives relating to sample preparations and testing methodologies. This is a fictional construct. In this field, methods are developed by molecular biologists, immunochemists, biochemists, and classical microbiologists. People involved in methods development are not the same as the people who develop sampling plans. Neither of the two groups follows FSIS and FDA directives and guidelines. Those are done by the practitioner's in the field. Dr. Bailey's construct is, therefore, a person with extraordinary skills in several arts.

It is not valid to simply assert that microbiologists or other practitioners have knowledge of statistics suitable for designing sampling plans. The preface to Jarvis (1987) states as follows:

"For many years I have been aware of a lack of understanding of statistical concepts by biologists in general and microbiologists in particular."

In other words a person of ordinary skills in the field is either a practitioner (user of methods), a method developer (to develop PCR, immunochemical, and clinical methods), a statistician (to develop sampling plans), a regulatory compliance person (to decide how to address applicable regulations and directives) or a quality systems professional (who conducts validation studies). Dr. Bailey's "person of ordinary skill" is necessarily a person with skills in many arts.

"9a. I have read at least sections 8.4.2 and 8.4.2.1 of B. Jarvis, *Statistical Aspects of the Microbiological Analysis of Foods,* 21 PROGRESS IN INDUSTRIAL MICROBIOLOGY 138·142 (1989) (hereinafter "Jarvis") and provide the following explanation of how Jarvis would have been understood in August of 2004 by one of ordinary skill in the art. By August 2004, a person of ordinary skill involved with the development of methods for the sampling, testing, and validation of food products for microbial contamination would have been aware of the importance of ensuring the statistical validity of such methods, and therefore would have

consulted a reference like the Jarvis publication, which addresses the statistical aspects of such methods."

RESPONSE BY INVENTOR:

Dr. Bailey continues on the basis of the false assumption that there is a person with ordinary skills in the art that has mastery of sampling method design, designing test systems, has expertise in statistics, and designs and conducts validation studies.

The Jarvis reference is an obscure reference that deals with statistical analysis of sampling methods. As the title of the chapter implies it is not an enabling reference. It does not teach how to conduct the methods, does not provide materials and methods, and does not report results. Furthermore, when it gets to the section that discusses compositing it does not cite the enabling references. It also misrepresents the teachings of Silliker, Gabis and Price regarding method 3 (section 8.4.2) who argue against compositing before pre-enrichment.

"9b. Section 8.4.2 describes the effect of subculture volume and incubation times in the conventional testing of samples for the presence or absence of microbes. See p. 138. As was the usual practice, the testing procedure involves both "pre-enrichment" and "selective enrichment stages." *Id.*"

RESPONSE BY INVENTOR:

Dr. Bailey relies on a series of misrepresentations of the references cited to make his case. To begin with he fails to explain that the Jarvis section cited here is about testing a single lot of food for *Salmonella*. The method in question is the FDA BAM method for *Salmonella*. The method requires the use of pre-enrichment for the samples, followed by enrichment in selective media, followed by detection using plating.

"9c. Regarding how the samples themselves are collected and prepared, in the introduction to the chapter, Jarvis simply discloses that the sample could be an "initial food homogenate (or culture)." See p. 117."

RESPONSE BY INVENTOR:

In my opinion, either Dr. Bailey either doesn't understand, or is misrepresenting, the referenced. On page 117 (APPENDIX A), Jarvis states (first paragraph): "In its simplest form, the dilution consists of serial dilution of the initial food homogenate (or culture)

followed by inoculation of an appropriate volume of each dilution into one or more tubes of culture (agar or broth)…". Within Jarvis, it is the subject of Sections 8.4.2 and 8.4.2.1 which are the prior art relevant to patent '584. Anyone with ordinary skills in the art would realize that a sample description for a given method, would not necessarily apply to a different method. In this regard, by citing page 117 Dr. Bailey is using a sample description for a dilution count method for indicators, and incorrectly implying that it is the sample description for the *Salmonella* method of Sections 8.4.2 and 8.4.2.1, which are the core of the argument (the only argument) in Jarvis.

9d. "During the pre-enrichment stage, an initial sample is dispersed in a pre-enrichment medium and then it is incubated, resulting in a "pre-enrichment culture." See Example 8.6, p. 139. The incubation of the sample in the pre-enrichment medium causes at least the target microorganism to grow. *Id.* Due to the incubation, the pre-enrichment culture has been enriched in the target microorganism, which in Example 8.6 discussed in section 8.4.2 is *Salmonellae.* In August of 2004, it was well known in the art that this type of enrichment would both resuscitate the target microorganism and cause it to grow to reach detectable levels and become substantially uniform within the liquid pre-enrichment culture."

RESPONSE BY INVENTOR:

Neither the definition, nor the function of pre-enrichment has changed from 1960s (Silliker), 1970s (Silliker, Gabis, Price), 1980s (Jarvis) to 2004 (patent '584), to 2007 (Jarvis, 2007). Pre-enrichment is a term of art that refers to an initial incubation of a sample in a media that will not allow rigorous growth but is instead designed to resuscitate and result in limited growth of the target organism to a point that when an aliquot of the pre-enrichment is taken to inoculate the enrichment media, there is 99% confidence that the aliquot taken will at least contain at least one viable cell of the target organism in the aliquot taken. In this regard I do not believe that Dr. Bailey is properly interpreting the text in Jarvis.

I have attached (APPENDIX B) a technical datasheet for lactose broth issued by PML Microbiologicals, a purveyor of microbiological media. Lactose Broth is the pre-enrichment media referenced by Jarvis, Price, Silliker and Gabis. The technical sheet for the lactose broth clearly shows that Dr. Bailey's interpretation is incorrect when he states: *"The incubation of the sample in the pre-enrichment medium causes at least the target microorganism to grow."* On the first page of the datasheet, second paragraph of the section

entitled Principle it states:

> ***This pre-enrichment medium is used to provide a higher ratio of Salmonella to non-Salmonella bacteria after incubation. Lactose is fermented by most non-Salmonella bacteria while Salmonella does not. A bactiostatic effect on competing microorganisms is created by the decrease of pH in the medium produced by the lactose fermenting bacteria. The Salmonella will have little growth during the initial 24 hours of incubation, while the non-Salmonella will be utilizing the lactose. As the products of fermentation build, the non-Salmonella are inhibited and the Salmonella begins its growth phase (24-48 hrs.) (Emphasis added)***

In my opinion Dr. Bailey is misleading the Examiner with his interpretation of pre-enrichment which does not conform to the commonly understood term of art.

9d. (continued) "Microorganisms in food products often are debilitated because of the prior handling and processing of the products, but are able to resuscitate and proliferate under favorable conditions. Pre-enrichment utilizes nutrients and conditions to effect such resuscitation and growth so that the pre-enrichment culture can be even further diluted at the collective enrichment stage. Specifically, according to Jarvis, only 1 ml of each pre-enrichment broth will be added to 100 m1 of the enrichment broth to form the composite."

RESPONSE BY INVENTOR:

Again, per the lactose broth technical sheet (APPENDIX B) the function of the lactose broth is to encourage the growth of the competitive flora in order to drop the pH, damage the acid sensitive bacteria, and provide a competitive advantage to *Salmonella* cells when they are transferred into the selective enrichment broth.

9d. (continued 2) "The table in Example 8.5, on page 141 of Jarvis, shows that it is necessary to incubate the pre-enrichment medium at least 12 hours to ensure a 99% probability that any 1 m1 of the pre-enrichment culture that is transferred to the composite includes more than one viable *Salmonella* organism. This means that each 1 ml of the pre-enrichment culture includes viable *Salmonella* organisms, i.e." the viable *Salmonella* organisms are detectable and uniformly distributed within the pre-enrichment culture."

RESPONSE BY INVENTOR:

Dr. Bailey references a table for Example 8.6 of Jarvis where Jarvis is attempting to illustrate his main point; i.e. that the function of pre-enrichment is to assure that the

portion transferred to the enrichment broth contains at least one viable cell. In this mathematical model, he assumes a concentration of 0.1 CFU/gram or 10 organisms per 100 grams.

Here, Dr. Jarvis also assumes a generation time of 30 minutes in Lactose Broth, an assumption which is inconsistent with the technical sheet of APPENDIX B, which states that Salmonella does not begin its growth phase until after 24 h. Furthermore, Jarvis does not consider the impact of the competitive organisms. Jarvis is engaged in performing theoretical mathematical calculations and reports his results in a table having a mathematical frame work. However, right under the table Jarvis explains the reality of pre-enrichment:

> ***"Once sufficient growth has occurred in pre-enrichment for at least 3 viable salmonellae/ml medium, the probability of transferring at least one viable organism in an inoculum of 1 ml or more will be > 0.95, assuming Poisson distribution, with m=3: 1'(x=0) = 0.05, therefore 1'(:c)-I)= 0.95. However, at lower numbers, the chances of transferring at least 1 viable organism will be affected not only by the initial contamination level (x) but also by the volume of medium inoculated into the enrichment culture. In methods which permit only resuscitation, not growth, during pre-enrichment, it is essential to use the entire pre-enrichment culture to inoculate double-strength enrichment medium (Hall, 1975), although selectivity may then be affected by the food constituents. Obviously the level of salmonellae in the pre-enrichment culture after any given incubation period will depend upon the cultural conditions used, the initial contamination level, the extent of sub-lethal cell damage and the relative numbers and types of competitive organisms in the sample."***

Dr. Bailey elects to use selective portions of the reference in an attempt to present his case and misdirect the Examiner. Dr. Bailey incorrectly states:

> ***"This means that each 1 ml of the pre-enrichment culture includes viable Salmonella organisms, i.e." the viable Salmonella organisms are detectable and uniformly distributed within the pre-enrichment culture."***

Please note that this is the same 1 ml of the pre-enrichment culture that aims to deliver at least one viable cell to the enrichment culture. Please also note that the limit of detection of food enrichments by PCR method is 10,000 CFU/ml. A presentation by Dr. Baily himself, an excerpt of which is included as APPENDIX C, shows an enrichment level of 10E4 (10,000 CFU/mL). It is common knowledge that the limit of detection for lateral flow

devices is in the range of 100,000-1000,000 CFU/ml.

9d. (continued) "Microorganisms in food products often are debilitated because of the prior handling and processing of the products, but are able to resuscitate and proliferate under favorable conditions. Pre-enrichment utilizes nutrients and conditions to effect such resuscitation and growth so that the pre-enrichment culture can be even further diluted at the collective enrichment stage. Specifically, according to Jarvis, only 1 ml of each pre-enrichment broth will be added to 100 m1 of the enrichment broth to form the composite."

RESPONSE BY INVENTOR:

Again, per the lactose broth technical sheet (APPENDIX B) the function of the lactose broth is to encourage the growth of the competitive flora in order to drop the pH, damage the acid sensitive bacteria, and provide a competitive advantage to *Salmonella* cells when they are transferred into the selective enrichment broth.

9d. (continued 2) "The table in Example 8.5, on page 141 of Jarvis, shows that it is necessary to incubate the pre-enrichment medium at least 12 hours to ensure a 99% probability that any 1 m1 of the pre-enrichment culture that is transferred to the composite includes more than one viable *Salmonella* organism. This means that each 1 ml of the pre-enrichment culture includes viable *Salmonella* organisms, i.e." the viable *Salmonella* organisms are detectable and uniformly distributed within the pre-enrichment culture."

RESPONSE BY INVENTOR:

Dr. Bailey references a table for Example 8.6 of Jarvis where Jarvis is attempting to illustrate his main point; i.e. that the function of pre-enrichment is to assure that the portion transferred to the enrichment broth contains at least one viable cell. In this mathematical model, he assumes a concentration of 0.1 CFU/gram or 10 organisms per 100 grams.

Here, Dr. Jarvis also assumes a generation time of 30 minutes in Lactose Broth, an assumption which is inconsistent with the technical sheet of APPENDIX B, which states that Salmonella does not begin its growth phase until after 24 h. Furthermore, Jarvis does not consider the impact of the competitive organisms. Jarvis is engaged in performing theoretical mathematical calculations and reports his results in a table having a mathematical frame work. However, right under the table Jarvis explains the reality of

pre-enrichment:

> ***"Once sufficient growth has occurred in pre-enrichment for at least 3 viable salmonellae/ml medium, the probability of transferring at least one viable organism in an inoculum of 1 ml or more will be > 0.95, assuming Poisson distribution, with m=3: 1'(x=0) = 0.05, therefore 1'(:c)-I)= 0.95. However, at lower numbers, the chances of transferring at least 1 viable organism will be affected not only by the initial contamination level (x) but also by the volume of medium inoculated into the enrichment culture. In methods which permit only resuscitation, not growth, during pre-enrichment, it is essential to use the entire pre-enrichment culture to inoculate double-strength enrichment medium (Hall, 1975), although selectivity may then be affected by the food constituents. Obviously the level of salmonellae in the pre-enrichment culture after any given incubation period will depend upon the cultural conditions used, the initial contamination level, the extent of sub-lethal cell damage and the relative numbers and types of competitive organisms in the sample."***

Dr. Bailey elects to use selective portions of the reference in an attempt to present his case and misdirect the Examiner. Dr. Bailey incorrectly states:

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9d. (3) Thus, at the pre-enrichment stage in Jarvis, the sample is dispersed in a pre-enrichment medium and then incubated to form a pre-enrichment culture, and the pre-enrichment culture has been incubated under conditions suitable to allow the target microorganism to reach detectible levels and is substantially uniform throughout the pre-enrichment culture. This procedure described by Jarvis is typical of known methods for the pre-enrichment and selective enrichment of salmonella.

RESPONSE BY INVENTOR:

Dr. Bailey again is misrepresents Jarvis. We repeat here the section of Jarvis cited above as explaining the reality of pre-enrichment under the table on Page 141:

> ***"Once sufficient growth has occurred in pre-enrichment for at least 3 viable salmonellae/ml medium, the probability of transferring at least one viable organism in an inoculum of 1 ml or more will be > 0.95, assuming Poisson distribution, with m=3: 1'(x=O) = 0.05, therefore 1'(:c)-I)= 0.95. However, at lower numbers, the chances of transferring at least 1 viable organism will be affected not only by the initial contamination level (x) but also by the volume of medium inoculated into the enrichment culture. In methods which permit only resuscitation, not growth, during pre-enrichment, it is essential to use the entire pre-enrichment culture to inoculate double-strength enrichment medium (Hall, 1975), although selectivity may then be affected by the food constituents. Obviously the level of salmonellae in the pre-enrichment culture after any given incubation period will depend upon the cultural conditions used, the initial contamination level, the extent of sub-lethal cell damage and the relative numbers and types of competitive organisms in the sample."***

We urge the Examiner to consider this pre-amble paragraph, taken from the previous passage on page 139, fourth paragraph, of the Jarvis reference:

> ***"Clearly, the possibilities for isolation of specific organisms will depend not only on the initial incidence of contamination but also on the manner in which the test is done. A too short period of pre-enrichment may result in no recovery of stressed organisms, thereby reducing the statistical probability of transferring resuscitated organisms to the enrichment broth. Too long a period in a non-selective pre-enrichment may provide opportunity for overgrowth by competitive organisms, leading to suppression of the organism sought. A hypothetical example of the effects of incubation time is shown in Ex. 8.6 and 8.7 and in Fig. 8.4."***

This preamble establishes two things: *first* that the table on page 141 is a hypothetical model; and *second* that Dr. Bailey once again misrepresents the Jarvis reference. The growth of *Salmonella* in the Lactose Broth is not a sure event, and the outcome is not predictable, and certainly the *Salmonella* after the pre-enrichment is not detectable, according to Jarvis.

9e. "At the selective enrichment stage, a 1 ml portion of the pre-enrichment culture (which has already been incubated) is transferred to a selective enrichment medium (in this example two separate selective enrichment media). See p. 139. Each selective enrichment medium is intended to foster further growth of the target microorganism. Then, loopfuls of the

selective enrichment media are streaked on selective diagnostic media, incubated again, and then tested. *Id.* The incubation after streaking on selective diagnostic media causes the target microorganism to grow on the selective diagnostic media."

RESPONSE BY INVENTOR:

Dr. Bailey's submission of the Jarvis reference stopped at page 142. However, material on page 143 (attached as APPENDIX D) shows that pre-enrichment does not result in the levels of organisms claimed by Dr. Bailey. Page 143 shows that pre-enrichment results in only 410 CFU/ml of the pre-enrichment. Considering that the pre-enrichment is diluted 1:10 dilution into enrichment, the initial concentration of *Salmonella* in the enrichment culture at the beginning of the incubation is a mere 41 CFU/ml, which is far below detectable levels.

Only after 18 hours of further enrichment does the level of *Salmonella* in the enrichment culture reach 670,000 CFU/ml, and then after 24 hours 43,000,000 CFU/ml. Both of these *Salmonella* levels (after 18 and 24 hours) are detectable by any method. Clearly, pre-enrichment did not accomplish enrichment to detectable levels, contrary to Dr. Bailey's assertions.

9f. "Section 8.4.2.1 describes a modification of the procedure discussed in section 8.4.1 by pooling (i.e., compositing) a plurality of samples at various stages. See p. 141. Method (2) in section 8.4.2.1 discusses wet-compositing samples after incubation. *Id.* Specifically, according to method (2), multiple pre-enrichment series (one for each of a plurality of samples) are prepared, and then after incubation 1 ml portions of each of the resulting pre-enrichment cultures are composited into a 100 ml enrichment broth. *Id.* Thus, according to method (2), a plurality of samples that have first been incubated to form pre-enrichment cultures (i.e., enriched in the target microorganism) are composited. Jarvis discloses that compositing pre-enrichment cultures after incubation (1) reduces workload and, (2) if a positive test result is obtained, permits tests to be repeated on stored pre-enrichment cultures to determine which of the pre-enrichment cultures resulted in the positive test. See pp. 141 and 142."

RESPONSE BY INVENTOR:

Dr. Bailey fails to mention that Jarvis describes taking a plurality of samples from a single lot. Also, simply identifying which pre-enrichment culture(s) contributed to a pooled positive test result is not helpful in that taking additional samples from a lot after a positive finding is prohibited by government agencies for the purposes of validating a test lot for

release into commerce. My method, in contrast, puts a statistically sufficient number of samples for validation into each separate enrichment, thereby eliminating the need to take additional samples and providing a means for retesting of the separate enrichments contributing to the pool which conforms to regulatory agency expectations and which can thereby result in release of the lot of food corresponding to the separate enrichment into commerce.

Jarvis does not say that such retesting is practical, but rather talks about this issue as a "theory". Jarvis says: " Method (2), at least in theory, permits the tests to be repeated on stored pre-enrichment cultures". Jarvis qualifies his statement because what he has retained are pre-enrichments, and as is well known in the art pre-enrichments are unstable. If pre-enrichment is allowed to continue past the optimum time point the target organism can be masked by competing microflora. Even if the pre-enrichment is stored in refrigerator psychrotrophic organisms in the pre-enrichment will continue their growth and, in all likelihood, mask the *Salmonella* which is unable to grow at low temperatures. Jarvis states (page 139, fourth paragraph):

> ***"A too short period of pre-enrichment may result in no recovery of stressed organisms, thereby reducing the statistical probability of transferring resuscitated organisms to the enrichment broth. Too long a period in a non-selective pre-enrichment may provide opportunity for overgrowth by competitive organisms, leading to suppression of the organism sought."***

Since the time period between pre-enrichment and obtaining a test result in Jarvis is 3-5 days, consistent with obtaining results by cultural methods, the retesting option is purely theoretical. Jarvis clearly implied that the tests done on the stored pre-enrichment cultures would be done using the same type of test that was used to test the enrichment broth. Obviously, since the broth after initial pre-enrichment required a second enrichment step before the test could be performed, so the stored pre-enrichments would also require this second enrichment step.

Patent '584 overcomes the limitations of using pre-enrichments by using fully enriched individual broths that are stable and permit immediate testing and validation of the corresponding lot for release into commerce. Even more significant, when a positive *Salmonella* finding occurs in Price, he presents no further options and all of the lot which was tested must be destroyed. In the patented method, several of the lots forming the modular composite may yield negative results upon retesting, and because the individual

enrichments were formed on the basis of collecting a number of portions that conforms to a statistical-based sampling plan a negative re-test result validates those particular lots for release into commerce.

10a. "I have read W.R. Price et al., *Salmonella Testing of Pooled Pre-Enrichment Broth Cultures for Screening Multiple Food Samples,* 23(4) APPLIED MICROBIOLOGY 679-682 (1972 (hereinafter "Price") and provide the following explanation of how Price would have been understood in August of 2004 and 2005 by one of ordinary skill in the art. Price describes a method of pooling pre-enrichment broth cultures into a single enrichment broth prior to plating and testing. See abstract, FIG. 1, and pp. 679-682. According to the method, samples are added to pre-enrichment broths. See p. 679 and FIG. 1. Figure 1and the accompanying text indicate that the food products being tested by Price were dried products of a particulate or powdery nature. Thus, Figure 1 shows that Price began with single samples of each food product test lot."

RESPONSE BY INVENTOR:

Dr. Baily is clearly implying that Figure 1 represents compositing of single samples from a plurality of test lots, which is a misinterpretation which must be denied by the Examiner. Price clearly explains that three types of dried foods were used to conduct his experiments. He acquired undisclosed quantities of egg albumin, cocoa, and nonfat dry milk powder. As explained on page 679, materials and methods section, he "blended the dry test food with one of the three dry inocula". The inocula contained various types of *Salmonella*. Necessarily it follows that Price prepared individual samples of dry test food from which he took multiple portions which were subsequently pre-enriched for his investigation of pooling. From Table 1 of Price (Page 680, middle of page) it is clear that in his investigation he examined positive enrichments pooled with various numbers of negative enrichments from the same dry test food, i.e. a single lot. This can be seen in the column headings, which are variously labeled as P (P standing for positive), P+5 N (N standing for negative), P+9N and P+14N under each dry test food type. There is no data presented in Price where samples or portions from more than one dry test food type are combined. Thus, contrary to the interpretation of Dr. Bailey Figure 1 of Price (Page 680, top of page) cannot be interpreted as combining samples from a plurality of test lots.

Price's work is not based on real samples taken from a lot of food to be tested. He attempted to mimic a real lot of food by dividing a single batch of dry food into artificially-inoculated and non-inoculated parts, taking a plurality of samples and inoculating each sample into an individual pre-enrichment. Price contains no discussion to suggest that he

is doing anything other than attempting to find a more economical way of testing a single lot of food. Price has three lots of different product that he uses in his experiment. But at no point does he attempt to combine enrichments representing these different lots of product. Rather, he maintains experimental separation between the various different product types and reports the results concerning each in separate columns of his tables.

10b2. "By August 2004, a person or ordinary skill would have understood that such particulate or powdery products could be mixed or blended until homogeneous, and thus a single sample would be representative for microbiological testing."

RESPONSE BY INVENTOR:

Dr. Bailey is making an unwarranted extrapolation. A person with skills in the art and scientific training would read the materials and methods section of Jarvis and interpret it as the factual basis of the experiments. It is absolutely incorrect to assert that when Price takes a quantity of a dry food, divides it into two portions, inoculates one portion, and then takes multiple samples from each portion to conduct the experiment it is anything but taking a plurality of samples from a single test lot. The inoculation of a portion of the quantity of dry food was a means to ensure that the test lot had positive samples when a plurality of samples were taken.

10b3. "As discussed in Section 2.52 of the "Compendium of Methods for the Microbiological Examination of Foods," 4th Ed., Eds. Frances Pouch Downes and Keith Ito, American Public Health Association, 2001 (referred to herein as the "Compendium", for products such as liquids, the product is mixed until homogenous before drawing a sample. However, for samples that cannot be adequately mixed or bulk solids or semi-solids, the Compendium teaches that multiple samples of aliquots should be taken from several areas of the product to ensure that a representative sample is obtained."

RESPONSE BY INVENTOR:

Dr. Bailey correctly states that the compendium teaches taking a plurality of samples from a single lot. But Dr. Bailey fails to mention that this is standard practice, and that the method employed by Price, Gabis and referenced by Jarvis (the FDA BAM method) required that each of the samples taken were to be pre-enriched individually. This is what Price did, this is what everyone before and after him did, and this is what Jarvis referenced in 1989, and 2007 (page 596, right column, bottom, section entitled 'iii. My preferred approach ('wet' compositing)').

10b4. "Thus, in my opinion, if the Price procedure were to be used to assay lots of solid or semi-solid food products (e.g., beef trim or ground beef), a person of ordinary skill would have recognized that multiple portions of the test lot would be sampled and composited to obtain a representative sample."

RESPONSE BY INVENTOR:

Dr. Bailey's assertion is false and goes directly against the teachings of Price. Not only did Price take multiple samples and pre-enriched them individually (page 679, materials and methods), but also on page 681 he teaches against compositing the plurality of samples (second full paragraph), and asserts Silliker (1969) on the same subject.

10b5. "As discussed below, the pre-enrichment broths described by Price are individually enriched by incubation, resulting in pre-enrichment broth cultures. See pp. 679-682, and FIG. 1. At the enrichment stage, equal portions of each pre-enrichment broth culture are pooled (i.e., composited) into a single selenite enrichment broth, thereby forming a first composite of the pre-enrichment broth cultures, and composited into a single tetrathionate enrichment broth, thereby forming a second composite of the pre-enrichment broth cultures. See FIG. 1 and p. 679. Following the enrichment stage, each enrichment broth is streaked onto plates and then identified colonies are biochemically tested. See FIG. 1 and p. 681. When the test is positive, the saved portions of each pre-enrichment culture are individually tested to determine which culture or cultures contributed *Salmonella* to the composite(s). See abstract, FIG. 1, and pp. 679 and 681. The individual tests could be done using the same type of test that is used to test the enrichment broth. A person of ordinary skill in August 2004 also would have understood that negative test results for the composited enrichment cultures described by Price would validate each of the samples making up the composite as negative for the target microorganism."

RESPONSE BY INVENTOR:

Here again Dr. Bailey is making an unwarranted extrapolation. He starts by describing Price as taking a plurality of samples from a single lot, and then he makes the unwarranted extrapolation of saying that anyone reading this would have taken plurality of samples from a plurality of lots and composited the samples from each lot, and then he returns to Price and continues on. A person with skills in the art would not read Price and expand it against his teachings. Nothing in Price suggests that testing methods involving multiple lots are being addressed.

As a matter of fact a scientist with proper training reads a research paper, assimilates the "materials & methods" section which is the enabling portion of each paper, for the purpose of gaining the ability to repeat the work. Anyone reading Price will attempt to understand his teachings, will understand the limitations of his work, and will not try to selectively choose portions and misrepresent other portions to fit one's preconceived objective as Dr. Baily is so aptly doing.

10c. "Price states that *"Salmonella* analyses were carried out as described in the Bacterial Analytical Manual (BAM) except for the pooling of pre-enrichment broths in the following paragraph." Thus, in Price, the samples that are included in the pre-enrichment broths are first prepared according to BAM (see paragraph 11 below for a discussion of BAM).

10d. "One of ordinary skill in the art in August 2004 and 2005 would have understood Price to require incubation of the pre-enrichment broths for at least the following three reasons. First, on page 682, Price refers to "the incubation time of the pre-enrichment cultures," thereby indicating that the pre-enrichment cultures have been incubated. Second, Price refers to "pooled pre-enrichment broth cultures." See title, abstract, and pp. 680-682. The use of the term "cultures" implies that the pre-enrichment broths were incubated. This is consistent with how the term "culture" was used in the art as evidenced by Jarvis, discussed above. Third, in August 2004, it was common to use two separate enrichment steps: (1) a "pre-enrichment" enrichment step in which a pre-enrichment broth is incubated to cause organism growth resulting in a pre-enrichment broth "culture," and (2) a second enrichment step that causes further organism growth.

10e1. "In August 2004, it was well known that the incubation of the pre-enrichment broth cultures disclosed in Price would cause the microorganism to reach detectable levels and become substantially uniform within each pre-enrichment broth culture at least because a portion of each pre-enrichment broth culture will be further diluted at the selective enrichment stage."

<u>RESPONSE BY INVENTOR</u>:

Dr. Bailey's statements are not true. In 2004 and today the status of levels of *Salmonella* at the pre-enrichment stage have not changed. It is still at a point where when 1 ml is taken to inoculate enrichment there should be a 99% chance that there is at least one viable organism in the one ml transfer portion (the dividability stage). Today, as I write this, the BAM method still relies on pre-enrichment followed by selective enrichment. <u>The logical question that needs to be asked is "If at the end of pre-enrichment *Salmonella* levels are</u>

detectable, why did Silliker, Gabis, Price, Jarvis, BAM, the compendium, etc. require a second enrichment step?" The answer is simple, at the end of pre-enrichment there simply are not enough *Salmonella* cells to be detected by any method including PCR.

It is also my opinion that Examiner's assertion that PCR can detect one cell (per Entis) is wrong. Entis merely says that "in theory" PCR can detect a single copy of a gene in a PCR reaction, but she drastically qualifies her statement by noting that PCR involves only microliter scale portions, so that much larger concentrations are practically required to get a single cell into a PCR tube. There are also the issues of inhibitors and interfering molecules, and the Poison distribution of the cells in solutions, that put the minimum detectable limits by PCR at about 10,000 CFU/ml.

10e2. "That is, because as little as 1 ml of each pre-enrichment broth culture would be transferred to each of the enrichment broths (see p. 182), each 1 m1 of each contaminated pre-enrichment broth culture would have to have a detectable amount of *Salmonella.*"

RESPONSE BY INVENTOR:

The term "detectable" is a term of art, it implies a level of a target organism in an enrichment culture that gives a positive signal for the said organism using a test method. Dr. Bailey is misconstruing the terms of art. Jarvis 1989 is seeking dividability, not detectability, per page 141 first paragraph:

> ***Once sufficient growth has occurred in pre-enrichment for at least 3 viable salmonellae/ml medium, the probability of transferring at least one viable organism in an inoculum of 1 ml or more will be >. 0.95, assuming Poisson distribution, with m=3: 1'(x=O) = .05….."***

10e3. "For every 1 m1 of each contaminated pre-enrichment broth to have a detectable amount of *Salmonella,* each pre-enrichment broth culture would have had to have been incubated long enough to allow any *Salmonella* therein to reach detectable levels and become substantially uniform. Otherwise, if the incubation period was not sufficient to allow *Salmonella* to reach detectable levels and become substantially uniform, it is possible that a 1 m1 portion of a contaminated pre-enrichment broth culture might not contain a viable *Salmonella* organism."

RESPONSE BY INVENTOR:

This is another circular argument confusing the 1 ml of the pre-enrichment broth having at

least one viable cell (dividability) with detectability. This is purely for the purpose of confusing the Examiner. No person with ordinary skills in the art would reasonably accept this argument.

10e4. "Furthermore, Price discloses that the saved portions of each pre-enrichment culture are individually tested to determine which culture or cultures contributed *Salmonella* to the composite(s). For the saved portions to be individually tested, any *Salmonella* therein must reach detectable levels and become substantially uniform."

RESPONSE BY INVENTOR:

Dr. Bailey conceded that: "10c. Price states that *"Salmonella* analyses were carried out as described in the Bacterial Analytical Manual (BAM) except for the pooling of pre-enrichment broths in the following paragraph."

Thus, in Price, the samples that are included in the pre-enrichment broths are first prepared according to BAM (see paragraph 11 below for a discussion of BAM). Bailey leaves out the fact that, per BAM, if anyone was going to test the saved pre-enrichments they would have had to transfer 1 ml to each of the two selective enrichment broths, followed by incubation, plating, etc. Again Dr. Bailey misleads the Examiner by confusing the fact that the pre-enrichment would have needed to contain only one viable target cell, whereas the patent requires detectable levels.

10f. "In August 2004 and 2005, it was well known in the art that enrichment broths (which could be selective enrichment broths) would be incubated to foster organism growth and that plates would also be incubated to foster organism growth so that colonies of the microorganism could be identified for testing.

10g. "In Price, two different enrichment broths (selenite and tetrathionate) are used (1) because one may be more effective than the other at fostering *Salmonella* growth in a particular sample, (2) because one may be more effective at inhibiting competing organism growth in a particular sample, and/or (3) for confirming the test results. Because the different enrichment broths are more/less effective at fostering *Salmonella* growth and inhibiting competing organism growth, when inoculated with the same material, the two broths will have different concentrations of *Salmonella* following incubation."

RESPONSE BY INVENTOR:

Here Dr. Bailey admits that two separate enrichment broths are needed, and are to be inoculated with the pre-enrichment broth. His problem lies in the fact that if the levels of *Salmonella* in the pre-enrichment broth were detectable why did Price, and the rest of the references (Silliker, Gabis, Jarvis, BAM, The Compendium) continue using the selective enrichment, a practice that has continued up to the present day? Dr. Bailey's third assertion, (3), is false. It is very simple. If you inoculate two enrichments, incubate for prescribed time, and then plate the purpose is for detection, not confirmation.

10h. "Price discloses that the pooling of pre-enrichment broth cultures "offers a simple, yet effective way to increase sample capacity in the *Salmonella* testing of foods," and that a portion of each pre-enrichment culture can be retained for later reference so that, if the test of the pooled pre-enrichment broths is positive, individual tests can be performed on the retained pre-enrichment cultures to determine which culture or cultures contributed *Salmonella* to the pool. See abstract, FIG. 1, and pp. 679 and 681."

RESPONSE BY INVENTOR:

Price claims that pooling of pre-enrichments of a plurality of samples from a single lot for testing for *Salmonella* in types of foods that have incidents of contamination, would result in reducing the number of enrichment cultures and detection plates. The results presented by Price in his paper *teach away* from using pooling in that a person with ordinary skills in the art would immediately recognize that false negative rates for pooling pre-enrichments of up to 28% (Table 2 pooled pre-enrichments for egg albumin) and 50% for pooling samples before pre-enrichment, are not acceptable for validating test lots for release into commerce by any regulatory agency or accreditation body. As an example the USDA FSIS Microbiological Laboratory Guide (MLG) 5.03 (in effect at the time the patent was submitted) specifies an upper acceptable limit of false negatives as less than 2%). Anyone with skills in the art recognizes, and Jarvis (1989, 2007) confirms the fact that the retesting of the pre-enrichment is to establish the extent of contamination, unlike patent'584 where the goal is to statistically enable release the negative lots according to a statistical-based sampling plan.

10i. "Thus, Price at least teaches that a plurality of pre--enrichment broth cultures (each corresponding to a sample) which have been incubated under conditions suitable to allow any target microorganism to reach detectible levels and become substantially uniform, are composited prior to testing and a portion of each pre-enrichment broth culture is saved and is individually tested, if the test of the composite is positive."

RESPONSE BY INVENTOR:

Dr. Bailey misrepresents Price. Price does not teach that pre-enrichment allows for *Salmonella* to reach detectable levels, for he would have plated the pre-enrichments and bypassed the selective enrichments.

11. "Food Sampling and Preparation of Sample Homogenate." BACTERIAL ANALYTICAL MANUAL (U.S.D.A. 2003), http://www.fda.gov/Food/ScienceResearch/http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalytucalManualBAM/ucm063335.htm (hereinafter "BAM") was a well-known FDA publication that describes a method of preparing samples (and particular food homogenates) for microorganism testing."

RESPONSE BY INVENTOR:

This has no bearing on the issues at hand.

12. "In August 2004, in view of Price's disclosure that *"Salmonella* analyses were carried out as described in the Bacterial Analytical Manual (BAM)," the skilled artisan would have looked to the most recent version of BAM available at that time, which was the 2003 version of BAM."

RESPONSE BY INVENTOR:

This has no bearing on the issues at hand.

13. "In August 2004 and 2005, serological testing would have been considered an "immunochemistry-based" testing method. See J. Coombs, DICTIONARY OF BIOTECHNOLOGY 310 (2nd ed. 1992)."

RESPONSE BY INVENTOR:

This has no bearing on the issues at hand.

14. "In August 2004 and 2005, it was well known that a "selective enrichment" broth fostered the growth of a particular target organism (e.g., *Salmonella)* while inhibiting the growth of competing organisms."

RESPONSE BY INVENTOR:

This has no bearing on the issues at hand.

15. "In August 2004 and 2005, it was well known that "selective plating" fostered the growth of a particular target organism (e.g., *Salmonella)* while inhibiting the growth of competing organisms."

RESPONSE BY INVENTOR:
This has no bearing on the issues at hand.

16. "By August 2004, the Compendium was a standard reference textbook for food microbiologists. Chapter 2 of the Compendium describes general concepts and procedures for sampling food products for microbiological testing. I am familiar with '584 patent claims 1, 2 and 17 (and claims dependent on them) which specify that the claimed method be applied to a plurality of "test lots" or "single-unit production lots." I also am aware that the term "test lot" is defined in the '584 patent as

> An assemblage of one or more specimens of a medium or process (e.g., assemblage of specimens of air, water, solids, or of products of a production process, etc.), where such assemblage can be sampled by taking portions of the one or more specimens thereof, and where the one or more specimens of the assemblage are operationally linked in a manner (e.g., proximitytime, process step, etc.) whereby information derived about sampled portions is operationally applicable to all specimens of the assemblage, and thus to the test lot. In particular instances, portions are synonymous with specimens.

"'584 patent, col. 5, lines 21-32. I am also aware that the terms "single production unit" or "single-unit production lot" are described in the '584 patent as a form of test lot, and "refers to a single unit of production (e.g., for beef trim a 'combo' or for almonds a 'bin') rather than as in the prior art, to a composite comprised of multiple, non-operationally linked single production units (production sublots)." '584 patent, co1. 5, lines 33-38. By August 2004, it was common practice for microbiologists to sample operationally linked production lots for microbiological testing. The statement in the above quotation from the '584 patent implying that the prior art employed a "composite comprised of multiple, non-operationally linked single production units (production sublots)" is an overstatement. Regardless of whether some processors combined production lots before samp1ing for microbiological testing, it is also clear that the use of single production units or lots for sampling was also commonplace. This is evidenced by Chapter 2 of the Compendium. In that chapter the authors discuss the importance of developing a "sampling plan" and of

obtaining 'representative samples" for microbiological testing. In Section 2.511, "Sampling Plan" is defined as "[a] design that indicates the number of units to be collected from each lot and the criteria to be applied in accepting or rejecting the lot." "Lot" is defined as,

> [t]he number of sample units produced in one batch or in some specified period of time such that the units will be about the same quality. Each lot or batch should consist of units of product of a single type, grade, class, size, and composition. The lot should be manufactured under the same conditions and at essentially the same time.

"Thus, Chapter 2 of the Compendium makes clear that, prior to August 2004, microbiologists utilized sampling plans that involved sampling a "number of units to be collected from each lot" and that a "lot" was understood to be a production batch consisting of operationally linked units. Accordingly, a person of ordinary skill in the art was aware that a "test lot" or "single-unit production lot" consisted of an assemblage of units or specimens operationally linked by "type, grade, class, size and composition" and "manufactured under the same conditions and at essentially the same time." Based on the teachings of Compendium Chapter 2 and my general experience, it is my opinion that the sampling of "test lots" or "single unit production lots" by taking a plurality of portions and combining (compositing) them, as specified in steps a) and b) of claims 1, 2 and 17 (and claims dependent upon them) was known and obvious to persons of ordinary skill in the art at the time.

RESPONSE BY INVENTOR:

Dr. Bailey equates the taking a plurality of samples from one lot to taking a plurality of samples from a plurality of lots. This is an unwarranted extrapolation that strikes at the heart of the difference between patent '584 and the prior art. To make this extrapolation is simply an effort to cause confusion and mask the difference between patent '584 and the prior art.

17. "I am familiar with the USDA FSIS Microbiology Guidebook Protocol for the sampling and analysis of raw ground beef for *E. coli* contamination, as reproduced as Section 35.243 of the Compendium ("the FSIS Protocol"). In my opinion, by August 2004 a person of ordinary skill in the art would have understood that the method described in the FSIS Protocol would be applied to a "test lot" or "single unit production lot" of ground beef, as those terms are defined in the '584 patent."

RESPONSE BY INVENTOR:

This observation by Dr. Bailey continues the effort to cause confusion and mask the difference between patent '584 and the prior art noted in the prior answer to issue 16.

18. "The FSIS Protocol refers to blending composite samples of ground beef with an enrichment medium defined as "mEC with novobiocin." In August 2004, a person of ordinary skill in the art would have understood that m.EC broth was a modified form of EC broth, a well-known culture medium for the detection of *E. coli* and that "mEC broth with novobiocin" was a selective enrichment medium for *E. coli."*

RESPONSE BY INVENTOR:

Dr. Bailey does correctly represent the FSIS testing protocol as stated in MLG 5.03, with effective implementation date of 10/25/02. The FSIS test method for the samples taken from a lot of food is exactly the reverse of the pooling/compositing process. FSIS teaches to take a plurality of samples from a single lot, and composite them into one composite sample. However, at the time of analysis, per MLG 5.03, Revision 03, Section 5.5, five 65 gram sub-samples are taken out of each bag, and each is individually enriched and tested. This is without any pooling. In other words, FSIS teaches de-compositing and teaches away from pooling.

19. "The FSIS Protocol describes a procedure for analyzing a batch or test lot of raw ground lots on a regular basis. The FSIS Protocol describes sampling, enriching and analyzing each lot of product being tested. In other words, there is no suggestion in the FSIS Protocol would be applied to multiple separate production lots or test lots of raw ground beef to produce a set of separate test lot samples, particularly in large operations producing multiple production lots on a regular basis. The FSIS Protocol describes separately sampling, enriching and analyzing each lot of produc.t being tested. [n other words, there is no suggestion in the FSIS Protocol that non-operationally linked production lots should be combined before sampling."

RESPONSE BY INVENTOR:

This is a misrepresentation of the FSIS Directive. The directive is aimed at FSIS inspectors, and prescribes a specific manner of collecting one set of sample from a day's production. No one with ordinary skill in the art would extend the protocol. This is a specified regulatory sampling for FSIS. The sample concept of taking multiple samples from a single lot is in Price, Jarvis, Gabis, BAM and Compendium. Thus, the FSIS reference merely overlaps the other references, rather than showing the way toward any modification or extension or combination. References which completely overlap one

another cannot be combined to teach something additional.

20. "I am familiar with two articles published J.H. Silliker and D.A. Gabis in the early 1970s: Silliker, J.H. et al., 1973. ICMSF Method . I. Comparison of Analytical Schemes for Detection of *Salmonella* Dried Foods. *Can. J. Microbiol., 19*:475 and Gabis, D.A. et al, 1974. ICMF Method Studies. II. Comparison of Analytical Schemes for Detection of *Salmonella* High-Moisture Foods. *Can.J. Microbiol., 20:663-669.* Silliker et al. and Gabis et al. describe methods for analyzing dried food products and high-moisture food products (such as meat, poultry and eggs) respectively for contamination with *Salmonella.* of these publications described taking multiple samples from the lot of food product being tested, those samples in pre-enrichment media, incubating those media, transferring aliquots to a selective enrichment media and testing for the presence of *Salmonella.* my , each of these publications described the sample-compositing and incubation steps a), b) c) of the claims of the '.584 patent. As discussed above in connection with Jarvis and Price, pre-enrichment procedures used by Silliker et al. and Gabis et al. were effective in growing target microorganism to detectable levels. In each instance, 1 ml aliquots of the pre-enrichment were transferred to selective enrichment broths for further enrichment and by conventional plating techniques. It is thus apparent that each 1 ml aliquot had to a detectable amount of the microorganism in positive samples. A person of ordinary skill by August 2004 would have understood that the Silliker et al. and Gabis et al. procedures would be applied to "test lots" or "single-unit production lots" of food products and that such "test lots" or "single-unit production lots" would consist of combinations or assemblages of separate pieces or specimens of the food product. A person or ordinary skill also would have understood that the Silliker et al. and Gabis et al. procedures would be applied to a plurality of separate "test lots" or "single-unit production lots," especially in large facilities producing a number of production lots on a regular basis."

RESPONSE BY INVENTOR:

Here, Dr. Bailey revives the argument that the presence of at least one viable cell in the 1 ml of the pre-enrichment media, as detectable level. Per my earlier comments I believe that this is a misrepresentation of the art and science. He also fails to mention that the Silliker 1969 reference used by Price in his article, which was reaffirmed by Price, specifically prohibits and teaches against dry compositing samples into a pre-enrichment. Price reports up to 50% false negatives and reaffirms Silliker on this issue. Furthermore, there is nothing that Dr. Bailey has presented which shows that the Price and Jarvis's has ever been extended to any other method in microbiology, or to plurality of samples taken from a plurality of lots in the manner of my patent claims 1 and 17.

21. "Silliker et al. and Gabis et al. describe testing for *Salmonella* using conventional plating technology. By August 2004 so-called "Rapid" methods for the detection of microorganisms had been developed and were being widely used. These Rapid methods were based on immunochemical or nucleic acid-based technology (such as PCR and nucleic acid probes). These methods were faster and often more sensitive than the conventional microbiological techniques utilized in the preceding decades. By August 2004, it would have been obvious to a skilled person to utilize these Rapid methods in combination with the sample compositing and wet-compositing procedures described, for example, by Jarvis, Price, Silliker et al. and Gabis et al. A person of ordinary skill in the art would have understood that pre-enrichment and enrichment procedures described in those publications could be streamlined because of the greater sensitivity of the Rapid methods. A number of Rapid methods are described in Chapter 10 of the Compendium."

RESPONSE BY INVENTOR:

This is irrelevant, none of these arguments pertain to the claim language.

22. I have read U.S. Patent No. 7,534,584 (584 Patent). The term "production assemblage" is not defined in the specification of the 584 Patent. There is no special industry definition of this term that would have applied in August 2004 and 2005. Thus, to construe the term production assemblage, the skilled artisan would have employed the common definition of "assemblage," which is "a collection of things." AMERICAN HERITAGE COLLEGE DICTIONARY 82 (3rd ed. 1998) *(Exhibit B)*. This common definition is consistent with the use of the term "production assemblage" in the specification."

* * *

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and may jeopardize the validity of the application or any patent issuing thereon.



Mansour Samadpour, Ph.D.

31 January 2011

Patent Owner Ex. 1007
Page 0026

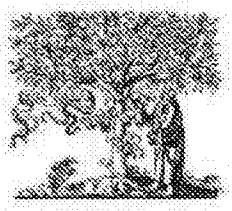
statistical aspects of the microbiological analysis of foods

B. JARVIS

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progress in industrial microbiology

volume 21



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CHAPTER 8

ERRORS ASSOCIATED WITH DILUTION COUNT AND PRESENCE/ABSENCE METHODS

In its simplest form, the dilution count method consists of serial dilution of the initial food homogenate (or culture) followed by inoculation of an appropriate volume of each dilution into one or more tubes of culture medium (agar or broth). Following incubation, the number of tubes showing evidence of growth (e.g. colonial growth in agar; turbidity in broth; production of acid and gas in MacConkey's Bile Salt broth in the test for presumptive coliforms, etc.) are recorded. The cultures showing no evidence of growth are assumed not to have received a viable inoculum ($p = 1$), i.e. to have received < 1 viable cell or cell aggregate; an assumption which may not always be correct. Similarly, any culture showing growth is assumed to have received at least one viable propagule ($p = 1$). If each viable propagule is assumed to be capable of producing a growth response, then the proportion ($P_{(x=0)}$) of cultures at any one dilution which receive no viable inoculum after inoculation with a mean inoculum level of m propagules is given by the first value of the Poisson expansion, i.e.:

$$P_{(x=0)} = e^{-m} \quad \text{(Table 3.2)}$$

Similarly, the proportion becoming turbid:

$$P_{(x>1)} = 1 - P_{(x=0)} = 1 - e^{-m}$$

8.1 Dube dilution series and Most Probable Number counts

When more than one culture is inoculated at each dilution level, and several dilution levels are inoculated, one would expect a graded response with all tubes positive at the lowest dilution and none, or only a few tubes, positive at the highest dilution tested. This is illustrated in Table 8.1 for dilutions from 10^{-3} to 10^{-8} of culture initially containing 2×10^5 viable cells/ml, and 10, 5 or 1 replicate tubes at each dilution level.

Figure 8.1 illustrates the shape of the expected dose-response curve, based on Poisson distribution. It has been pointed out by several workers (e.g. Eisenhardt and Wilson, 1943) that the response curve may be less steep. This could arise through systematic technical error (Meynell and Meynell, 1965) and as a result of contagious distribution, reflecting clumping, sublethal damage, etc.

LACTOSE BROTH
INDUSTRIAL APPLICATIONS

PRODUCT DESCRIPTIONS:
Bottled Media: B8445 (225 mL), B8457 (90 mL, wide mouth), B8458 (100 mL, wide mouth), B8460 (500 mL, Boston Round)

PURPOSE:
Lactose Broth is used for the detection of lactose-fermenting, Gram-negative coliforms, as a pre-enrichment broth for *Salmonella* species, and in the study of lactose fermentation of bacteria in general. It is used extensively in testing water, foods, dairy and pharmaceutical products.

Sublethally injured salmonellae may be present in low numbers in dried or processed foods. The recovery of *Salmonella* may be hindered by the presence of other bacteria as well as components of the food sample. Lactose Broth, a nonselective medium, supplies a pre-enriched environment, which allows for repair of cell damage while diluting toxic or inhibitory substances, and providing nutritional advantage to *Salmonella* over other bacteria.

PRINCIPLE:
Beef Extract and Peptone provide carbon and nitrogen sources for general growth requirements, while lactose provides a carbohydrate source.

This pre-enrichment medium is used to provide a higher ratio of *Salmonella* to non-*Salmonella* bacteria after incubation. Lactose is fermented by most most non-*Salmonella* bacteria while *Salmonella* does not. A bactiostatic effect on competing microorganisms is created by the decrease of pH in the medium produced by the lactose fermenting bacteria. The *Salmonella* will have little growth during the initial 24 hours of incubation, while the non-*Salmonella* will be utlizing the lactose. As the products of fermentation build, the non-*Salmonella* are inhibited and the *Salmonella* begins its growth phase (24-48 hrs.)

FORMULAS:

Approximate, per liter of deionized filtered water.

(1) Lactose Broth

Enzymatic Digest of Gelatin	5.0 g
Beef Extract	3.0 g
Lactose	5.0 g

Final pH: 6.9 ± 0.2 at 25° C

PRECAUTIONS:*
For laboratory use only. Observe approved biohazard precautions.

Storage: Upon receipt store at 2-25°C. Media should not be used if there are signs of contamination, deterioration (discoloration), or if the expiration date has passed. Media can be inoculated up to the expiration date and incubated for the appropriate incubation period.

Limitations:
Due to nutritional variation, some strains may grow poorly or fail to grow in this medium

PROCEDURE:*

Method of Use:
Lactose Broth is used for the pre-enrichment phase for the testing of food and other materials, to enhance the recovery of *Salmonella.* Consult the appropriate references for specific procedures for each type of material being tested.[1-4,6]

1. Transfer a 25 g or 25 ml sample of test material into a container. Add 225 ml of sterile Lactose Broth. Mix as necessary to make
   a homogeneous suspension. Incubate at 35°C for 24 hours.

2. Transfer 1 ml of suspension to appropriate enrichment broths such as Tetrathionate Broth and Selenite Cystine Broth. Incubate at 35°C for 24 hours.
3. Transfer a loopful of suspension to appropriate selective agar media, such as Hektown Enteric Agar, XLD Agar and Bismuth Sulfite Agar. Incubate at 35°C for 24 hours

Material Required but Not Provided: Standard microbiological supplies and equipment such as loops, needles, incubator, and incinerator are not provided.

QUALITY CONTROL:

Microorganism	ATCC® #	Response
Enterococcus faecalis	29212	Good Growth
Escherichia coli	18739	Good Growth
Pseudomonas aeruginosa	9027	Good Growth

User Quality Control: Check for signs of contamination and deterioration.

BIBLIOGRAPHY:

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3. U.S. Food and Drug Administration, *Bacteriologicl Analytical Manual*, 8th ed., AOAC International, Gaithersburg, MD., 1995.
4. Cunnif, P. (ed.), *Official Methods of Analysis AOAC International*, 16th ed. AOAC International, Gaithersburg, MD.,1995.
5. Franson, M. A. H. (ed.), *Standard Methods for the Examination of Water and Wastewater*, 20th ed., American Public Health Association, Washington D. C., 1998
6. U.S. *Pharmacopeia and National Formulary, USP 27, NF 24*, The USP convention Inc., Rockville, MD 2004.

*For more detailed information, consult appropriate references.

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Data #1009
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Revision Date: 06/20/2007

New Technology for Microbiological Testing of Foods

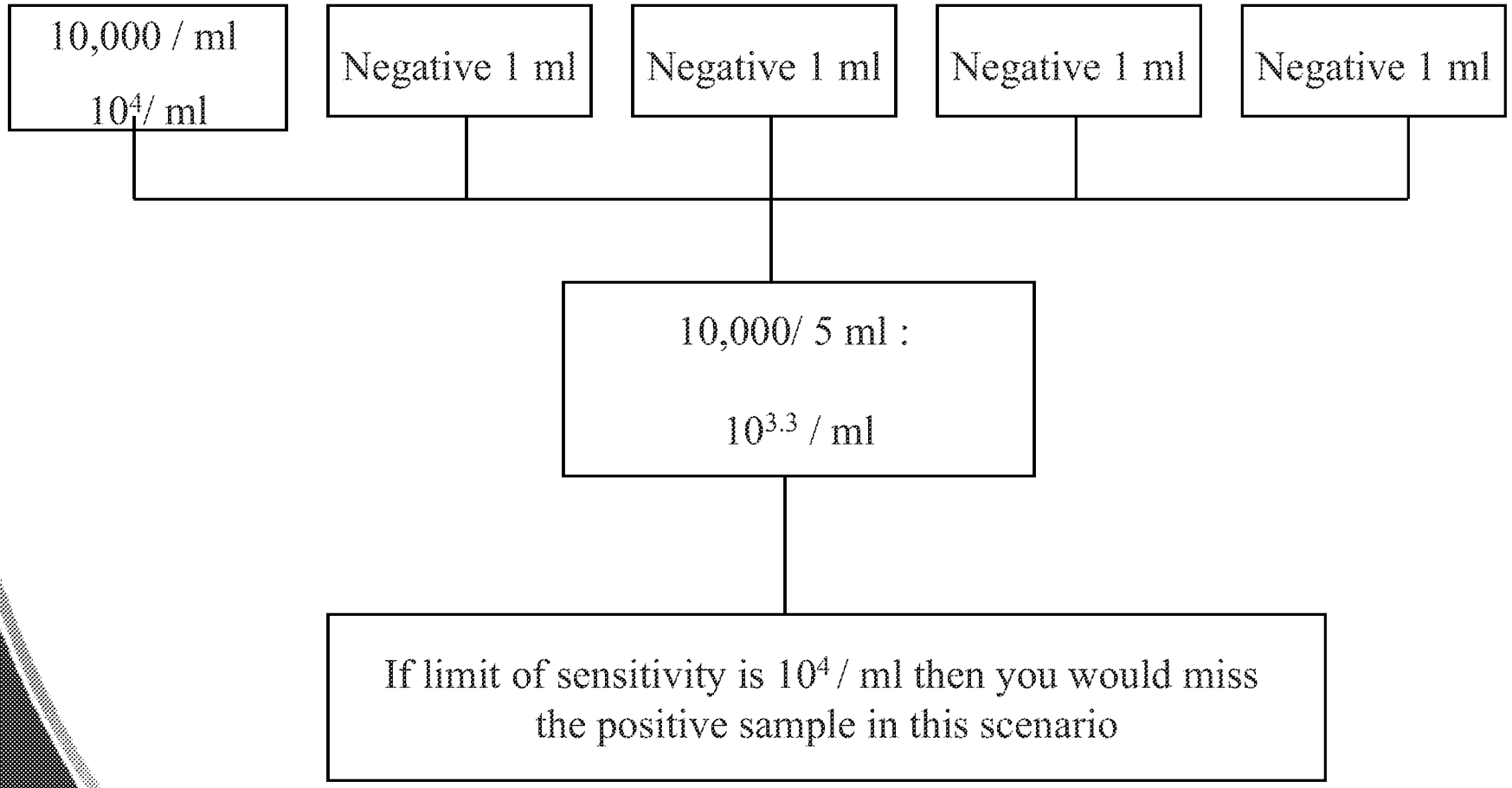
Stan Bailey, PhD

President, International Association for Food Protection

Director Scientific Affairs - Industry, bioMerieux

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Pooled sample protocol



National Beef Ex. 1007
Page 1216

statistical aspects of the microbiological analysis of foods

B. JARVIS

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progress in industrial microbiology

volume 21



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Amsterdam — Oxford — New York — Tokyo 1989

Assume:

(1) initial mean contamination level = α salmonellae/g sample,
(2) pre-enrichment for 12 h (α_0 fx. 6.6), yielding 410 α salmonellae/ml,
(3) 1 ml pre-enrichment medium inoculated into 10 ml selective enrichment medium,
(4) lag time (2) in enrichment medium = 4 h; doubling time = 60 min = 1 h,
(5) enrichment period 18 or 24 h and
(6) 0.02 ml (1 loopful) inoculated onto diagnostic medium.

Then, from fx. 6.6, after 12 h pre-enrichment the medium would contain 410 α salmonellae/ml. Since pre-enrichment is diluted 1 in 10 into selective enrichment medium the initial level of salmonellae would be 410 α/10 = 41 α/ml = α_0.

Then after 18 h, assuming synchronous growth and other factors as above:

$$\alpha = 41\alpha_0 \; 2^{(18-4)/1} = 41\alpha_0 \; 2^{14} = 6.72 \times 10^5 \text{ salmonellae/ml}$$

Similarly, after 24 h:

$$\alpha = (41\alpha_0) \; 2^{(24-4)/1} = 41\alpha_0 \; 2^{20} = 4.3_0 \times 10^7 \text{ salmonellae/ml}$$

Now if initial contamination level (α_0) = 0.04 (i.e. 1 salmonella/25 g sample), a 0.02-ml loop would be expected to transfer 536 or 34,400 salmonellae after 18 and 24 h incubation, respectively. Hence the probability of transferring at least one viable salmonellae to the isolation medium would be => 0.99.

However, had the initial mean inoculum level after 2 h pre-enrichment, with recovery but no growth, been only 0.01α_0 (fx. 6.6, 2 h incubation), then for α_0 = 0.04/ml the expected numbers transferred with a 0.02 ml loop after 18 h and 24 h enrichment culture would be about 0.01 and 0.86 salmonellae, respectively. Hence, the probability of transferring at least one viable salmonella would be = 0.01 and = 0.58, respectively.

It is essential, therefore, to ensure that an adequate inoculum is transferred from the pre-enrichment culture; otherwise the probability of detecting salmonellae will be low. These effects are illustrated diagrammatically in Fig. 6.4.

Example 6.8. Probability for selection of a specific colony from a diagnostic plate culture

What is the probability for picking at least one *E. coli* colony from a diagnostic plate containing 20 typical coliform colonies if numbers of non-*E. coli* colonies are (a) 10 and (b) 20?

Since the population is finite and (relatively) small, removal of any one colony will reduce the residual number from which to select another colony. Hence, the hypergeometric distribution is the most appropriate. The probability of selecting no *E. coli* colonies is described by the equation:

$$P_{(x=0)} = [s! \; (N-s)!]^2 / [u! \; (u+s)! \; N!]$$

where:

N = number of typical coliform colonies
s = number of colonies picked for identification (= integer of 20)

and,

u = number of non-*E. coli* colonies on the plate.

Since:

$$P_{(x>0)} = 1 - P_{(x=0)}$$

Exhibit I

Via Electronic Submission
Attorney Docket No.:66090-008US2

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mansour Samadpour
Serial No.:	95/001,294
Filed:	05 April 2010
For:	MODULAR COMPOSITING-MULTIPLE LOT SCREENING PROTOCOLS FOR DETECTION OF PATHOGENS, MICROBIAL CONTAMINANTS AND/OR CONSTITUENTS
Examiner:	Brenda G. Brumback
Art Unit:	3991
Attorney Docket No.:	66090-008US2
Date:	31 January 2011

Attn: Mail Stop "Inter Partes Reexam"
Central Reexamination Unit
Commissioner for Patents
P. 0. Box 1450
Alexandria VA 22313-1450

DECLARATION OF WARREN MIRTSCHING UNDER 37 C.F.R. § 1.132
(IN SUPPORT OF RESPONSE UNDER 37 C.F.R. § 1.111)

Madam:

I, Mr. Warren Mirtsching, being duly sworn, say:

1. I am familiar with the above-identified patent application, and am aware that it discloses and claims novel methods comprising sampling, testing, and validating test lots for microbial contamination. This declaration is to corroborate the declaration of Dr. Samdapour concerning showing a nexus between the inventive method and commercial success and additionally to corroborate skepticism amongst clients and others in the art for the validity, accuracy and safety of Dr. Samadpour's method. In addition, this declaration provides evidence of copying by competitors and others in the field. As such, according to 37 C.F.R. § 1.116(e), this declaration is necessary to respond properly to the action closing prosecution.

2. I am familiar that the detailed claimed method comprises: collecting a plurality of portions from each of a plurality of test lots, the test lots each comprising an assemblage of one or more specimens, wherein each test lot is separately sampled by taking said plurality of portions thereof; combining the collected plurality of portions corresponding to each of the separate test lots to provide a corresponding set of separate test lot samples, wherein each separate test lot sample is attributed to a particular corresponding separate test lot; incubating the set of separate test lot samples under conditions suitable to allow levels of a target microbial agent or organism that is present in one or more of the separate test lot samples to reach detectable levels and become uniform, or substantially uniform, throughout the respective one or more separate test lot samples, to provide a set of separate test lot samples enriched for the target microbial agent or organism; removing portions of each enriched separate test lot sample, and combining the removed portions to provide a modular composite sample; and testing of the modular composite sample, using a suitable detection assay, for the target microbial agent or organism, wherein when such testing is negative all of said separate test lot samples are validated, and wherein when such testing is positive, individual separate test lots may nonetheless yet be validated by further testing of a portion of the respective enriched separate test lot sample using the same or a more sensitive protocol and obtaining a negative test result.

3. ~~My position~~I am the Chief Operating Officer in the Meat Division at Institutes of Environmental Health, which I have held since May 2008~~, relates to the Safety and Quality at the Greely Laboratory~~. I have a Bachelor of Science Degree in in Animal Science from Texas A&M University. My detailed curriculum vitae is attached hereto as APPENDIX A.

4. Previously, in 2004, I was employed by Swift and Company in charge of food safety. At this time Swift and Company decided to investigate whether the wet composting technique developed by Dr. Samadpour could be used for testing meat products produced by Swift.

5. Intially, I was concerned that Dr. Samadpour's approach would not give the accuracy of testing to reach a sensitivity to identify the pathogen, due to a "dilution effect". Gerome Lawler, then the Director of Food Safety at Swift, was also highly skeptical of Dr. Samadpour's wet compositing approach. He questioned whether it was sensitive enough to meet Swift's requirements.

6. To confirm that Dr. Samadpour's wet compositing approach would work, we spent a great deal of effort conducting side-by-side cmparsions at Swift's Greely plant for about six months, from early to mid-year 2004. The tests were accomplished by comparing the standard old manner that we had been using with Dr. Samadpour's method. During the test period we ran about 100 tests per day by the standard old method, and about 400 tests of individual combos that went into about 100 composites as described in Dr. Samadpour's method.

7. Our extensive, detailed testing found that Dr. Samadpour's technique, in fact, worked. That is, it was sensitive enough to meet the requirements prescribed by Swift. In addition, Dr. Samadpour's technique allowed individual combo bins to be validated for release to commerce even if composite testing of the overall production lot tested positive. The techniques used prior to Dr. Samadpour's technique obligated that a positive test results in the entire production lot of 10,000 pounds be diverted to a low-value use, but implementing Dr. Samadpour's approach allowed 2000 pound single-lot units to be saved.

8. Major customers of Swift and Company initially refused to accept product that was tested using Dr. Samadpour's methods. In particular, Tyson Company, and their Director of Safety Dr. Dean Danielson, were highly skeptical. Dr. Danielson challenged the entire concept of wet compositing as proposed by Dr. Samadpour. Tyson initially stated they would not accept product from Swift that had been qualified with Dr. Samadpour's new form of testing. We were required to supply them with actual results from the Swift tests which proved that the process was sufficiently sensitive. In addition to Tyson Company, Cargill initially would not accept Dr. Samadpour's technique. To convince Cargill that Dr. Samadpour's technique was a valid and accurate method for testing production lots, Swift had to put a substantial amount of effort into proving to them that Dr. Samadpour's techinque worked.

9. After Dr. Samadpour's technique was tested internally, Swift and Company decided to implement the method at its major processing facilities, including the plants in Grand Island, NE; Greely, CO; Dumas, TX; Hyrun, UT; Omaha, NE; and Nampa, ID. These implementations were completed in 2007, and Swift has been using Dr. Samadpour's invention since then.

10. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and that these

statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and may jeopardize the validity of the application or any patent issuing thereon.

5

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Warren Mirtsching

31 January 2011

APPENDIX A
CURRICULUM VITAE

Warren Mirtsching
Safety and Quality

Warren Mirtsching

5221 Keystone Creek Court
Ft. Collins, CO 80528
Home: (970) 206-4512 **Fax: (970) 206-1415** **Cell: (970) 692-4061**

CAREER SUMMARY

A proven food processing executive, leader, and strategist. Demonstrated ability to change organizational structures and lead others in new creative directions. Well versed in and capable of implementing food safety and quality systems. Experienced in consumer package items for both retail and foodservice trades. Designed and implemented multiple product and packaging changes to processed and fresh food for global distribution customer base. Comfortable and able to establish working relationships at all levels from the manufacturing floor to executive management. Able to adapt quickly to new environments and challenges. Experienced at evaluating facilities, organizational effectiveness and skill sets, driving productivity improvements through "Lean" process systems and methodology. Working as a leader and participant across functional lines for the betterment of the organization, acute at assessing competitive best practices scenarios due to network of peers, industry, and scientific fields.

PROFESSIONAL SUMMARY

IEH Inc. **May 2008 to present**
COO Meat Division
Laboratory and Consulting Services

IEH Inc is a laboratory and consulting service located in Seattle, WA. (www.iehinc.com) Current responsibilities are focused on client/customer service for prevention and crisis management related to the food industry. Development of consistent operating practices to improve revenue generation while enhancing and broadening customer service. Grow the customer base through the use of industry knowledge for the improvement of Food Safety Systems. Collaborate with associated companies to leverage the services provided.

JBS SWIFT & COMPANY **July 2007 to March 2008**
Oversee Food Safety, Quality, & Regulatory Compliance

DWT 16458829v1 0066090-068US2 DWT 16336565v1 0066090-068US2

JBS of Brazil purchased Swift & Company in July 2007 and redefined the organization, making JBS the world largest beef processor and a true global entity. I was retained to oversee all Food Safety, Quality, and Regulatory Compliance issues for the Beef group in North America.

- Implemented manpower reduction which generated a $1.7 million savings annually.
- Initiated deep dive into company expenses to assist in a 5% reduction in vendor cost of doing business.
- Supported the integration of new Brazilian management with business concepts in order to reduce their learning curve.
- Completed the licensing of a patented micro sampling device.
- Implemented USDA Grading trial on vision technology for improvements to consistency and value enhancement in the total supply chain.
- Completed the communication of the new single combo testing methods which will reduce the industry's product loss/devaluation by 80%.

SWIFT & COMPANY **1997 to July 2007**

Senior VP, Food Safety & Quality
North American Red Meat 2006 to July 2007

Senior Food Safety and Quality Executive for Swift & Company's $8 Billion revenue generating business in North America, 2nd largest red meat company in the world. Responsible for Food Safety, Quality, Regulatory and Trade Compliance, planning (annual and strategic), budgeting, capital spending, and interfacing with Suppliers, Customers, and Consumers.

- Implemented vision grading of beef carcasses to improve revenue opportunities identifying $5 million annually.
- Solidified Swift's Food Safety Leadership role through the reduction of E. coli incident rates by 80%.
- Managed the development of Swift's Beef Export Verification through vendor and customer relationships using Quality System Assessment process.
- Pioneered the implementation of single combo lot for trimming saving $750,000 from testing cost and logistics.

VP, Food Safety and Quality
North American Red Meat 2002 to 2006

Selected to fill the combined position of both Beef and Pork VP Food Safety and Quality. Accountable to bring the Beef and Pork separate organizations together under a consolidated and effective direction. Integrate varied practices into a business strategy which supported Sales, Marketing, Operations, and other support groups. Develop best practices and revenue generating ideas with sustainable growth and continuous improvement processes.

- Developed "SwiftTrace" traceback & traceforward systems integrating existing IT framework to capture an annual $4.0 million net revenue return with a major foodservice company. Applicable to domestic and international markets for a competitive advantage. Expandable system to implement Country of Origin Labeling Laws.
- Implemented Matrix system for the consolidation of multiple management programs into one; consistency of specifications and new product development system for all species.
- Implemented best practices/guidelines/policies which reduced product/process claims by $1.4 million annually.
- Implemented long term contracts for lab support and sanitation practices with vendors recognizing a $1.2 million cost reduction.
- Using best practices and consolidation of efforts, gained $1.5 million reduction of recall insurance over a three year period.
- Identified and implemented industry's first "Double pasteurization system" in conjunction with "Multiple Hurdles linked to Standard Operating Procedures".

VP, Food Safety & Quality
ConAgra Beef Company/Monfort 1998 to 2002

Implement continuous improvement processes and develop best practices in multiple functions within ConAgra Beef Company/Monfort. Direct P&L accountability for Cow plant Operations, Trim & Grind Sales, Research & Development, Food Safety & Quality, Logistics and Scheduling.

- Implemented a strategy to maximize trim/grind complex by $4.2, $2.8, and 2.3 million annually through aggressive customer and product management.
- Developed "Strongest Chain of Quality" and "Strongest Chain of Tenderness" marketing programs which brought beef business into a positive customer perception.
- Identified the use of "Operational Critical Control Points" for the consistency of education, communication, and production of ground beef over four different facilities.
- Instrumental in Customer Confidence related to product quality perception.

Director, Beef Quality Assurance
Monfort 1997 to 1998

Responsible to convert the product and processing quality of Monfort Beef into a competitive advantage. Implementation of consistency and detailed execution to change the customers' perception of beef quality and services. Develop a culture within the organization which eliminated waste and rework, thereby improving competitive costs.

- Drove consistency of product specifications and disciplines into the facilities eliminating $1.5 million in waste and rework.
- Earned the respect of customers and competitors who believed that Monfort's beef was not acceptable and had to be priced low.

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- Managed the organization and development of personnel to be accountable and responsible for their decisions.
- Earned the respect of the organization and therefore promoted to VP over the entire Beef group.

EXCEL CORPORATION (Div of Cargill) **1977 – 1997**

General Manager, Cargill Processed Meat Products – Ft. Branch, IN 1992-1997

Chief executive for Excel's $100 million revenue generating processed meats facilities, supplying a variety of processed meat items for foodservice and retail markets. Responsibilities included P&L for the facilities, customer and consumer satisfaction, planning (annual & strategic), budgeting, capital spending, employee retention, safety, transportation, and daily operating issues.

- Negotiated the purchase of a family owned brand name consumer packaged goods company in Indiana.
- Successfully integrated two facilities into the Cargill system.
- Implemented reduction in supply vendors through consignment inventory and consolidated pricing.
- Harmonized raw material supply to match finished product values.
- Developed and implemented formulation & packaging changes to enhance the value of hams, bacon, sausage, roast beef, lunch meats, and hotdogs.
- Integrated new product formulations to maximize the consumer preference of finished packaged goods.
- Implemented technology to enhance shelf life of the packaged goods by 50% through sound science applications and detailed execution.
- Trained the management staff to engage in responsibilities which allowed for total employee growth of accountability.

General Manager
Excel Pork Customer Service & Processed Meats – Wichita, KS 1988-1992

Selected to fill a newly created dual role accountable for developing and implementing Excel's Fresh Pork Customer Service program and growing Excel's market share in the processed meat business. P&L responsibility for the processed meat division, including sales, marketing, manufacturing, engineering, and administration.

- Chaired the Corporate Total Quality Management Committee.
- Negotiated a multi-year contract with major Processed Products marketer for 40 million pounds annually.
- Linking supply chain management to leverage product position and revenue.
- Oversaw the development and operations of packaged consumer goods to retail and foodservice accounts such as: hams, bacon, and fresh sausage.

General Manager

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Beef Slaughter and Fabrication – Plainview, TX 1983-1988

P&L accountability for the $900 million Beef slaughter and fabrication business unit with 1400 employees, including operations, engineering, quality assurance, distribution and administration. Oversaw the implementation of double shifting the plant, adding ground beef, and waste water revenue program in record setting time.
Simultaneously, oversaw Cargill's Brazilian operations in Maringa and Jose Bonafacio.

Plant Fabrication Manager – Dodge City, KS 1982 - 1983
(construction of fabrication and startup)
Plant Fabrication Manager – Wichita, KS 1980-1982
MBPXL Corporation – Plainview, TX 1977- 1980
Meat packing – acquired by Cargill in 1979
Various positions including: Assistant Plant Manager; Fabrication Superintendent; General Foreman; Packing Supervisor; and Technical Services Coordinator

EDUCATION

Texas A&M University, College Station, TX – BS in Animal Science 1977
Commercial Option

PERSONAL DEVELOPMENT

ISO Quality Systems – 2004 - present
Team Building – 1999 - 2001
Sportsmind – Team Building 1995
Total Quality Management 1988 – 2008
Minnesota School of Management for Marketing 1989

COMMITTEES AND ASSOCIATIONS

Beef Industry Food Safety Council (BIFSCO) 1998 – present
North American Meat Association 1998 – present
National Cattlemen's Beef Association 1998 – present
American Meat Institute 1997 – present

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Via Electronic Submission
Attorney Docket No.:66090-008US2

Exhibit J

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mansour Samadpour
Serial No.:	95/001,294
Filed:	05 April 2010
For:	MODULAR COMPOSITING-MULTIPLE LOT SCREENING PROTOCOLS FOR DETECTION OF PATHOGENS, MICROBIAL CONTAMINANTS AND/OR CONSTITUENTS
Examiner:	Brenda G. Brumback
Art Unit:	3991
Attorney Docket No.:	66090-008US2
Date:	31 January 2011

Attn: Mail Stop "Inter Partes Reexam"
Central Reexamination Unit
Commissioner for Patents
P. O. Box 1450
Alexandria VA 22313-1450

DECLARATION OF JAMES BYRON UNDER 37 C.F.R. § 1.132
(IN SUPPORT OF RESPONSE UNDER 37 C.F.R. § 1.111)

Madam:

I, Mr. James Byron, being duly sworn, say:

1. I am familiar with the above-identified patent application, and am aware that it discloses and claims novel methods comprising sampling, testing, and validating test lots for microbial contamination. This declaration is to corroborate the declaration of M. Samdapour concerning skepticism amongst clients and others in the art for the validity, accuracy and safety of Dr. Samadpour's method. As such, according to 37 C.F.R. § 1.116(e), this declaration is necessary to respond properly to the action closing prosecution.

2. I am familiar that the detailed claimed method comprises: collecting a plurality of

portions from each of a plurality of test lots, the test lots each comprising an assemblage of one or more specimens, wherein each test lot is separately sampled by taking said plurality of portions thereof; combining the collected plurality of portions corresponding to each of the separate test lots to provide a corresponding set of separate test lot samples, wherein each separate test lot sample is attributed to a particular corresponding separate test lot; incubating the set of separate test lot samples under conditions suitable to allow levels of a target microbial agent or organism that is present in one or more of the separate test lot samples to reach detectable levels and become uniform, or substantially uniform, throughout the respective one or more separate test lot samples, to provide a set of separate test lot samples enriched for the target microbial agent or organism; removing portions of each enriched separate test lot sample, and combining the removed portions to provide a modular composite sample; and testing of the modular composite sample, using a suitable detection assay, for the target microbial agent or organism, wherein when such testing is negative all of said separate test lot samples are validated, and wherein when such testing is positive, individual separate test lots may nonetheless yet be validated by further testing of a portion of the respective enriched separate test lot sample using the same or a more sensitive protocol and obtaining a negative test result.

3. As an executive in the food industry I had been a competitor of IEH from November 2005 until I joined IEH in February 2010. From 2005 until June 2009, I was Executive Director of Sales for SDI – a biotech company and manufacturer of food safety test kits with annual food safety test kits sales of $5 million. From June 2009 until January 2010, I was Vice-President of Sales & Marketing for Food Safety-Net Services of San Antonio Texas. I currently serve on the American Meat Institute Scientific Advisory Committee and I am involved in various food industry trade associations. At IEH my role is Vice-President of Technology Transfer & International Business. Prior to my involvement in the food industry, my career advanced through positions of increasing responsibility serving 25 years working in the industrial chemicals, electric utilities, and Nuclear power industries. Previous employers include Nalco Company of Naperville IL and The President & Fellows of Harvard University, Cambridge Massachusetts. My detailed curriculum vitae is attached hereto as APPENDIX A.

4. While in my Executive Director of Sales at SDI one of my responsibilities was to be aware of all emerging technology associated with food testing, especially new technology being developed either by food testing laboratories or by competitor test kit manufacturers. In December 2007, during my tenure with SDI, I received a phone call from Dr. Samadpour, IEH to inform me of his patent application related to his inventive testing method, wet modular

composite pooling, and to offer to make the technology available to SDI under a commercial agreement. At the time I took the call and related the information to a Marketing Manager responsible for SDI Food Safety business, Dr. Orla Cloak, PhD Microbiology, University of Ulster, BSc(hons) Major Microbiology, Minor Chemistry University College Dublin, for further review. At the time, the 'idea' of wet modular composite pooling was not an obvious testing strategy, in fact it was scoffed by Dr. Cloak when presented by IEH as she believed that detection limits would be compromised because of the small amount of enriched sample from a 'positive' sample when combined with 'negative' samples would lead to false negative results. Because of the lack of understanding related to the potential benefits to be gained from the technology the opportunity was ignored. Training of new food safety kits sales people at SDI included discussion of the very small amount of enriched sample used in PCR analysis and it was stated that the small aliquot of sample made it possible to 'miss' or 'not transfer' E. coli cells to thermocycle step and hence a false negative would be possible. This training was provided by Dr. James Stave, Chief Science Officer, VP Research & Development, Ph.D. in Microbiology from the University of Maryland and his B.S. in Biology from Michigan Technological University. It was considered by SDI scientists that the perceived challenges of PCR technology would be further challenged by dilution associated with wet modular composite pooling.

5. Since 2007, the industry has learned that testing using Dr. Samadpour's Wet modular composite pooling technique does not compromise or reduce the accuracy of testing. Wet modular composite pooling *increases* the number of lots that are tested, by reducing (by a factor of up to 5) the 'lot size' of product tested and thus incurs an increase in the associated testing cost. The outcome is that while the test lots are smaller (1/5), and more lots are tested (5x), increased cost of testing is insignificant relative to the overall savings from having to destroy or divert less total product. This breakthrough has significant economic impact for the food production industry, both in the value of product preservation (increase number of lots, smaller lot sizes, reduces product loss from positive test results), and in the additional benefit of allowing up to 5 times (500%) more testing of product while only increasing the total cost of testing by as little as ~15%.

6. The following is a summary of my accounting of the industry response to Dr. Samadpour's discovery, IEH's development of validation data related to the discovery, and the response from the food industry, food testing laboratories, and food test kit manufacturers.

7. The IEH discovered advantages from wet modular composting pooling methods

were initially met with strong skepticism and open ridicule from the food testing market, including but not limited to trade associations, food manufacturers, food testing laboratories, and test kit manufacturers. The idea of making one single composite from several enrichments was a complete departure from industry practice and not a single test kit or food testing laboratory was using this approach. I experienced the skepticism and obvious disapproval of Dr. Samadpour's inventive technique first hand due to my position at both a leading test kit manufacturing company and at a leading food testing laboratory.

8. In fact, prior to IEH developing this wet modular composite pooling technology, the idea of such wet pooling of such enriched samples (each corresponding to a separate underlying test lot) was not used within the food testing industry. As recently as January 2008, the prevailing industry behavior was to challenge and cast doubt upon the science behind the IEH methods, a behavior that is still perpetuated to this day among some personalities within the industry. Eventual recognition of the scientific validity of Wet Pooling was achieved only after years of effort by IEH wherein they were able to demonstrate the superiority of wet modular composite pooling, satisfying both industry leaders and government regulators that the method had acceptable sensitivity, specificity, and limits of detection as compared with conventional methods.

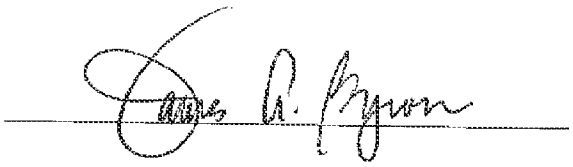
9. Food manufacturers that have adopted the use of Wet Modular Pooling as provided within US Patent 7534584 have benefitted from substantial economy improvement in overall costs (less product being destroyed/diverted) in addition to efficiency gains in their production operation and enhanced food safety program performance (statistically more robust testing).

10. It has long been my personal view, as both a former-executive for a Test Kit manufacturing company and as a former-executive in a Food Safety Testing Laboratory, that reaching a commercial agreement with IEH to gain access to this protected inventive technology is important for food safety in addition to being a wise business decision for a test kit or food testing laboratory company. Not surprising, and fully consistent with the novelty and efficacy of IEH's modular composite pooling methods, there are those in the industry that want to practice this method, but wish that it wasn't patented (e.g., the present Third Party Requestor in this re-examination). In fact, I am aware that in 2009, representatives from particular entities held a meeting in Colorado in an attempt to coordinate efforts to avoid legal complications associated with implementing modular wet composite pooling. While this is understandable, this merely illustrates not only the novelty and non-obviousness of the method, but also the importance and

promise of the method, and in no way detracts from the validity of U.S. Patent 7,534,584. This is precisely the type of invention that I understand the patent laws are designed to protect.

11. I am aware that in 2009 some participants in the industry adopted a practice of copying and using the technology encompassed within US Patent 7534584 without the benefit or protection of having a commercial agreement in place with the patent owner; and without the permission or consent of the patent owner. I also know, from public documents that Food Safety-Net Services of San Antonio in 2009 copied Dr. Samadpour's inventive technique by engaging and performing validation studies of Wet Pooling for the Vidas test kit manufactured by BioMerieux (the Third Party Requestor in this inter partes re-examination).

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and may jeopardize the validity of the application or any patent issuing thereon.



James Byron

31 January 2011

APPENDIX A
CURRICULUM VITAE

JIM BYRON

405 Trolley Way, West Chester, PA 19382
jimbyron828@gmail.com 484.356.7283

Business professional effective in leading organizational re-direction strategic planning, including 'boots on the ground' leadership and coaching skills used to establish and lead a sales & marketing organization in a challenging, technical and growth driven environment. As a strong collaborator, this candidate possesses a creative talent for establishing measurable goals and metrics that inspire loyalty and high performing professionalism in teams. Consistent history of team leadership delivers results that exceed (or reverse negative) established revenue and profit trends while meeting business objectives for team development and business growth. Proven track record as an effective Contributor, Leader, Coach, Teacher, Manager and Professional Salesperson are indicators of future performance in your company.

Professional Experience

IEH Laboratories & Consulting, Seattle Washington – February 2010 to Present

Vice President of Technology Transfer & International Business - Consulting directly with the company owner, responsible for strategy formulation and business plan execution to support company growth goals exceeding 30% CAGR. Directly engaged within the market to facilitate plan execution, collaborating with IEH organization at the "C" level to establish performance metrics and facilitate alignment of company resources.

Food Safety Net Services, San Antonio Texas – July 2009 to February 2010

Vice President of Sales & Marketing - Food Safety Net Services is a leading provider of laboratory testing, consulting, auditing and specialized services to the food industry; operating a nationwide network of strategically located laboratories in six US cities. Exceptionally well networked and recognized as a leader within the industry, VP of Sales & Marketing is responsible for developing and driving impactful marketing strategies, including development of key client and strategic supplier-partner relationships. Responsible for leading company into new market segments and increasing compound annual growth rate to exceed 30%.

Strategic Diagnostics Inc, Newark Delaware – December 2005 to July 2009

Executive Director of Sales

- Global accountability for 4 diverse sales teams serving focus markets of Antibodies Life Science and Food Safety, in addition to Water Quality & Environmental, and Agricultural markets
- 100% retooling of company sales organization and sales management processes including hiring, metrics for performance evaluation, recognition programs, and direct coaching and development of selling skills

- Led & Managed reversal of long established low/no/negative sales trends; 2007 sales exceed $27 million
- Revenue Growth in focus markets 5.7% (2006), 27.7% (2007)
- Gross Profit Increased 2.99% (2006) and 18.84% (2007)

Nalco Company, Naperville Illinois – January 1990 to November 2005

2004-2005 **National Accounts Executive**

- Closed major deals (multi-year/multi-million dollar) including GSK, Exelon Energy, Alliant Energy
- Directed local sales/service resources to assure 100% post-sale customer satisfaction

2002-2004 **Services Development Manager, North America**

- Key Contributor to Strategic Planning associated with Global Corporate Reorganization
- "Change Leader" for Corporate Re-Organization – Led implementation of global sales/service restructuring
- Reorganization transitioned $440 million dollars annual US sales revenue to new business model
- Established 8 direct report managers in US geographic regions with >150 individual contributors
- Success became the model for transitioning worldwide organization impacting > $2.7 billion dollars

2001-2002 **District Sales Manager, St. Louis Missouri**

- 16 direct sales reports, $13.5 million dollar annual sales, 10 states
- 2001 Revenue Increase 8%, 2002 Revenue Increase 13%
- 2001 Gross Margin Improvement 4%, 2002 Gross Margin Improvement 9%

1997-2001 **Area Sales Manager, Chicago Illinois**

- Revitalized poorly performing area to increase sales from $3.6 to $5.8 million in 3 years (61% growth)
- Increased workforce retention, reduced representative turnover rate from 72% to <16%
- Business focus prioritizes personnel development, customer satisfaction, sales growth, margin improvement.
- Personnel responsibilities include hiring, promotion, terminations, performance appraisals, policy & salary administration, coaching & training sales personnel.

1990-1997 **Account Manager, Minneapolis Minnesota**

- Election to Nalco Chairman's Club Membership, Salesman of the Year 1995
- Percent Sales Increase Club 1992, 1993, 1994, 1995, 1997, 1998, 1999, 2000, 2001
- Total Dollar Sales Increase Club 1992, 1993, 1994, 1995, 1997, 1998, 1999, 2000, 2001
- New Accounts Club 1991, 1993, 1994, 1995, 1997, 1998, 1999, 2000, 2001
- Council of Sales Professionals 1995, 1998, 1999
- Focus industries include Nuclear & Fossil Power, Chemical Production, Food Production, Paper, Refining, and Aluminum & Steel.

President & Fellows of Harvard University, CMC, Boston Massachusetts – 1983 to 1990

1985-1990 **Engineering Coordinator**

- Developed, Implemented, & Managed systems for project management including schedule, weekly/monthly status/progress publication, supervision of trades people (electricians, mechanics, laborers), facilitated advancement of 'stuck' milestones to assure successful project completion
- Major projects included:
  - $ 15 million underground electric utility distribution installation

- $ 4 million steam boiler upgrade/retube & water polishing projects
- $ 2 million diesel generator protection project – lube oil management

1983-1985 Maintenance Coordinator

Associations & Professional Development Programs

- AMI American Meat Institute, Scientific Advisory Council
- Leadership & Coaching: Situational Leadership, Delivering Customer Advantage through Advanced Coaching, Becoming a Leader Manager
- Sales: Target Account Selling, SPIN Selling, Versatile Sales Person, Counselor Sales Person (Certified Trainer)
- Quality: Six Sigma Qualitrak Quality Training

Strategic Account Manager Association Annual Conference, SAMA

Compete Through Service Symposium, Arizona State University, WP Carey School of Business

Commonwealth of Massachusetts Licensed Boiler Operator, Fireman 2nd Class

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Education

Boston College, Chestnut Hill Massachusetts. B.A., Business Administration, 1989, Cum Laude, AΣN.

Journal of Applied Microbiology 2003, 95, 1016–1025 doi:10.1046/j.1365-2672.2003.02070.x

Evaluation of the use of pooled serum, pooled muscle tissue fluid (meat juice) and pooled faeces for monitoring pig herds for *Salmonella*

R.H. Davies[1], P.J. Heath[2], S.M. Coxon[2] and A.R. Sayers[3]

[1]Department of Food and Environmental Safety, Veterinary Laboratories Agency (VLA) Weybridge, Surrey, [2]Laboratory Testing Division, VLA Bury St Edmunds, Suffolk, and [3]Epidemiology Department, VLA Weybridge, Surrey, UK

2003/0005: received 27 March 2003, revised and accepted 3 July 2003

ABSTRACT

R.H. DAVIES, P.J. HEATH, S.M. COXON AND A.R. SAYERS. 2003.

Aims: Monitoring for *Salmonella* in slaughter pigs is important to enable targeted control measures to be applied on problem farms and at the abattoir. The aim of this study was to determine whether pooled serum and meat juice could be used to identify finishing pig herds with a high prevalence of infection.

Methods and Results: Samples of meat juice, serum, caecal contents, carcase swabs and pooled faeces from pig pens were taken from 20 commercial pig finishing farms and comparisons were made between the results of *Salmonella* culture, individual ELISA tests on serum and meat juice and pooled samples of serum and meat juice. *Salmonella* was isolated from samples from 19 of 20 farms. None of the ELISA tests showed a statistically significant correlation with caecal carriage of *Salmonella* or contamination of carcases. Serum mean optical density (O.D.) from pools of five, 10 or 20 sera showed a significant correlation with the *Salmonella* status of farm pen faeces. All pooled serum O.D. and sample/positive control ratio results correlated significantly with the results of the conventional individual sample ELISA. There was a statistically significant correlation between the incidence of *Salmonella* in farm pen pooled faeces and the prevalence of *Salmonella* in caeca of slaughter pigs.

Conclusions: The results show a generally poor correlation between serological and bacteriological results but pooled serum or meat juice samples could be used as a cheaper substitute for serological screening of farms for *Salmonella* than individual samples.

Significance and Impact of the Study: The availability of a cheaper test should allow the costs of *Salmonella* monitoring of pig farms to be reduced or allow more regular testing to enhance the designation of farm *Salmonella* risk status.

Keywords: culture, ELISA, pigs, pork, *Salmonella*, surveillance.

INTRODUCTION

In many countries, especially those in which significant quantities of raw, smoked or lightly cooked ground pigmeat products are consumed, pork is a source of a substantial proportion of human *Salmonella* infections (Berends *et al.* 1998; Anon. 2002). The situation in the UK is less clear, but some outbreaks, including a small number of deaths, have been associated with contaminated pigmeat (Maguire *et al.* 1993; Smerdon *et al.* 2001) and newly identified phage types of *S.* Typhimurium, such as U310, have been specifically linked with pigmeat products (Ward and Threlfall 2001). An abattoir survey has shown that at least 23% of pigs are carrying *Salmonella* in their caeca at slaughter (Davies *et al.* 2001).

Theoretically the increasing vertical integration of the pig industry and centralization of slaughter in larger abattoirs,

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where specialized quality assurance and hygiene control operations are in place, should reduce the opportunity for dissemination of *Salmonella* infection and contamination. Unfortunately, poor monitoring of farm inputs, increasing farm size, dietary changes and lack of specific *Salmonella* controls has meant that *Salmonella* has become more of a problem for the modern integrated pig industry than in the smaller independent units it has replaced (Blaha 2001).

The starting point for control is monitoring to define the extent and distribution of infection. Bacteriological monitoring gives the best indication of the distribution of *Salmonella* on farms and facilitates the introduction and monitoring of control measures (Bager and Baggesen 1993), but this must be carried out sufficiently rigorously to be meaningful, which may be expensive. Serological testing is less expensive for each individual sample, whereas bacteriological samples can be pooled in composites of up to 20 subsamples. Pooled bacteriological samples have been very effective for monitoring large groups of poultry (Kradel and Miller 1991; Aho 1992), but are more difficult to achieve in a representative way for routine use on pig farms. Serology may provide a more sensitive indication of persistent pig herd infection than limited bacteriology (Wiuff *et al.* 2002).

Following large outbreaks of *Salmonella* in humans and problems exporting contaminated pigmeat, a model *Salmonella* control policy was set up in Denmark (Mousing *et al.* 1997). This was based on serological monitoring using a Mix-ELISA test capable of detecting groups B and C *Salmonella* serotypes, which are predominant in pigmeat production (Nielsen *et al.* 1998).

This report describes a series of experiments designed to evaluate the use of pooled serum and meat juice samples in comparison with the conventional ELISA test carried out on individual samples and bacteriological results from caeca, carcase swabs and pooled pen faeces from the farm of origin of the batches of pigs tested. The objective of this study was to determine whether pooled serum or meat juice could act as an effective indicator of herd *Salmonella* infection status and so offer the possibility of providing an effective monitoring programme at minimum cost.

MATERIALS AND METHODS

Sampling methods

Twenty pig herds were chosen at random from the regular suppliers of two abattoirs. The previous *Salmonella* status of these herds was unknown. From the 20 herds a total of 430 serum, 421 juice, 421 carcase swab, 418 caecal and 369 pooled pen faecal samples were collected for testing. The aim was to attempt to test and compare samples from at least 20 pigs from each farm and 20 (or maximum available number if <20) pooled faeces samples from finishing pens on the farm of origin. There was some variability in this resulting from missing or inappropriate samples at slaughter and the sampling of some extra pigs occurred in an attempt to compensate for this.

Sampling on farm. Pigs to be slaughtered and sampled at abattoir the following day were identified on each farm and pooled pen faeces samples taken.

Each pen faeces sample (25–30 g) consisted of approx. 15 'pinch-sized' samples taken from the surface of fresh faecal material in separate parts of the dunging area before mucking out. If large pens had more than one dunging area each area was sampled. Pen samples were collected by hand using a new disposable plastic glove for each pen sample. One pooled pen sample was taken per pen, however if the pen contained over 30 pigs then an additional sample was taken for each additional 30 pigs. The faecal samples were submitted to the laboratory for examination within 3–4 h of collection and cultural examination was undertaken on the same day of receipt.

Sampling at abattoir. At the sticking point an individual, unique tag was attached to the ear of each pig to be sampled. The tag numbers were recorded on a working sheet for transfer to the submission form after sampling was complete, this ensured that the four samples to be taken could be identified to each individual pig.

Blood samples. Blood samples were taken into universal bottles when the pigs were bled out, labelled with each pig's unique identification number and allowed to stand at room temperature for the blood clot to retract. At the laboratory a serum sample was removed from each blood and stored at −20°C until batch tested.

Carcase swabs. Carcase swabs were taken from the uniquely identified pigs before entry to the chiller. A single gauze swab was used to rub vigorously over a 10 × 10 cm square of skin at three sites on each pig – the belly, ham and jowls, according to a Danish adaptation of a US protocol (Sørensen *et al.* 2001). The swab was returned to a uniquely identified sterile container and gloves were changed before taking each carcase swab sample.

Muscle samples. A sample of diaphragm muscle was taken from each pig to fill the whole of the top of a labelled meat juice sample tube. Each tube was closed and placed in an insulated sample box. At the laboratory the samples were frozen, then thawed. Meat juice was taken into uniquely identified tubes and stored at −20°C until batch tested.

Caecum. For each pig the caecum was ligated and removed from the carcase into a labelled mini-grip bag.

National Beef Ex. 1007
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The carcase swab, neck muscle and caecum samples were transported to the laboratory in an insulated container with ice blocks. At the laboratory 25 g of caecal contents was aseptically removed for pre-enrichment culture.

Laboratory tests

Culture for Salmonella. The pooled pen faeces samples, the carcase swabs and the individual pig caecum contents were added to buffered peptone water (BPW, Merck, Poole, UK) and incubated at 37°C for 16–20 h. After incubation 0·2 ml of the BPW broth was dispensed into the depth of a semisolid Diassalm (Merck) plate and all inoculated plates were incubated at 41·5°C for 24 h. On day 3 the Diassalm culture plates were examined for suspect *Salmonella* growth and where this was seen a sample of the agar was inoculated onto a Rambach agar (Merck) plate, which was incubated at 37°C for 18–24 h. After incubation the Rambach plates were examined for suspicious *Salmonella* colonies, which were confirmed serologically and biochemically. A subculture of each confirmed *Salmonella* isolate was submitted to VLA Weybridge, Bacteriology Department, for full serotyping and phage typing where applicable.

Salmonella *ELISA*. Serum and meat juice samples were tested by the Guildhay VetSign *Salmonella* ELISA Kit (VP020; Guildford, UK), which incorporates lipopolysaccharide (LPS) antigens from the groups C_1 and B salmonellas: *S.* Choleraesuis and *S.* Typhimurium. Tests were conducted according to the manufacturer's instructions. Briefly, serum and meat juice samples from individual farms were tested individually and as pools of samples containing five, 10 and 20 individual samples. For making the pools, 100 μl of each serum or juice was pipetted into a single tube of their respective pools of five and mixed thoroughly. From these four pools of five, 100 μl was pipetted from each of two pools to make the two pools of 10 and mixed thoroughly. From each of the two pools of 10 samples, 100 μl was taken into another tube to make the pool of 20 and mixed thoroughly. Where the herd sample batch size varied, (range: 19–22), this resulted in a fourth pool of four to seven samples, one of nine to 12, and a single final pool of 19 to 22. After thorough mixing of the individual and pooled samples they were tested by ELISA. Individual and pooled samples of serum and meat juice were diluted 1 : 400 and 1 : 30, respectively, according to kit instructions, before testing. Positive results were those, which gave a ratio of sample O.D. to positive control O.D. [sample/positive (SP) ratio] of 0·25 or greater.

Statistical analyses. All analyses and correlations were made on the basis of using the herd as a unit rather than individual animals. The correlations and descriptive statistics were calculated with STATISTICA (StatSoft, Inc. 2001).

RESULTS

Table 1 shows a comparison of mean optical density (O.D.) and sample/positive (SP) ratio readings from individual and pooled serum samples from the 20 sampled herds. In total, 430 serum samples were tested individually and in pools of five, 10 and 20. The mean O.D. for the individual tests carried out on serum was 0·18 (range: 0·08–0·48) and the SP was 0·23 (range: 0·02–0·72). A total of 109 of the 430 samples (25·3%) (range: 0·06–0·82) were positive for *Salmonella* antibodies. These results would place seven herds in the equivalent of Danish category 1, 11 herds in category 2, and two herds in category 3, i.e. the worst *Salmonella* status herds requiring serious remedial action.

The mean results from the various poolings were broadly equivalent to the mean values of individual results. The mean O.D. for five pools was 0·21 (range: 0·07–0·82) and the SP was 0·33 (range: 0·02–1·18). For the 10 pools the mean O.D. was 0·21 (range: 0·08–0·83) and the mean SP was 0·33 (range: 0·05–1·19) and in the case of the pools of 20 the mean O.D. was 0·22 (range: 0·07–0·96) and the mean SP was 0·37 (range: 0·07–1·39).

In addition, an attempt was made to allocate herds, on the basis of ranges of 20s, into categories <0·1, 0·1–0·4 and >0·4 SP 20, as these categories appeared to correlate with low, moderate and high prevalences of *Salmonella* in caeca or pooled faeces from herds identified in bacteriological tests (Table 1). This classification produced only five herds in the best category, seven herds in the middle category and eight herds in the worst category. In terms of comparisons with meat juice ELISA results a worst farm mean SP 20 result of >0·5 would produce the same two category 3 equivalent herds. A higher SP 20 cut-off, eg. 1·0, could be chosen if a smaller category 3 equivalent was required.

The results relating to serology carried out on meat juice are presented in Table 2. In total, 421 samples were collected and all yielded sufficient tissue fluid for testing as individuals and as pools. The mean O.D. for the individual tests carried out on meat juice was 0·35 (range: 0·07–0·70) and the SP was 0·41 (range: 0·03–0·87). A total of 182 of the 421 (43·2%) samples gave positive meat juice ELISA (MJE) results. This would place only two (10%) farms in category 1, eight (40%) farms in category 3 and 10 (50%) farms in category 2, if the Danish categorization was applied.

The mean results from the various meat juice poolings were slightly higher than those relating to individual samples. The mean O.D. for individual meat juice was 0·35 and the SP 0·41. This compared with 0·45/0·48 for 'five pools', 0·47/0·51 for '10 pools' and 0·46/0·48 for '20 pools'.

Table 3 shows the results of bacteriological testing of carcase swabs, caeca and pooled pen faeces. A total of 442

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Table 1 *Salmonella* ELISA for pig serum from 20 farms: mean results by farm

	Individual sera					Pooled sera							
						5s		10s		20s		IS%	SP 20s
Herd ref.	O.D.	SP	*N*	*N* +ve	% +ve	O.D.	SP	O.D.	SP	O.D.	SP	cat.	cat.
P0134	0·12	0·12	22	3	13·6	0·12	0·17	0·12	0·15	0·12	0·17	≤17%	0·1–0·4
P0135	0·18	0·21	22	5	22·7	0·19	0·20	0·20	0·22	0·18	0·18	18–50%	0·1–0·4
P0182	0·09	0·10	22	1	4·5	0·09	0·08	0·11	0·17	0·08	0·05	≤17%	<0·1
P0183	0·12	0·26	21	7	33·3	0·13	0·26	0·14	0·30	0·18	0·47	18–50%	>0·4
P0189	0·12	0·11	22	4	18·2	0·15	0·25	0·13	0·19	0·16	0·28	18–50	0·1–0·4
P0193	0·08	0·02	19	0	0·0	0·09	0·08	0·10	0·08	0·07	0·02	≤17%	<0·1
P0217	0·40	0·53	22	15	68·2	0·40	0·56	0·47	0·66	0·39	0·55	>50%	>0·4
P0276	0·23	0·37	22	9	40·9	0·23	0·66	0·28	0·86	0·32	1·05	18–50%	>0·4
P0277	0·22	0·33	22	8	36·4	0·22	0·63	0·21	0·59	0·23	0·68	18–50%	>0·4
P0306	0·09	0·03	22	1	4·5	0·07	0·02	0·09	0·08	0·10	0·09	≤17%	<0·1
P0320	0·26	0·36	20	10	50·0	0·28	0·33	0·30	0·37	0·35	0·44	18–50%	>0·4
P0328	0·08	0·03	22	0	0·0	0·08	0·05	0·09	0·07	0·09	0·08	≤17%	<0·1
P0329	0·08	0·03	22	0	0·0	0·08	0·05	0·08	0·05	0·09	0·07	≤17%	<0·1
P0353	0·21	0·26	20	6	30·0	0·19	0·21	0·21	0·24	0·19	0·20	18–50%	0·1–0·4
P0382	0·15	0·15	20	2	10·0	0·13	0·11	0·14	0·12	0·15	0·14	≤17%	0·1–0·4
P0399	0·20	0·23	22	7	31·8	0·18	0·45	0·18	0·47	0·19	0·50	18–50%	>0·4
P0400	0·18	0·19	22	6	27·3	0·21	0·59	0·18	0·47	0·18	0·46	18–50%	>0·4
P0466	0·48	0·72	22	12	54·5	0·82	1·18	0·83	1·19	0·96	1·39	>50%	>0·4
P0498	0·21	0·26	22	4	18·2	0·29	0·36	0·21	0·23	0·22	0·26	18–50%	0·1–0·4
P0516	0·15	0·23	22	9	40·9	0·16	0·39	0·17	0·14	0·22	0·32	18–50%	0·1–0·4
Overall mean result	0·18	0·23	430	109	25·3	0·21	0·33	0·21	0·33	0·22	0·37	NA	NA

Key: IS, individual sample; Herd ref., pig herd reference code; O.D., optical density; SP, sample/positive control O.D. ratio; *N*, no. samples tested; *N* +ve, no. samples positive; % +ve, percentage of samples positive; 5s, samples tested as pools of five individual samples; 10s, samples tested as pools of 10 individual samples; 20s, samples tested as pools of 20 individual samples; IS% cat., categorization of herds according to Danish classification: ≤17% = category 1, 18–50% = category 2, >50% = category 3; SP 20s cat., categorization by SP level of pool of 20 individual samples: <0·1 = theoretical lowest *Salmonella* risk category, 0·1–0·4 = theoretical medium *Salmonella* risk category, >0·4 = theoretical highest *Salmonella* risk category.

carcase swabs were taken of which seven [1·7% (range: 0–9·1%)] were positive for *Salmonella*, all *S.* Typhimurium. The positive carcase swabs largely, but not exclusively, came from herds with higher numbers of caecal positives.

A total of 420 caecal samples were tested and 102 [24·3% (range: 0–65·0)] contained *Salmonella*. About 17·4% of samples contained *S.* Typhimurium and other serotypes obtained were *S.* Derby, *S.* Reading, *S.* Newport and *S.* Indiana. One farm had *S.* Agona in pen samples but this was not found in the 20 caecal samples examined and one farm had *S.* Enteritidis. A wide range of *S.* Typhimurium phage types (DT104, 208, 193, U308A, U310, U302, untypable) were found both in caeca and in pooled pen faeces samples.

In only one farm no *Salmonella* was found in either caecal or farm pen samples. Serum and meat juice from this farm was also negative by ELISA.

A total of 369 samples of pooled pen faeces were taken from the farms of which 121 [32·8% (range: 0–75·0)] contained *Salmonella* and 23·6% (range: 0–75·0) contained *S.* Typhimurium. There was a good correlation between the serotypes and phage types found in caeca and carcases of pigs with those found on farm.

Table 4 shows a correlation matrix which displays the various correlations between serology and bacteriology. None of the serological tests, including the standard serum and meat juice ELISA results (individual serum, individual juice) showed a statistically significant correlation with either carcase contamination or caecal carriage of *Salmonella*. O.D. readings of five, 10 and 20 pools of serum (5s, 10s and 20s) showed a significant correlation with the percentage of positive pen faeces whereas those from the standard serum and meat juice ELISA did not. There was no such correlation for pooled meat juice, largely because the meat juice readings were higher than serum so were less 'specific' when compared with bacteriology. This is likely to be a feature of the dilution factor applied to serum and less dilution of serum or greater dilution of meat juice should result in closer correlations.

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Table 2 *Salmonella* ELISA for pig meat juice from 20 farms: mean results by farm

	Individual meat juice					Pooled meat juice							
						5s		10s		20s		IS%	SP 20s
Herd ref.	O.D.	SP	*N*	*N* +ve	% +ve	O.D.	SP	O.D.	SP	O.D.	SP	cat.	cat.
P0134	0·18	0·26	21	7	33·3	0·27	0·25	0·28	0·26	0·08	0·00	17–50%	<0·1
P0135	0·24	0·28	21	6	28·6	0·28	0·32	0·30	0·34	0·30	0·35	17–50%	0·1–0·4
P0182	0·07	0·03	22	0	0·0	0·09	0·01	0·08	0·00	0·11	0·03	≤17%	<0·1
P0183	0·18	0·13	20	5	25·0	0·28	0·19	0·19	0·11	0·17	0·09	17–50%	<0·1
P0189	0·37	0·38	21	10	47·6	0·53	0·43	0·73	0·62	0·76	0·64	17–50%	>0·4
P0193	0·18	0·23	19	7	36·8	0·23	0·20	0·23	0·19	0·22	0·18	17–50%	0·1–0·4
P0217	0·48	0·70	22	16	72·7	0·58	0·76	0·60	0·80	0·57	0·75	>50%	>0·4
P0276	0·55	0·69	22	16	72·7	0·99	0·88	0·92	0·81	0·83	0·73	>50%	>0·4
P0277	0·62	0·79	22	12	54·5	0·95	0·84	1·04	0·93	0·92	0·81	>50%	>0·4
P0306	0·31	0·48	22	9	40·9	0·44	0·46	0·51	0·55	0·32	0·30	17–50%	0·1–0·4
P0320	0·32	0·65	20	12	60·0	0·44	0·67	0·42	0·64	0·42	0·64	>50%	>0·4
P0328	0·19	0·16	22	5	22·7	0·15	0·20	0·14	0·18	0·17	0·24	17–50%	0·1–0·4
P0329	0·10	0·05	22	0	0·0	0·09	0·08	0·09	0·07	0·08	0·06	≤17%	<0·1
P0353	0·26	0·50	20	13	65·0	0·29	0·40	0·30	0·42	0·29	0·40	>50%	0·1–0·4
P0382	0·19	0·22	19	4	21·1	0·19	0·23	0·18	0·20	0·17	0·19	17–50%	0·1–0·4
P0399	0·63	0·51	21	14	66·7	0·65	0·55	0·71	0·62	0·74	0·64	>50%	>0·4
P0400	0·70	0·57	22	16	72·7	0·65	0·55	0·63	0·54	0·82	0·72	>50%	>0·4
P0466	0·64	0·87	19	12	63·2	1·01	1·68	1·12	1·89	1·14	1·92	>50%	>0·4
P0498	0·27	0·32	22	8	36·4	0·26	0·36	0·31	0·39	0·30	0·35	17–50%	0·1–0·4
P0516	0·51	0·41	22	10	45·5	0·63	0·53	0·64	0·55	0·72	0·62	17–50%	>0·4
Overall mean results	0·35	0·41	421	182	43·2	0·45	0·48	0·47	0·51	0·46	0·48	NA	NA

For *Key* see Table 1.

Figure 1 shows a scatter plot comparing serum O.D. 10s (which had the highest correlation coefficient) with pen faeces results. This shows that although the majority of farms lie close to the line a small number of substantial outliers means that only approx. 25% of the variation in one variable can be directly attributed to the other.

Table 5 shows the correlation between pooled serum and meat juice and the percentage positive individual samples by standard ELISA. All of the correlation co-efficients are significant ($P < 0·05$), especially serum SP with serum percentage of positive and meat juice OD with juice percentage of positive. An example correlation between pooled serum (SP 20) with serum percentage of positive is shown in Fig. 2.

Figure 3 shows a correlation between the percentage of positive pen faeces and the percentage of positive caeca. For all *Salmonella* the correlation co-efficient (r) is 0·51 ($N = 20$, $P = 0·022$) and for *S.* Typhimurium $r = 0·5$ ($N = 20$, $P = 0·026$). Both of these are significant correlations but the scatterplot shows there are substantial numbers of outliers.

The correlation coefficient between the mean farm O.D. results for individual unpooled serum samples and individual unpooled juice was 0·61 ($P = 0·005$) with 95% confidence interval (0·22–0·83). For the farm mean SP values it was 0·77 ($P < 0·001$) with 95% confidence interval (0·50–0·90). Scatterplots are shown in Figs 4 and 5.

DISCUSSION

The large amount of work carried out in development and operation of the Danish *Salmonella* control policy has shown that a pragmatic approach to categorize herds on the basis of *Salmonella* antibody seroprevalence can be an effective tool for identifying highly infected herds (Mousing *et al.* 1997; van der Wolf *et al.* 2001a), and this approach has been adopted in several other countries worldwide (Ludewig and Fehlhaber 2001) and in other target species (Hoorfar *et al.* 1997; Feld *et al.* 2000).

Although the individual serum or meat juice *Salmonella* ELISA test has a poor correlation with bacteriology on an individual pig basis (Christensen *et al.* 1999; Clouting and Davies 2001; Davies *et al.* 2001; Corrégé 2002) it can be used to flag up herds which are more likely to be in need of improved *Salmonella* control. This can then be confirmed by bacteriological sampling at farm level and a control plan imposed. It is also possible to organize logistic slaughter

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Table 3 Bacteriological results from carcase swabs, caeca and bulk pen faeces for pigs from 20 farms

	Carcase swabs					Caecum					Faeces				
Farm ref.	*N*	*N* +ve	Typh. *N* +ve	% +ve	Typh. % +ve	*N*	*N* +ve	Typh. *N* +ve	% +ve	Typh. % +ve	*N*	*N* +ve	Typh. *N* +ve	% +ve	% Typh. +ve
P0134	22	2	2	9·1	9·1	21	11	8	52·4	38·1	19	14	9	73·7	47·4[c,b,m,j]
P0135	21	0	0	0·0	0·0	21	4	4	19·0	19·0	19	7	7	36·8	36·8[f]
P0182	22	0	0	0·0	0·0	22	0	0	0·0	0·0	20	6	0	30·0	0·0[g]
P0183	21	0	0	0·0	0·0	21	3	3	14·3	14·3	20	2	2	10·0	10·0[c,p]
P0189	20	0	0	0·0	0·0	20	3	2	15·0	10·0	15	1	1	6·7	6·7[f,b,d,e]
P0193	19	0	0	0·0	0·0	19	8	0	42·1	0·0	20	4	0	20·0	0·0[j]
P0217	22	1	1	4·5	4·5	22	7	3	31·8	13·6	15	8	2	53·3	13·3[n,e]
P0276	22	0	0	0·0	0·0	20	8	8	40·0	40·0	20	9	9	45·0	45·0[a,b]
P0277	22	1	1	4·5	4·5	22	4	4	18·2	18·2	20	5	5	25·0	25·0[f]
P0306	20	0	0	0·0	0·0	20	13	13	65·0	65·0	20	5	5	25·0	25·0[f,j,l]
P0320	20	0	0	0·0	0·0	20	6	6	30·0	30·0	19	12	11	63·2	57·9[c,e,n]
P0328	22	0	0	0·0	0·0	22	0	0	0·0	0·0	17	2	2	11·8	11·8[n]
P0329	22	0	0	0·0	0·0	22	0	0	0·0	0·0	20	0	0	0·0	0·0[g]
P0353	20	0	0	0·0	0·0	20	2	2	10·0	10·0	20	15	15	75·0	75·0[c,p]
P0382	19	0	0	0·0	0·0	19	0	0	0·0	0·0	18	1	1	5·6	5·6[c]
P0399	22	0	0	0·0	0·0	22	3	3	13·6	13·6	15	0	0	0·0	0·0[g]
P0400	22	0	0	0·0	0·0	22	9	8	40·9	36·4	17	7	5	41·2	29·4[b,h]
P0466	19	1	1	5·3	5·3	19	7	2	36·8	10·5	16	11	3	68·8	18·8[b,d,j,l]
P0498	22	2	2	9·1	9·1	22	11	5	50·0	22·7	19	10	8	52·6	42·1[c,b,j,n]
P0516	22	0	0	0·0	0·0	22	3	3	13·6	13·6	20	2	2	10·0	10·0[c,e,m,j]
Overall mean results	422	7	7	1·7	1·7	420	102	73	24·3	17·4	369	121	87	32·8	23·6

Key: Salmonella types found in batch at slaughter or on farm: [a]Typh. 104; [b]193; [c]208; [d]U302; [e]U308A; [f]U310; [g]Agona; [h]Enteritidis; [j]Derby; [l]Indiana; [m]Newport; [n]Reading; [p]Typh. Untypable (Typh.= *S.* Typhimurium).

Table 4 Correlations of serum/juice with carcase/caecum/faeces

	Correlations* (% +ve)					
Variable	Carcase	Carcase Typh.	Caecum	Caecum Typh.	Faeces	Faeces Typh.
Individual serum	0·20	0·20	0·14	0·15	0·44	0·29
SOD 5s	0·43	0·43	0·25	−0·01	*0·53*	0·16
SSP 5s	0·34	0·34	0·25	0·12	0·39	0·13
SOD 10s	0·35	0·35	0·22	−0·01	*0·54*	0·15
SSP 10s	0·26	0·26	0·24	0·15	0·43	0·15
SOP 20s	0·32	0·32	0·21	0·02	*0·48*	0·14
SSP 20s	0·23	0·23	0·22	0·17	0·34	0·15
Individual juice	0·10	0·10	0·42	0·40	0·44	0·43
JOD 5s	0·14	0·14	0·31	0·34	0·20	0·13
JSP 5s	0·28	0·28	0·33	0·23	0·43	0·20
JOD 10s	0·18	0·18	0·31	0·31	0·18	0·09
JSP 10s	0·30	0·30	0·33	0·21	0·40	0·16
JOD 20s	0·07	0·07	0·19	0·18	0·12	0·02

*Marked correlations are significant at $P < 0·05$ and are italicized.
S, serum; J, meat juice; O.D., optical density; SP, sample/positive ratio.

based on herd ELISA results and this can help reduce carcase and meat contamination (Swanenburg *et al.* 2001). Recent changes in the Danish interpretation of results have been made in an attempt to increase the sensitivity of the test and weight results so that a closer approximation to the herd infection status at slaughter is obtained (Alban *et al.*

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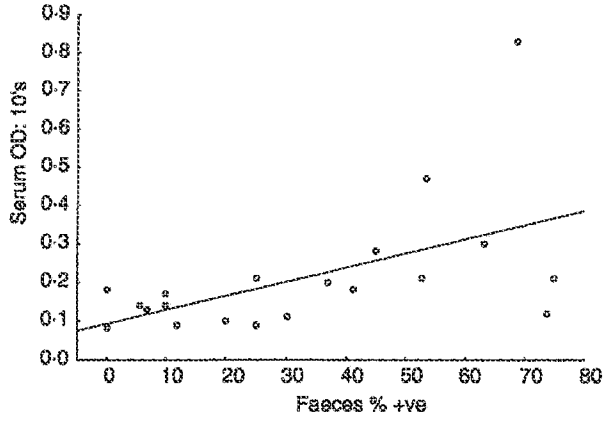


Fig. 1 Scatterplot of serum O.D. (pool of 10) and percentage positive pooled pen faeces

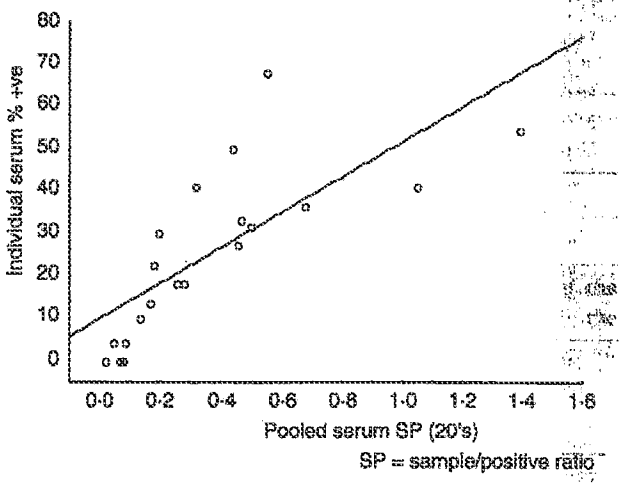


Fig. 2 Scatterplot of individual serum percentage positive compared with pools of 20

Table 5 Correlations of serum/juice pooled results with percentage of positive individual sera

	Correlations of positive individuals	
Variable	Serum (%)	Juice (%)
SOD 5s	0·70	0·51
SSP 5s	0·77	0·69
SOD 10s	0·74	0·52
SSP 10s	0·76	0·67
SOD 20s	0·71	0·49
SSP 20s	0·75	0·64
JOD 5s	0·69	0·77
JSP 5s	0·74	0·71
JOD 10s	0·74	0·75
JSP 10s	0·70	0·68
JOD 20s	0·66	0·75
JSP 20s	0·70	0·68

S, serum; J, meat juice; O.D., optical density; SP, sample/positive ratio.

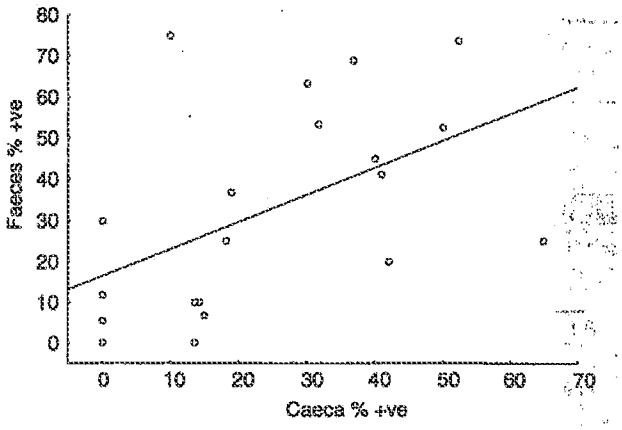


Fig. 3 Correlation between percentage positive farm faeces and positive caeca

2002). With any serological test for enteric bacterial pathogens there will always be problems of sensitivity and specificity; however so, it will never be possible to predict with certainty the likelihood of a batch of pigs carrying or excreting *Salmonella* at slaughter. This means that any controls can only effect a reduction rather than elimination of the problem.

A wide range of *S.* Typhimurium phage types were found both in caeca and in pooled pen faeces samples in the current study. *Salmonella* Typhimurium U310 and U308A appear to be spreading rapidly in the pig industry at present and it will be in pig farmers' best interests to carry out more bacteriological sampling of replacement breeding stock to help identify the sources of these waves of new *Salmonella* strains which sweep through the industry. It is also important to retain some national bacteriological monitoring or the emergence of new potentially zoonotic strains may be missed. Pigs carrying *S.* Enteritidis, a major human pathogen and the number one serotype worldwide, would also not be detected by the ELISA. There is no serological test, which will detect all *Salmonella* serovars (van Winsen *et al.* 2001) so the ELISA test may miss new emerging serovars which lie outside the antigenic detection range. It is likely therefore that *S.* Newport (group C_2/C_3) which is currently causing serious problems in the US may be poorly detected if it became established in pigs. (The single *S.* Newport positive pig gave negative ELISA results in the current study.) Similarly, of the top 10 human serovars

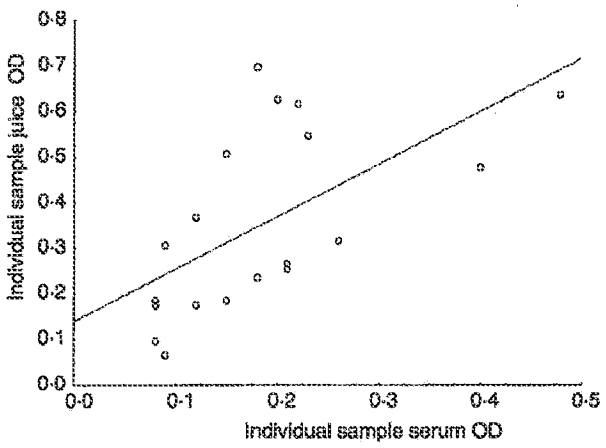


Fig. 4 Correlation between individual mean farm meat juice and serum O.D. results

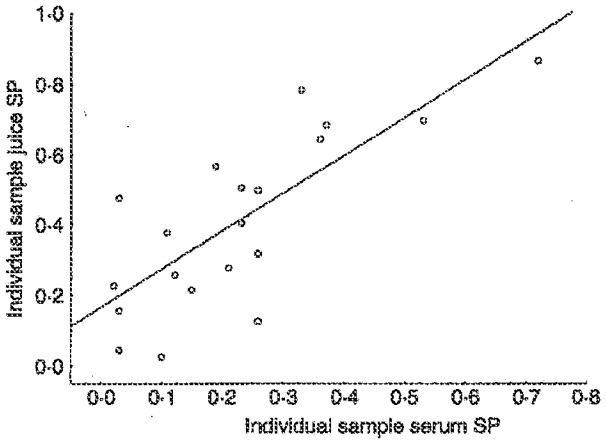


Fig. 5 Correlation between individual farm meat juice sample/positive ratios (SP) and individual serum SP

found in the UK, *S.* Enteritidis would not be detected and *S.* Hadar and *S.* Blockley are likely to be poorly detected. The test can be extended to include additional serogroups but care must be taken to ensure the sensitivity and specificity of the expanded test is not compromised (Proux *et al.* 2000; Clouting and Davies 2001).

Another problem with ELISA testing is the proliferation of tests and the deficiency of validation and harmonization (van der Wolf *et al.* 2001b). In an international ring trial there were considerable differences in the performance of various ELISA test kits and in-house tests (van der Heijden 2001) and continual changes in methodology and interpretation are being made in many of these, which may also effect harmonization between test laboratories (Wiuff *et al.* 2000; Alban *et al.* 2002). The commercial test used in the current study was found in previous unpublished work by the authors to be sufficiently robust for general use provided appropriate quality assurance (QA) procedures are applied. It is recommended that some samples are tested in duplicate (as in this study) to ensure reliability and detection of test errors but single well testing may be acceptable for monitoring trends (Steinbach *et al.* 1999).

Comparisons between the ELISA results for serum and meat juice showed a statistically significant correlation but the variability amongst farms was relatively large. The divergence is greater than that previously found when Danish or in-house ELISAs were used (Nielsen *et al.* 1998; Clouting and Davies 2001). Some of the discrepancy may be due to the dilution rate recommended for use with juice from diaphragm muscle and previous observations have suggested that 1 : 40 may be more appropriate (Carol Clouting, VLA Weybridge, personal communication). There are also some farms where samples appear to diverge widely from expected serum/juice ratios. The reasons for this are unclear but it is possible that factors such as stress and the state of hydration of pigs may have an influence on meat juice ELISA results.

This study has shown that pooling serum or meat juice would be a viable surveillance option. Testing one pooled sample per farm gave results, which compared well with testing 20 individual samples. If an SP category of <0·1 was used to distinguish the 'best herds' and >0·4 to distinguish the worst then there would be four 'best category' herds and nine 'worst category' herds. This would compare with the individual ELISA prevalence and could easily be further categorized such that farms could be ranked in a different way to that currently used in the Danish Scheme. Thus the 1% of herds with highest O.D. 20 results could be selected for further action. Herd designations should preferably be obtained on a 4-month rolling mean basis by taking one bulk sample of 20 muscle sections each month, i.e. 12 bulk samples per farm per year, and weighting the most recent sample as double the previous samples to provide a 'most current status'. Pooling meat juice from 20 pigs into one test carried out monthly would allow inclusion of samples from 240 pigs per year for the cost of little more than 12 tests. The representativeness would be further enhanced by using weighted rolling means (Alban *et al.* 2002). It would then be possible to select the proportion of herds with the highest mean O.D. or SP results, which is deemed appropriate and affordable for further monitoring and control actions.

The results of this work are in agreement with other studies, which suggested that the mean antibody titre was the best parameter to compare pig herds (Steinbach and Staak 2001). The current study was carried out on only one batch of pigs from each of only 20 farms so is likely to give a less accurate indication of farm status than the same methods used in ongoing monitoring. It is therefore

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recommended that further work is carried out on samples from a larger number of farms over a longer period.

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Exhibit L

COMPENDIUM OF METHODS FOR THE

MICROBIOLOGICAL EXAMINATION OF FOODS

FOURTH EDITION

EDITED BY
FRANCES POUCH DOWNES
KEITH ITO



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Chapter 2

Sampling Plans, Sample Collection, Shipment, and Preparation for Analysis

Thaddeus F. Midura and Raymond G. Bryant

2.1 INTRODUCTION

The objective of this chapter is to enable the user to obtain representative samples of a food lot, submit the samples to the laboratory in a condition that is unchanged microbially from the time of sampling, and prepare the samples for analysis. The procedures described in this chapter apply generally to collecting, labeling, transporting, storing, and preparing samples for analysis. For specific information and discussion about sampling and analytical procedures for canned foods, see Chapters 61 and 62. Other foods also may require special sampling and preparation procedures depending upon the specific microorganisms involved. For such procedures, see the chapters covering the relevant food types and specific microorganisms.

2.2 GENERAL CONSIDERATIONS

A representative sample that is appropriately transported to the laboratory, and prepared for examination, is the first priority in the microbiological examination of any food product. Laboratory results and their interpretation are valid only when appropriate samples are examined. Samples must be representative of the entire lot of material under evaluation, must be the proper type for the determination to be made, and must be protected against extraneous contamination and improper handling, especially at temperatures that may significantly alter the microflora. Refrigeration often must be provided to prevent destruction or growth of organisms in a sample. Unfrozen samples must be refrigerated, preferably at 0° to 4.4°C, from the time of collection until receipt at the laboratory. To avoid contact of the product with melting ice, a sealed eutectic coolant is preferable for use in the shipment container. Samples collected while frozen should be kept solidly frozen. When dry ice is used, the containers should have tight closures to prevent pH changes in the sample caused by the absorption of carbon dioxide. As a general rule, samples should be examined within 36 hr after sampling. Perishable items that cannot be analyzed within 36 hr should be frozen or retained at refrigerated temperatures, depending upon the type of product, reason for analysis, and type of microorganisms sought. Nonfrozen samples of shellfish should be examined within 6 hr after collection; they cannot be frozen.[3]

Samples must be clearly and completely identified with the following information: sample description, collector's name, name and address of the manufacturer, lot number, dealer or distributor, and date, place, and time of collection. Frequently the temperature at the time of collection is also useful to the laboratory for the interpretation of results. Further, it is often desirable that the reason for testing be given, e.g., samples may be collected as part of a quality control or surveillance program, as official samples to determine conformity to regulatory standards, or as part of a foodborne disease investigation.

2.3 EQUIPMENT, MATERIALS, AND REAGENTS

1. **Instruments for opening containers.** Sterile scissors, knives, scalpels, can openers, or other hand tools as required.
2. **Sample transfer instruments.** Sterile multiple- or single-use spatulas, scoops, spoons, triers, forceps, knives, scissors, tongue depressors, drills and auger bits, corers, dippers, metal tubes, and swabs as required.
3. **Sample containers.** Sterile multiple-use containers, either large or small-mouthed designs, nontoxic, leakproof, and presterilized polyethylene bags or other suitable sterile, nontoxic containers as appropriate.

   Nonsterile, nontoxic, single-service vials, polyethylene bags, or bottles that are clean and dry and do not have a viable bacterial count in rinse tests in excess of one organism per mL of capacity are acceptable transport containers. Sterile, evacuated sampling equipment also can be used. Sterile glass containers usually are not desirable because of possible breakage and consequential glass contamination of the sampling environment.
4. **Thermometers.** Thermometers that measure –20 to 100°C with graduation intervals not exceeding 1°C. A metal dial type or digital electronic unit is preferred due to the risk of glass thermometers breaking. Thermometers should be sanitized by dipping in a hypochlorite solution not less than 100 mg/L, or other equivalent microbicide, for at least 30 sec before being inserted into foods.
5. **Microbicide.** Medium strength (100 mg/L) hypochlorite solution or other approved disinfectant.
6. **Labeling supplies.** Pressure-sensitive tapes and labels, tags of adequate size to hold sample information, indelible marking pens.
7. **Sample shipping containers.** For frozen or refrigerated samples, rigid metal or plastic containers that are insulated and equipped with a tight-fitting cover. Each container

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should have ample space for the refrigerant so that samples will remain at the desired temperature until arrival at the laboratory.

Containers for nonperishable samples should be made of sturdy corrugated cardboard or other material capable of withstanding abusive shipping conditions.

Refrigerant or dry ice is to be added as needed for perishable samples.

8. **Balance.** A balance with 2000 g capacity having a sensitivity of 0.1 g with a 200 g load is acceptable.
9. **Blenders and mixers.** Mechanical blenders with several operating speeds or rheostat speed control with sterile glass or metal blending jars and covers or a Stomacher (Tekmar Company, Cincinnati, Ohio) are examples of acceptable devices.
10. **Diluents.** Sterile Butterfield's phosphate buffer (Chapter 63). Sterile 0.1% peptone water (Chapter 63). Sterile sodium chloride solutions (Chapter 63). Special diluents required for specific microorganisms and special analytical conditions (specific chapters).

2.4 PRECAUTIONS

Adequate precautions should be taken to prevent microbial contamination of samples from external sources, including the person taking the samples, air, sample containers, sampling devices, and the shipping vehicle. When foods are packaged in small, sealed containers, collect the unopened containers rather than portions from each container.

The sampling operation should be organized in advance with all the needed equipment and sterile containers at hand. For collecting samples, use an instrument appropriate to the physical state of the food.

Protect sampling instruments from exposure and contamination before and during use. When removing sampling instruments from the food container to the sample container, do not pass them over the remaining presterilized instruments. When opening the sterile sampling container, open it sufficiently to admit the sample, then close and seal it immediately. Do not touch the inside of the sterile container lip or lid. Do not allow the open lid to become contaminated. Do not hold or fill a sampling container over the top of a bulk food container when transferring a sample. Fill the sample container not more than 3/4 full to prevent overflow and to allow proper mixing of sample in the laboratory. Do not expel air when folding or whirling plastic sample bags. Submit an empty sterile sampling container similarly opened and closed as a control.

The sample collector must keep their hands away from the mouth, nose, eyes, and face. Hands must be washed immediately before beginning the sampling and during sampling if the hands become contaminated. Sterile plastic gloves may be useful to limit contamination during the procedure.

Contaminated sampling equipment must be placed into proper containers for ultimate disposal and/or sterilization. Labels must never be moistened with the tongue. Use direct labeling of the container or pressure-sensitive labels.

Chapter 1 gives additional safety precautions to ensure proper handling and preparation of samples for microbiological analysis. Food samples may contain infectious microorganisms or toxic materials that may be potentially hazardous. The best protection against hazards is the use of good sampling techniques and treating each sample as if it were contaminated.

2.5 PROCEDURES

2.51 *Sampling Plans*

Sampling plans were first developed in 1923 by engineers at the Western Electric Company, but were not immediately adopted.[21] After World War II the Department of Defense developed Military Standards for attribute and variables sampling plans[21,22,23,24] (definitions in Section 2.511). These plans considered single and multiple sampling based on the history of the producer performance. Levels of sampling (i.e., tightened, normal, and reduced) that reflected prior producer performance were established for these plans. Special categories of sampling plans have been adapted to agricultural products.[5,6,14,19,20,25]

A comprehensive set of single sampling attribute plans has been published for food microbiology.[7,12] Typical points of sampling where sampling plans may be applied are shown in the following chart:

Points of Sampling
raw materials
↓
production line
↓
producer's warehouse
↓
retail storage or sales outlet
↓
international port—export or import

This discussion of sampling plans will be clarified if certain basic concepts are stated at the outset. First, the lot to be sampled must be defined. When a given quantity of product is surveyed and units or portions of this lot are taken for examination (including microbiological analyses), the procedure is called "sampling." Sampling can be applied in a way that ensures that results obtained will be statistically valid. In this case, a "sampling plan" is developed, where selection of samples taken from a lot is carried out in a manner that ensures that each sample has the same chance of being chosen. For example, assume that a lot defined as "1000 packages in a warehouse" is to be sampled. Each package is assigned a number. If the 1000 items are stacked equally in 10 rows, then 1 to 100 would be assigned to the units in the first row, 101 to 200 to the second, and so on until all packages are numbered. A random number table is used to choose the required numbered containers for sampling.[12,17] In this example, if we select five as the required number of packages, then five typical random numbers (lot size being 1000) could be 586, 973, 99, 838, and 737. In addition to random sample selection, a sampling plan will also contain instructions that specify the number of packages to be taken and the basis for accepting or rejecting the lot. Thus, a sampling plan as employed in this context states the number of units required to be randomly collected from a lot and lists the acceptance and rejection criteria. Before using a sampling plan, it is usually prudent to consult a professional statistician to ascertain that the lot of food to be sampled meets the criteria required by that particular sampling plan.

2.511 *Definitions Used in Sampling Plans*

The following statistical terms are frequently used in the sampling literature.[9,12,13,24,25]

1. **Acceptance number(s)**—The maximum number(s) of defectives in an attribute sampling plan for which a lot will be accepted.

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2. **Acceptance quality level (AQL)**—The maximum percent defective (or the maximum number of defects per 100 units) that for purposes of sampling inspection can be considered satisfactory as a process average.[23]
3. **Analytical unit**—The amount of sample actually analyzed.
4. **Attribute**—A qualitative characteristic of a sample unit, e.g., the results of an analysis are positive or negative for *Salmonella*.
5. **Average outgoing quality limit (AOQL)**—The maximum possible percent defective that will result after a rectifying inspection of the lot has been performed. In such an inspection, 100% of rejected lots are screened to remove defective items, provided such screening is 100% effective.[24] Typically, AOQL values do not exceed 5%.
6. **Binomial distribution**—Distribution of a population in which a proportion (p) of the units in the population has a certain characteristic, and a proportion (q = 1–p) does not. Thus, each individual unit falls into one of only two classes, either p or q.
7. **Consumer protection**—The ability of a sampling plan to identify unacceptable lots. This is measured as the complement of the probability of acceptance for Limiting Quality (LQ) lots.[25] The consumer protection is often set at 90%.
8. **Consumer's risk**—The risk a consumer takes that a lot will be accepted by a sampling plan even though the lot does not conform to requirements.[25] This risk is known as a Type I risk, and in many standards[8,21,23,25] this risk is nominally set at 10%. A second type is a consumer beta risk, which is the risk that the lot will be rejected even though it is actually satisfactory.
9. **Continuous distribution**—A distribution of a population of measures that take on a continuum of values. A variables sampling (Section 2.515) plan may be applicable.
10. **Control chart**—A graphic device that can be used to monitor repeated sampling from a manufacturing or measurement process.
11. **Destructive testing**—A testing process that results in destruction of the unit or sample under test.
12. **Discrete variate**—A random variable consisting of isolated points that can have a finite or infinite number of values. These values can be used in an attribute sampling plan. (Sections 2.512, 2.513, 2.514).
13. **Estimate**—Any value computed from sample data and used to infer a corresponding population (lot) value, e.g., the sample mean (average).
14. **Estimated process average**—The average percent defective or average number of defects per 100 units of product found at the time of original inspection.[24]
15. **Frequency distribution**—The mathematical description of the way members of a population are distributed. The information about the distribution is used to calculate the probability of lot acceptance or rejection. The discrete random variable takes on a countable number of values, and the probability distribution is defined by a probability mass function. A continuous random variable is defined by a density function.
16. **Homogeneity of variances**—The equality of variances among populations, which must sometimes be tested.
17. **Homogeneous**—A product having a uniform texture or content.
18. **Limiting Quality (LQ)**—Percent defective or defects per 100 units.[25] A lot having a 10% probability of acceptance is referred to under many common standards[8,21,23,25] as a lot having a quality level equal to LQ.
19. **Lot**—The number of sample units produced in one batch or in some specified period of time such that the units will be about the same quality. Each lot or batch should consist of units of product of a single type, grade, class, size, and composition. The lot should be manufactured under the same conditions and at essentially the same time.
20. **Lot inspection by attributes**—Inspection whereby either the sample unit is classified as defective or nondefective with respect to a requirement or set of requirements (when on a "defective" basis), or inspection whereby defects in each sample unit are counted with respect to a requirement or set of requirements (when on a "defect" basis).[25]
21. **Lot quality**—A measure of the characteristic being controlled. The results of lot inspection are often expressed as percent defective units (e.g., a unit containing *Salmonella* is defective). Less frequently, the quality will be expressed in terms of the variable measured (weight/unit, coliform/g).
22. **Operating characteristic (OC) curve**—A graphical representation of the relation of the probability of lot acceptance to lot quality (usually expressed as percent defective units). This curve will depend on the number of units required in the sampling plan and the acceptance number. The curve also shows the lot quality associated with the consumer's risk and the producer's risk. It thus describes the consequences of the sampling plan (decision rule) for accepting lots of different quality.
23. **Population**—Any finite or infinite collection of individuals (samples or units) on which decisions are to be made.
24. **Probability**—An estimate of the frequency of occurrence of an event, e.g., probability of n sample units out of a population being positive for *Salmonella*, expressed as a value from 0 to 1. For a sampling plan, assumption of a particular probability distribution (i.e., binomial or Poisson) allows the estimation of the computation of the probability of lot acceptance versus lot quality in an operating characteristic curve.
25. **Producer's risk**—The risk that a producer takes that a lot will be rejected by a sampling plan even though the lot conforms to requirements.[25] This is called an alpha risk, and in many plans[8,21,23,25] this risk is set at 5%. Another type is the producer's Type I risk, which is the risk that the lot will be accepted even though it does not conform to requirements.
26. **Proportion defective units (P)**—The number of defective units divided by total units in a lot. Proportion defectives P or percent defectives (100P) are often plotted as the abscissa on an OC curve.
27. **Random sample**—A sample that was chosen in such a way that every sample or unit in the lot had equal chance of being selected. This is often achieved with the aid of a random number table.
28. **Representative sample**—In the widest sense, a sample which is representative of a population, and not merely a portion of it. Some confusion arises if "representative" is regarded as meaning "selected by some process that gives all samples an equal chance of appearing to represent the population." In fact, a representative sample is one that is considered typical of a population in respect of certain characteristics, however chosen. To obtain a truly representative sample, there are four requirements: (a) determine the location of sampling points critical to the population; (b) establish a sampling method representing the population characteristics; (c) select the sample size; and (d) specify the frequency of sampling.

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29. **Sample unit**—The smallest definable part of a lot, also called a unit. For example, a singleton or a package is a sample unit. When lots are bulk packaged in bins, barrels, bags, etc., then the sample unit is arbitrary and may depend on the sampling device. The use of the word in this context should be differentiated from the analytical sample unit specified by the analytical method.
30. **Sampling plan**—A design that indicates the number of units to be collected from each lot and the criteria to be applied in accepting or rejecting the lot. A *single sampling plan* requires the lot to be judged on the basis of one set of sample units (Section 2.512). A *double sampling plan* (Section 2.513) is a sampling inspection in which the inspection of the first sample leads to a decision to accept, to reject, or to take a second sample. The inspection of a second sample, when required, then leads to a decision to accept or reject. Two or more such collections would mean that a multiple sampling plan was being used. Another procedure where units are drawn one by one (or in groups) and drawing at any stage determines whether to accept, reject, or continue sampling is called a sequential sampling plan.
31. **Sample size**—The number of samples selected from a lot. The sample size should accurately describe the population, and should be the most economical possible to reach a certain level of accuracy. If the testing method is nondestructive, it may be possible to conduct sampling of 100% of the units in a lot. Typically, microbiological testing destroys product, and thus 100% sampling is not possible. Sample size can be determined by using sampling plan master tables, such as those found in Military Standard 105D, published by the American National Standards Institute.[23]
32. **Stratified random sampling**—A procedure for sampling where the lot is divided into parts or strata that differ with respect to the characteristics under study. In some cases, this is a way of improving the estimate of lot quality.
33. **Zero defective tolerance**—An evaluation system implying that a lot must be free of the undesirable characteristic or defect. All units in the lot must be tested in order to ensure zero defectives. This can be performed only where the test is nondestructive. The requirement of zero defects is definitely not possible in microbiological sampling plans since these cannot be applied to all units in a test lot.

2.512 Single Sampling (Two-Class) Attribute Plans

Single sampling procedures are useful in food inspection since, in many production processes, the lot can be sampled and tested only once. In addition, the results of microbiological tests are clearly defined as attributes. For example, an attribute such as the presence or absence of a microorganism, e.g. *Salmonella*, is frequently reported. In other instances, a certain level of organisms may be acceptable. For example, a unit may be acceptable if it has less than 3 *E. coli* colonies /g of sample.

Single sampling attribute plans also have the advantage that the true distribution of the variable in question (e.g., *E. coli*/g) is not required. Single sample plans and the multiple sample plans in the next section (2.513) can be evaluated by using the hypergeometric, binomial, or Poisson distributions. The choice of distributions used to compute the probability relationships[8,9] depends on the number of units (N) in a lot. When N is large relative to the sample size (n), or when the number of defective samples in a lot is small, a Poisson distribution can be assumed, serving as an approximation of the binomial distribution. The type of lots acceptable for single sample procedures are assumed to be large, of homogeneous quality, and to satisfy the conditions above.

Steps for choosing and applying an attribute sampling plan are as follows:

- Select the measurements of interest.
- Define the sampling units that constitute a lot.
- Determine a value of consumer's and/or producer's risk to ensure the lot quality (LQ or AQL) desired.
- Obtain an estimate of process average.
- Compute or select a plan that meets the risk and lot quality requirements.
- Calculate the OC curve.
- Apply the plan on a group of randomly selected units from a lot.
- Maintain records on the process average (if you are a producer) and make changes in the plan as necessary.

Figure 1 presents the operating characteristics (OC) curves for seven single sampling plans. These OC curves for the sampling plans will be used to help illustrate the process of choosing a plan. The reader should refer to Dodge and Romig,[8] Duncan,[9] and International Commission on Microbiological Specifications for Foods[12] for presentations of complete sets of plans where OC curves for additional values of n or c are given. Figure 1 has an acceptance number (c) of zero, or zero tolerance, which means that any positive results on a test lot will result in the rejection of that specific lot.

As an example, consider that a lot N > 1000 units is to be analyzed for coliform MPN/g and a unit is to be called defective if it

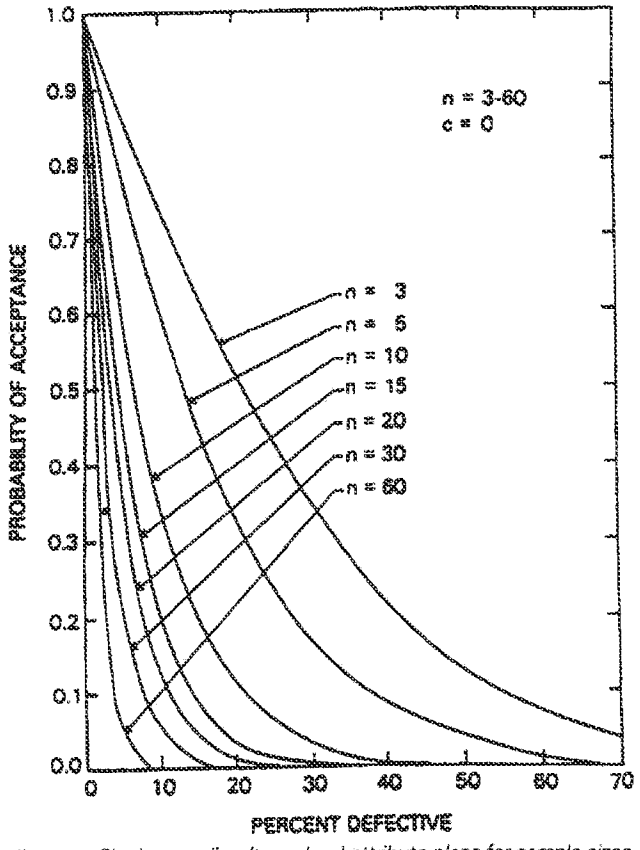


Figure 1. *Single sampling (two-class) attribute plans for sample sizes n = 3, 5, 10, 15, 20, 30, 60, and c = 0.*

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has MPN ≥ 100/g, and to be called acceptable or nondefective if it has an MPN < 100/g. A sampling plan is wanted to define the number of units (n) to sample where the number of defectives (c) equals zero and the probability (consumer's risk) of accepting lots with 8% or more defective units is 10%. Using these criteria, the sampling plan to be chosen (Figure 1) is n = 30 and c = 0.

Since microbiological tests are destructive (the sample unit cannot be used after testing), cost of sampling may be balanced with the cost of the risk. For example, a plan may be designed to inspect incoming raw materials and, on the other hand, may be designed to sample consumer products before releasing lots for sale. The measurement (i.e., coliform MPN/g) may be the same, but the choice of plan will be affected by other factors such as (a) processing conditions, (b) potential health hazard of the products, (c) persistence of organisms under different storage conditions, and (d) type of plan used by regulatory agencies to inspect the same lots. The n = 15, 20, 30, and 60 with c = 0 plans in Figure 1 are in common use[11,12] to test for *Salmonella*. The n = 3 and 5 with c = 0 plans could be used for screening raw materials.

Some analytical procedures are sufficiently sensitive to measure the presence of a single organism when the sampling units are pooled. A positive test indicates that one or more of the units was positive from n pooled units. This produces the same decision for a plan (e.g., n = 5, c = 0) as if the units had been analyzed separately. Pooling of sampling units cannot be done when c > 0 or when a positive sample is defined quantitatively (e.g., *E. coli* colonies/g). Calculations with OC curves are found in the references.[8,9]

It should be noted that as sample size n increases, the OC curve becomes idealized, with a probability of acceptance being 100% with 0 defects, and falling to a probability of 0% when the fraction of defects is > 0. Also, as the acceptance number c decreases, the OC curve shifts to the left, evidence that with a given number of defective units, the probability of accepting the lot decreases.

2.513 Multiple Sampling (2-Class) Attribute Plans

Sampling plans of this type are designed to inspect lots based on multiple samplings. The lot is accepted or rejected based on sequential decisions. As an example, consider a double sampling plan (Figure 2) of $n_1 = 10$, $c_1 = 0$ and $n_2 = 6$, $c_2 = 1$. This plan requires that 10 units be analyzed and the lot be rejected if any unit is defective. If only 1 of the 10 units sampled from the same lot is defective, then 6 additional units are tested. If one or more of the 6 units are defective, then the lot is rejected; otherwise it is accepted. The operating characteristic curve for this plan is given in Figure 2. In this example, if there were 24% defectives in the lot, the plan would accept 10 of 100 lots and the average outgoing quality would be 5% defectives.

Figure 2 shows the operating characteristic curve for the double sampling plan and one for a single sampling plan (n = 7, c = 0) that have the same average (5%) outgoing quality limit (AOQL). Although the AOQLs are equal in the two plans, it can be seen that the probability of accepting a defective lot is lower for the single sampling plan. In addition, the choice of taking 15 units (c = 0) for a single sampling results in a lowering of the AOQL from 5% to 2.5%. Thus, in choosing sampling plans, one must consider the probability of acceptance of a defective lot that will be tolerated.

A double or other multiple sampling plan requires that the lot of units be available if extended sampling is necessary. This is not practical in many types of foods. However, the advantage of multiple sampling plans is that lots of good quality (i.e., low coliform count) will on average require fewer samples to be tested than if tested by a single sample plan giving equal protection. For example, a two-stage or double sample plan of $n_1 = 10$, $n_2 = 6$ might require that just the first 10 units be analyzed. Thus, the average sample number tested would be decreased.

Further information on the design of multiple sampling plans can be found in Dodge and Romig[8] and Duncan.[9] A plan can be devised if the desired average quality limit (LQ), consumer (AQL) and/or producer's risk has been selected. Dodge and Romig[8] present the operating characteristic curves for double sampling plans and several outgoing quality limits (0.1 to 10%, p. 112). The figures allow a quick assessment of risks for each plan. The diagram shown in Figure 3 is an interpretation of double sampling plans.[8]

2.514 Three-Class Attribute Plans

Three-class attribute plans were developed by Bray et al.[7] in conjunction with the ICMSF[12] to be used with methods recommended by Thatcher and Clark[17] who stated[12] that the "test is concerned primarily with plans that may be applied to lots presented for acceptance at ports or similar points of entry." It was assumed that very little, if any, information would be known about the quality of lots and, thus, attribute plans, two-class plans (Section 2.513), and three-class plans would be applicable. These plans are

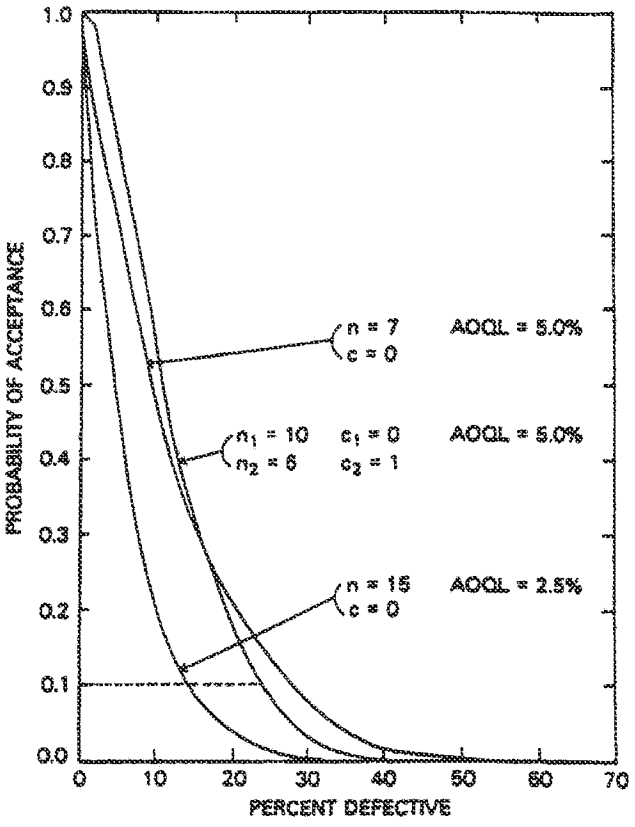


Figure 2. *Operating characteristic curves for two single and one double sampling plan with average outgoing lot quality limit 2.5 and 5.0%. The c = 0 for a single sampling plan indicates that a lot will be rejected if a single unit of the sample is positive. Rejection of the lot will occur if two or more units of the first or second sample are positive for the double sampling plan.*

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also useful for inspecting lots within a country or corporation where more information is known about a lot.

Three-class attribute plans differ from those described in Section 2.513 by having two microbiological limits that create three classes of product. Bray et al.[7] noted that the choice of limits (e.g., for coliform MPN, a quality or sanitation indicator) is difficult. Nonetheless they state that "most scientists can provide two numbers: one below which they have little or no concern and a higher value above which they clearly begin to have critical concern. If we denote the lower level by m and the larger one by M, then the set of values in the range [m, M] can be considered safe." Observations falling in the regions defined by the two limits are called acceptable, marginal, and unacceptable. The acceptable results have values equal to or less than m ($\leq$ m). Marginal observations fall between greater than m and equal to or less than M (e.g., [m, M]). Unacceptable results are greater than M. Three-class attribute plans can be specified by a sample size n, the number of units allowed (c_1) between limits $\geq$ m and $\leq$ M, and the number of units allowed (c_2) equal to or above M. It is assumed that all units $\leq$ m are acceptable. However, as stated above, the value of M is the decision point for this type of sampling plan. The value of $c_2 = 0$ was set in all plans discussed in *Microorganisms in Foods 2*[12] and in the discussion below. Thus, the sampling plans are noted as n, c where c is the number of marginal samples allowed. The three-class attribute sampling plans found in *Microorganisms in Foods 2*[12] present a detailed description of the type of microbiological methods to be recommended, potential risk of contamination, and subsequent hazard for a wide group of products and the choice of limits m, M for these products. Suggested sampling plans are presented for fish, fishery products, vegetables, dried foods, frozen foods, milk, milk products, raw meats, processed meats, shelf-stable canned foods, and fresh or frozen raw shellfish. Different measurements are of interest for each product and include aerobic plate count, coliforms, fecal coliforms, *Salmonella*, *V. parahaemolyticus*, *Staphylococcus aureus*, *B. cereus*, *C. perfringens*, and *C. botulinum*. Limits [m, M] were chosen for product-measurement combination and plans were established based on risk.

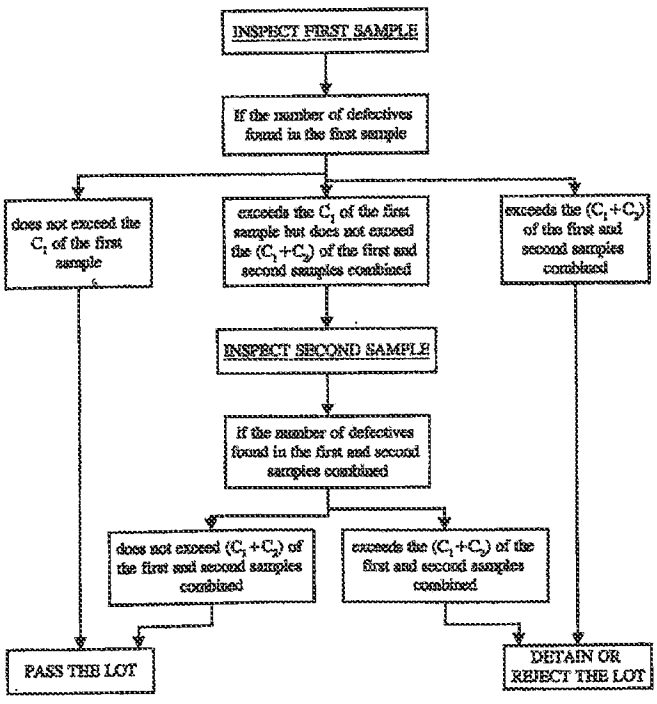


Figure 3. Interpretation of a double sampling plan.

The ICMSF[12] categorized the types of microbiological hazards and conditions to which a lot of food would be exposed. This categorization is an aid to the microbiologist when considering a choice of sampling plan. These hazards were defined as follows:

1. No direct health hazard, utility (e.g., general contamination, reduced shelf life, and spoilage).
2. Health hazard low, indirect (indicator).
3. Health hazard moderate, direct, limited spread.
4. Health hazard moderate, direct, potentially extensive spread.
5. Health hazard severe, direct.

These hazards are linked with three conditions of risk (reduction, no change, or increase) that reflect how a food was expected to be handled and consumed after sampling. The combination of the types of hazards and conditions of risk yields 15 cases where suggestions of sampling plans and limits could be presented for products, although only a few cases would be realistically applicable for a given product and measurement.

Sampling plans for hazards 1, 2, and 3 are three-class plans in which n = 10, c = 1; n = 5, c = 3; n = 5, c = 2; and n = 5, c = 1. In these cases (of the 15 defined cases) limits [m, M] are set to define marginal quality. When the hazards are more severe (i.e., 4 and 5) two-class plans in which n = 60, c = 0; n = 30, c = 0; n = 20, c = 0; n = 15, c = 0; n = 10, c = 0; and n =5, c = 0 are suggested. As noted in Section 2.513, the two-class plans above mean that n units are analyzed and the lot is accepted only if all units are negative for some characteristic (e.g., *Salmonella*). The operating characteristic curves are given for the two-class plans above in Figure 1.

The approach of the ICMSF[12] on three-class plans was to define risks, suggest limits for a wide variety of specific cases, and suggest plans to be employed. This approach differs from other texts on sampling plans in that the mathematical tools for computing and comparing plans are presented, and the reader must make a choice of various conditions. Those desiring to compute their own three-class plan should consult Bray et al.[7]

Operating characteristic contours presented as two-way tables are used rather than operating characteristic curves for three-class attribute plans. These contours reflect the fact that the true marginal percent can vary from 0 to 100% at the same time the proportion of defective units (value $\geq$ M) varies from 0 to 100% with the obvious restriction that the sum of percent good units, percent marginal units and percent bad units must be 100%.

The choice of an ICMSF sampling plan is straightforward. First, specify the food and measurement (e.g., freshwater fish and fecal coliforms). Second, specify the risk. ICMSF[12] suggests case 4 in this example, which reflects low indirect health hazard at reduced risk. The sampling plan is n = 5, c = 3. The last step is to choose limits m and M for the product to be sampled. ICMSF[12] recommends m = 4 and M = 400 for freshwater fish. The complete plan is listed (e.g., n = 5, c = 3; m = 4, M = 400), once the product and measurement are specified.

To derive a three-class plan, one must consider (a) the assignment of risk to the product, (b) the choice of sampling plan based on a comparison of probability of acceptance versus percent marginal units (assuming we will not accept any bad units), and (c) the values of m and M for the product. The choice of risk and the setting of m and M may differ depending on the purpose of the sampling. One set of FDA sampling plans for *Salmonella* has values of n = 15, c = 0; n = 30, c = 0; n = 60, c = 0.[11]

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2.515 Variables Sample Plans

A variables plan can be used when the probability density function of a measurement is known, and a majority of published plans are computed when the distribution of the variable or its transformation is normal (Gaussian). An advantage of the variables plans over single sampling plans is that fewer samples are required, resulting in a lower cost to achieve the same protection as a single attribute plan. Disadvantages include the calculations involved in evaluating a lot, the different calculations required for each variable (coliform, *E. coli*, etc.), and the requirement that the probability distribution must be known or assumed for each measurement. The last two requirements may prove to be the most inconvenient. A series of three or four variable (e.g., aerobic plate count, coliform, *E. coli, and S. aureus*) measurements may be required on a product. The transformation that ensures a normal distribution may not be known, and the estimates of variance are often different for each measurement. For these reasons, variables sampling plans are not widely used in the food industry for microbiological measurements. For further information about derivation of variables sampling plans, refer to Duncan,[9] Kramer,[14] and MIL-STD 414.[22]

2.516 Sampling Procedures for Low Contamination Levels

The microbiologist often encounters a situation where production lots of a product with low incidence of a pathogen (low number of contaminated units) must be sampled (e.g., 1 positive sample unit in 500). How many sample units should be taken to have a high probability of detecting the pathogen? Suppose 200 sample units have been analyzed and all are negative. What can be reported about the lot? Clearly, the whole lot may not be free of the pathogen. The same situation also arises in sterility testing. In both of these situations, it is assumed that the pathogen or contaminant can be detected if present in an analytical sample (e.g., 25 g) and the results will be reported as positive or negative. Other necessary assumptions are given in the discussion of single sampling attribute plans (Section 2.512).

Table 1 lists the number of sample units (n) needed to detect a positive result at a given level of fraction positive analytical units in a lot. This table can be used to determine how many units should be analyzed in order to detect a positive at a given fraction positive level. Suppose a regulator knows that the fraction positive of a certain pathogen is 0.04. How many sample units should be obtained in order to find a positive unit with probability 0.95? In Table 1, we go down the left hand (fraction positive samples) to 0.04 and over two columns to (1-Pr) = 0.95. Pr is the probability that all n units are negative. The required sample size is 75.

Let us examine the situation from another point of view. Suppose a producer knows that pathogens can be found in his product with a frequency of 6 in 1,000 units. The regulator takes 30 sample units per lot. What is the probability (Pr) that all 30 samples will be negative? The answer is $Pr = e^{-(30)(0.006)} = 0.84$. Thus, the regulator has a 0.16 chance of detecting a positive from a lot with a frequency of defective units of 0.006. Simply stated, 84% of the time that 30 samples are collected from this defective lot, no defects will be found, while defective units will be found 16% of the time.

Another situation arises when a sample of n units is examined and all are negative for the pathogen in question. What can be said about the rest of the lot in terms of any units being defec-

Table 1. Number of Samples Needed to Detect a Fraction Positive[a] with Probability 0.90, 0.95, and 0.99 Where at Least One Positive Result Occurs

Fraction positive[b]	Number of analytical units to be tested (n)		
samples (P)	Probability (1-Pr) 0.90	0.95	0.99
1.0	3	4	4
0.9	3	4	5
0.8	3	4	6
0.7	4	5	7
0.6	4	5	8
0.5	5	6	9
0.4	6	8	12
0.3	10	10	16
0.2	12	15	23
0.1	23	30	46
0.09	26	34	51
0.08	29	38	58
0.07	33	43	66
0.06	39	50	77
0.05	46	60	92
0.04	58	75	115
0.03	77	100	154
0.02	115	150	230
0.01	230	299	461
0.001	2,303	2,996	4,605
0.0001	23,026	29,963	46,052

[a] Can be computed from Thorndike Table, p. 35 of Dodge and Romig.[8]

[b] The fraction of positive units (e.g., 90 positives in 100 analytical units).

Table 2. Fraction Positive Samples When the Probability[a] Is That All n Samples Are Negative

Number of analytical	P = Fraction positive samples Probability (Pr)		
negative units (n)	0.10	0.05	0.01
3	0.77[b]	1.00	1.50
5	0.46	0.60	0.92
10	0.23	0.30	0.46
15	0.15	0.20	0.31
20	0.12	0.15	0.23
25	0.092	0.12	0.18
30	0.077	0.10	0.15
35	0.066	0.086	0.13
40	0.058	0.075	0.12
45	0.051	0.067	0.10
50	0.046	0.060	0.092
100	0.023	0.030	0.046
200	0.012	0.015	0.023
300	0.0077	0.012	0.015
400	0.0058	0.0075	0.012
500	0.0046	0.0060	0.0092
1000	0.0023	0.0030	0.0046

[a] Can be computed from Thorndike Table, p. 35 of Dodge and Romig.[8]

[b] Rounded to two significant digits.

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tive? Table 2 shows one way of expressing the result. The fraction positive (defective units) per lot can be related to the probability, Pr, when all n sample units examined are negative. If 100 sample units were negative, then there is only 1 chance in 10 (Pr = 0.10) that the fraction positive exceeds 0.023 per lot. Conversely, the probability is 9 chances in 10 that the fraction positive is 0.023 or lower. Stated yet another way, there is a probability of 10% (at most) that 230 sample units in 10,000 have a pathogen. So even in a lot size of 10,000, there is some chance (although only 10%) that a positive unit will reach a consumer. There is an additional, though small, probability that the consumers will receive the unit. This is because the distribution of microbial contaminants in the lot is not uniform. The fact that contaminants are not evenly distributed throughout the lot increases the chances of not detecting them, and thus increases the probability of accepting the defective lot.

If the samples were homogeneous and the organisms could be assumed to be randomly distributed throughout the lot, then an estimate of concentration could be obtained (Chapter 6). For example, assuming that one *Listeria monocytogenes* organism occurs in each 10 mL of milk, if n samples of milk are taken from a well-agitated bulk milk tank, then the concentration of pathogen could be estimated. If the analytical sample is 1 mL, how many sample units are required to obtain at least one positive sample with probability 0.95? From Table 1 we see that 30 samples should be examined.

Let us further suppose that 2 of the 30 results are positive upon examination. Based on the results of a single dilution, the estimate of concentration of *L. monocytogenes*/mL = ln(n/# negatives) = ln(30/28) = 0.069. Thus, sampling plans are useful not only for lot inspection, but in determining risk and in predicting a specified level of organisms associated with a given probability, although this is limited to lots where the organism is evenly distributed. In addition, the concentration can also be estimated in some restricted cases.

2.517 Summary

The discussion of sampling plans in this section has been modified for application to microbiological measurements. A general survey of plans is presented, but some of these procedures do not lend themselves directly to microbiological sampling in foods. The brief discussion of multiple sampling plans does not mean that these procedures may not be found useful. The sampling for microorganisms is destructive, the results may be delayed for several days because of incubation requirements, and the foods may be perishable. Thus, single sampling plans are generally best suited to most situations.

Variables sampling plans are subject to additional disadvantages beyond those stated in Section 2.515. The general level of statistical knowledge in the typical food facility may prevent the use of these plans. Calculations needed to choose and apply the variables plans may prove burdensome. The two- and three-class attribute plans are easy to apply and thus should aid in the spread of statistics in microbiological sampling of foods. Additionally, the ICMSF[12] has presented extensive suggestions of specific plans and microbial criteria to be used on a wide variety of products. This reduces the amount of work necessary in calculations and time spent in plan selection. Thus, the single sampling two-class and three-class attribute plans have the most utility in sampling of foods for microbiological analysis. Individuals who wish to derive their own sampling plans are advised to use Dodge and Romig,[6] Duncan,[9] and MIL-STD 105.[23]

2.52 Sampling Procedures

It is necessary to consider the physical state of the product to be sampled (e.g., dry, semisolid, viscous) and the reason for sampling and testing before taking samples so that the number of units will be representative and/or statistically significant for the intended use.[12] The sampling procedures described in this section are those that generally apply. Unusual sampling procedures that may be required for specific foods are given in the chapters dealing with particular microorganisms or commodities.

2.521 Finished Products

Consumer packages of foods should be sampled from original unopened containers of the same processing lot. Record processing information and product code of samples on forms submitted with samples. The practice of submitting unopened containers prevents contamination that might be introduced by opening at the sampling location. In addition, it allows laboratory examinations to be performed on products and packages or containers as they are offered to the public.

2.522 Bulk Liquid Material

If the products are in bulk form or in containers of a size impractical for submission, aseptically transfer a representative sample portion to a sterile, leakproof container. Before drawing a sample, aseptically mix the food mass to ensure that the sample is as homogeneous as possible. If adequate mixing or agitation of the bulk product is not possible, multiple samples should be drawn from the bulk container. Do not fill sample containers over bulk containers of food. Care must be taken to select sample containers with enough capacity to accommodate the needed sample volume when the container is 3/4 full. Avoid the use of glass sample containers. Thermometers used in bulk food containers should be sanitized before use.[4] Cool perishable samples to 0° to 4.4°C quickly if they are not already refrigerated. Metal thermometers are preferred since breakage of a mercury thermometer would contaminate the product.

When appropriate, a temperature control sample should be prepared and submitted with the samples to be tested. If a temperature control sample is needed for frozen samples, use a container of ethylene glycol. The sample should be sealed in its container so that the container will not break or leak and introduce extraneous contamination. If the sample is to be examined for a regulatory purpose, the sample container must be sealed so that it cannot be opened without breaking the seal. In addition, an empty sterile sample container should be submitted as a container control.

2.523 Bulk Solids or Semisolids

Sampling of dry or semisolid foods should be done with sterile triers, spoons, or spatulas. Sterile tongue depressors may be substituted for spatulas. Aliquots from several areas of the food under examination should be taken to ensure a representative sample. Care should be taken to protect this type of sample from excess humidity.

2.524 Frozen Bulk Materials

Frozen bulk foods may be sampled with sterile corers, auger bits, and other sharp sampling instruments. A presterilized auger bit or hollow tube may be used to obtain enough material for analysis. Frozen samples should be kept frozen until arrival at the laboratory. Thawing and refreezing of samples must be avoided.

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A suitable procedure for obtaining test units of frozen foods (particularly from larger samples) is to use an electric drill combined with a funnel.[1] The sterile auger bit is inserted through a sterile plastic funnel (which has been cut off so the hole is just slightly larger than the bit) held against the frozen sample (Figure 4). The frozen shavings are conveyed to the surface and collected in the funnel. The shavings then can be placed in a sterile sample container. For larger solid food samples—frozen or unfrozen, test units should be taken aseptically from several areas using sterile knives and forceps. These portions should be mixed as a composite to provide a sample representative of the food to be evaluated.

2.525 *Line samples (in-process samples)*

1. **Liquids.** Sterile metal tubes or dippers may be suitable sampling instruments at certain plant locations for liquid food samples. Disposable plastic transfer pipets can also be used. A special line sampling technique involves the use of a disposable sterile hypodermic needle and syringe. The needle is inserted into a rubber closure of a stainless steel nipple. The nipple can be clamped on or permanently located at any desired spot.[10]

   Sampling cocks on holding tanks and product pipelines may be used, but disinfection and material flow-through of the sampling cock must be assured before collecting the sample.
2. **Solids.** Sampling of solid line samples may be accomplished using the same equipment and procedures as would be used for bulk solid or semisolid products. Automatic sampling devices are available for powdered products and other solid products not requiring refrigeration. When automatic samplers are used, the manufacturer's directions must be strictly followed.
3. **Special purpose samples (foodborne outbreaks, consumer complaints).** In some instances samples are tested as part of a foodborne disease outbreak investigation or on the basis of a consumer complaint. Consult a clinical or public health laboratory. Such situations may be the subject of legal proceedings, and laboratory personnel may be required to testify concerning the results of their examinations. If the record of sample collection and handling is incomplete, or if samples are received in a partially decomposed state or having been temperature abused, the laboratory results may be of little or no value.

   In outbreak situations, collect all suspect foods. If there are no leftover foods, try to get samples of items prepared in a similar manner subsequent to the suspected food. Collect ingredients or raw items used in the suspect food, if available. All of these foods should be held under suitable conditions until an analysis of the attack rate data and other facts can define more accurately the suspect food(s).[12] The original containers in which the foods were found also should be collected, labeled, and submitted for examination. Other specimens as well as foods from outbreak investigations are essential. Human specimens may include stools, vomitus, and serum, depending upon the suspected cause of illness. These specimens should be collected in sterile leakproof containers and should be properly identified as to the patient's name, type of specimen, and date of collection. Transport media is sometimes indicated. Aseptic techniques always should be used to obtain samples even if the foods have been mishandled grossly.
4. **Samples for water activity measurements.** Collect samples intended for water activity in sealable, vapor tight containers. Obtain small, unopened, retail-size packages if possible. When sampling from bulk containers, work quickly to minimize changes in water content.
5. **Samples for pH measurements.** Samples to be tested for their pH and/or titratable acidity should be collected into tightly sealed containers. If the material to be tested is undergoing fermentation or some other gas producing reaction, use a vented container or flexible plastic bag with ample space for expansion.



Figure 4. Funnel collection apparatus.

2.526 *Storage and Shipment of Samples*

When it is necessary to store samples prior to shipment, a storage area for frozen (–20°C) and for refrigerated (0° to 4.4°C) samples should be available. Waterproof labels should be used on sample containers to prevent loss of labels. Direct labeling of containers is preferred.

Whenever possible, samples should be submitted to the laboratory in original unopened containers.

Samples should be delivered to the laboratory as rapidly as possible. The condition, time, and date of arrival at the laboratory should be recorded. The samples should be packed to prevent breakage, spillage, or change in temperature. Since laboratory examination of food samples requires preparatory work, the laboratory should be given advance notice, if possible, of the number and types of samples being submitted. If the product is in a dry condition or is canned (flat or normal), it need not be refrigerated for shipment. Swollen containers should be shipped with coolant. The product label may also indicate whether refrigeration is required. Samples not requiring refrigeration or freezing may be packed in a cardboard box using appropriate packing material to prevent breakage. Refrigerated products must be transported in an insulated shipping container with sufficient refrigerant to maintain the samples at 0° to 4.4°C until arrival at the laboratory. Water frozen in plastic containers or cold packs serves well for 0° to 4.4°C shipping and should last 48 hr under most conditions. Do not use loose ice as this may cause product contamination if the container breaks or leaks. Dry ice may be used for longer transit times if the sample is separated from the dry ice packing material to avoid freezing. Refrigerated products should not be frozen, as destruction of certain microorganisms

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will occur. Frozen samples can be kept frozen by ensuring that the samples are shipped with dry ice. Frozen samples collected in plastic bags, however, must not come in direct contact with dry ice as the plastic bag will become brittle and subject to rupture from the extreme temperature. Use paper or another suitable material to protect the sample. Such samples should be transported to the laboratory by the fastest possible means. Mark the shipment of samples as "Perishable," "Packed in Dry Ice," "Refrigerated Biologic Material," or "Fragile," as appropriate. Label the shipment according to federal postal service rules and Department of Transportation regulations.[26,27]

2.527 Preparation of Homogenates

Use aseptic technique. Prior to opening, swab the exterior area of the container with 70% ethanol to destroy microorganisms that might contaminate the sample.

Samples should be examined promptly. When initiation of analysis must be postponed, store frozen samples at -20°C until they are to be examined. Refrigerate unfrozen perishable samples at 0° to 4.4°C for not more than 36 hr.

Store nonperishable, canned (normal, flat), or low-moisture foods at room temperature until ready for analysis. Frozen samples should be thawed at refrigeration temperatures (≤4.4°C) for no longer than 18 hr in the original container in which received.[4] If the sample must be removed from the original container, it should be done aseptically. Alternatively, higher temperatures may be used for a short period of time, but the temperature must remain low to prevent destruction of microorganisms (<40°C for ≤15 min). Frequent shaking of samples is necessary when samples are thawed by the alternate procedure. A thermostatically controlled water bath with agitator is recommended for the rapid thawing of samples.

Liquid or semiliquid samples in containers that have an airspace can be mixed by rapidly inverting the sample container 25 times. Sample containers that are 2/3 to 3/4 full should be shaken 25 times in 7 seconds over a 30 cm arc. The interval between mixing and removing the test portion should not exceed 3 min. To ensure a homogeneous sample when no airspace is present, aseptically open the container and pour the product from the filled container back and forth into a sterile container three times.

Dry samples should be aseptically stirred with a sterile spoon, spatula, or other utensil to ensure a homogeneous sample. Test portions of nonviscous liquid products (i.e., viscosity not greater than milk) may be measured volumetrically using a sterile pipette (11 mL into 99 mL, or 10 mL into 90 mL, or 50 mL into 450 mL). If the pipette becomes contaminated before completing the transfer, replace it with a sterile pipette. Do not insert the pipette more than 2.5 cm below the surface of the sample. The pipette should be emptied into the diluent by letting the column drain from the graduation mark to the rest point of the liquid in the tip of the pipette within 2 to 4 sec, then touch the lower edge of the pipette tip against the inside of the neck of the dilution container. Do not blow out the last drop or rinse the pipette in the dilution fluid.[4] When measuring products having a viscosity similar to milk, the last drop should be blown from the pipette. For viscous liquid products, the test portion for the initial dilution should be aseptically weighed (11 ± 0.1 g) into a sterile 99 mL dilution blank (or 10 ± 0.1 g into 90 mL, or 50 ± 0.1 g into 450 mL). This provides a 1:10 dilution.

Test portions of solid or semisolid foods should be 50 ± 0.1 g. The 50 ± 0.1 g test portion should be weighed aseptically (using sterile forceps or spatulas) into a sterile tared blender cup[11,12]; then add 450 mL of sterile diluent. A variety of diluents may be used depending upon the nature of the product. Those most commonly used are Butterfield's phosphate buffer and 0.1% peptone water. When analyzing for specific organisms, other diluents may be appropriate, e.g., 3% NaCl for *Vibrio parahaemolyticus*. (Refer to the chapters on specific groups of organisms for other diluents.) When analyzing fatty foods or lump-forming powder, wetting agents such as Tergitol Anionic-7 (1% weight/volume) may be included in the diluent to promote emulsification. Blend for 2 min at low speed (approximately 8000 rpm) to disperse the material.[11] The blending time may vary depending on the type of food.[12] Some blenders may operate at speeds lower than 8000 rpm. It is preferable to use a higher speed for a few sec initially. No more than 15 min should elapse from the time the sample is blended until all dilutions are in appropriate media.

Optionally, if the entire food sample is less than 50 g, weigh to the nearest 0.1 g portion approximately one-half of the sample into a sterile tared blender cup. Add sufficient sterile diluent to make a 1:10 dilution (i.e., add an amount of diluent equal to nine times the weight of the test portion in the blender cup). The total volume in the blender cup must cover the blades completely. Blend as described above.

If the sample is not homogeneous, weigh 50 g from a representative portion of the package into a sterile, tared blender cup or analyze each portion of food separately Proceed as described above. Caution should be exercised in the blending step to prevent excessive heating. The amount of heating may vary with foods of different consistencies and may be expected to increase if blending times greater than 2 min are required. Chilled diluent (i.e., tempered in an ice-water bath) may be employed to decrease the chances of excessive heating.

Stomaching is acceptable as an alternative to blending in preparing the food sample homogenate.[16] In this procedure, the food sample with diluent is placed in a clean, preferably sterile plastic bag. The plastic bag is positioned within the stomacher and pummeled for 1 to 2 min. Because the sample is contained in a plastic bag, the developers recommend that samples with bones or other sharp or protruding objects not be prepared by stomaching. Thirty foods were evaluated using this procedure to determine the usefulness in a regulatory agency's laboratory. Results indicated that only certain food homogenates should be prepared using this procedure.[2]

In some solid food products, the microbial flora is restricted primarily to the surface area. More accurate enumeration of these microorganisms may be obtained by rinsing the sample with sterile diluent rather than by blending. This can be accomplished by placing the sample in a suitable sterile container (plastic bag or sealable bottle) and adding a volume of sterile diluent equal to the weight of the sample. The container is then shaken in a manner similar to that used for preparing an initial dilution of a liquid food sample. Each mL of "rinse" thus prepared represents 1 g of sample.

See Chapter 6 for preparation of further dilutions and plate count procedures. Use media recommended in the specific chapter for the organisms of interest.

2.528 Preparation of Samples for Water Activity Testing

The ideal preparation of samples for water activity measurements is to grind the material to a fine consistency before testing. It is important to avoid heat build-up in the sample during the grind-

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ing process. Certain emulsions such as oil/water emulsions may be difficult to measure unless the water phase can be separated by low temperature cycling or centrifugation. Judgement must be used in evaluating the accuracy of measurements done on the water phase of an emulsion. In any case, the prepared sample should be added to the test chamber of the water activity meter quickly to avoid an exchange of moisture with the air. If prepared samples will not be tested promptly, store them in vapor tight, sealed containers. Avoid storage in high humidity environments.

When performing the analysis, transfer sample portions to the test instrument sample holder and follow the manufacturers instructions. A reliable reading may take from 5 minutes to several hours depending on the type of instrument in use. Proper maintenance and calibration of water activity instruments requires some skill and experience. High quality training and support is available from the instrument manufacturers. A complete description of water activity testing can be found in the work of Troller and Christian,[18] and the symposium series by Rockland and Beuchat.[15] See Chapter 64 for a further discussion of water activity measurement.

2.529 *Preparation of Samples for pH Determinations*

Many types of liquid samples require very little preparation for pH determination. Semisolid samples, mixtures of solids and liquids, emulsions, and various types of marinated foods in oil, require special preparation steps. Semisolid samples can be blended to a thick paste and a small amount (no more than 20 mL/100 g) of distilled water added to provide a more fluid test portion. Mixtures of solids and liquids can be tested either by blending the mixture to a paste and measuring directly or by separating the solid and liquid portions using a U.S. standard #8 sieve. For marinated products in oil, separate the oil from the product, blend the solids (adding a small amount of distilled water if necessary) and test the paste. When attempting to determine the pH of emulsions, it may be necessary to separate the water phase for testing by low temperature cycling or centrifugation.

Temperature effects on the pH electrodes and the actual hydrogen ion activity will modify the pH readings from electronic pH meters. Instruments that have temperature compensation adjust the response of the electrodes. The ionic activity of the sample, however, cannot be corrected for. For accurate results, the standardization and actual determinations should all be done at the same temperature and within the range of 20° to 30°C. See Chapter 64 for a further discussion of pH measurement.

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Exhibit M

COMPENDIUM OF METHODS FOR THE

MICROBIOLOGICAL EXAMINATION OF FOODS

FOURTH EDITION

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FRANCES POUCH DOWNES
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Chapter 35

Pathogenic *Escherichia coli*

Jianghong Meng, Peter Feng, and Michael P. Doyle

35.1 INTRODUCTION

Escherichia coli, a member of the family Enterobacteriaceae, is a part of normal flora of the intestinal tract of humans and a variety of animals. Included in the Enterobacteriaceae family are some of the most important enteric pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. Although most of *E. coli* do not cause gastrointestinal illnesses, certain groups of *E. coli* can cause life-threatening diarrhea, and severe sequelae or disability.[21]

E. coli are classified on the basis of antigenic differences (serotyping) and virulence factors (virotyping).[78] Two *E. coli* surface components form the primary basis for the serological classification system: the O antigen of the lipopolysaccharide (LPS, O) and the flagella (H, for 'hauch,' the German term for flagella). The O antigen identifies the serogroup of a strain, and the combination of O antigen and H antigen identifies its serotype. For example, two strains identified as O157:H7 and O157:H19 react with the same anti-O antibody and are of the same serogroup but react with different anti-H antibodies and are different serotypes. More than 170 different serogroups have been identified. Isolates lacking flagella are non-motile and designated NM in the serotype formula. There is some, but not a definite, correlation between serogroup and virulence. For example, antigen O86 is commonly associated with *E. coli* strains that are members of the resident colonic microflora and *E. coli* of this serogroup rarely cause disease, whereas serogroup O55 is rarely associated with the resident microflora and is frequently associated with disease. Some *E. coli* strains also possess a capsular (K) antigen that also is used for classification.

The characteristics that form the basis for the virotyping system include patterns of bacterial attachment to host cells, effects of attachment on host cells, production of toxins, and invasiveness. Both classification schemes (i.e., serotyping and virotyping) used in combination are a useful approach for grouping *E. coli* strains; however, absolute categorization of strains can not always be made on the basis of antigenic differences and virulence factors.[68] Furthermore, the genetic codes for many of these virulence traits reside on plasmids or phages or chromosomal pathogenicity islands; hence, the capacity to be a pathogen consequently may be transferred from one strain of *E. coli* to another. *E. coli* strains, that have virulence properties associated with more than one pathogenic group, have been reported.[45] Currently, diarrheagenic *E. coli* are categorized into six virotypes. A brief description of the salient features and examples of foodborne outbreaks caused by *E. coli* of the different virotypes follows.

35.11 *Enterotoxigenic* E. coli *(ETEC)*

ETEC strains resemble *Vibrio cholerae* in that they adhere to the small intestinal mucosa via surface fimbriae (type 1 pili and colonization factor antigens) and produce symptoms not by invading the mucosa but by elaborating one or two enterotoxins, heat-labile toxin (LT) and heat-stable toxin (ST). The enterotoxins act on intestinal mucosal cells to cause diarrhea. ETEC strains cause traveler's diarrhea and severe, often fatal, diarrhea in infants in developing countries. Epidemiologic investigations have implicated contaminated food and water as the most frequent vehicles of ETEC infection. Although ETEC is rarely considered as a possible cause of diarrhea in the United States, several foodborne outbreaks have been documented since the first such outbreak was reported in 1970s.[21,77] ETEC outbreaks also have occurred aboard cruise ships. Foods that were involved in these outbreaks include cheese, curried turkey mayonnaise, crabmeat cocktail, and drinking water.[21] More recently, two outbreaks of ETEC in Rhode Island and New Hampshire were reported.[11] The outbreak in Rhode Island had 47 cases of ETEC infection that occurred on an airline flight, whereas 121 persons became ill after eating a buffet dinner served at a mountain lodge in New Hampshire. ETEC serotype O6:NM was isolated from patients in both outbreaks. Carrots that were grown in the same state and used in salads were implicated as the vehicle. In 1994, ETEC O153:H43 was identified as the cause of two foodborne outbreaks.[77] One outbreak was associated with eating pan-fried potatoes in Milwaukee, Wisconsin, whereas corn bread dressing was implicated in another outbreak in Louisiana. Interestingly, the two ETEC strains causing these outbreaks were both serotype O153:H43, produced ST enterotoxin, and were resistant to tetracycline, ampicillin, sulfisoxazole, and streptomycin; however, they were not related based on the epidemiologic investigation and molecular subtyping data generated by pulsed-field gel electrophoresis. In June 1998, potato salad contaminated with an ETEC strain was identified as the vehicle of the largest ETEC outbreak in the United States. As many as 4,500 people were sick after attending 300 parties in Illinois. In Japan, a large outbreak of ETEC affecting more than 800 students occurred at four elementary schools in 1996.[65] Tuna paste was implicated as the vehicle food and *E. coli*

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O25:NM was isolated from the food and stool specimens of the patients.

35.12 Enteroaggregative E. coli (EAEC)

EAEC strains cause persistent diarrhea, particularly in children.[15] EAEC are defined as *E. coli* strains that do not secrete enterotoxins LT or ST and that adhere to HEp-2 cells in an aggregative adherence pattern. The first description of EAEC as a cause of diarrhea among children was reported in Santiago, Chile, in the 1980s. Subsequent studies have supported the association of EAEC with diarrhea in other developing countries.[67] EAEC, however, are not strictly confined to the developing world, and have been isolated from diarrhea patients in the United Kingdom. Recently, Smith et al.[81] reported four foodborne outbreaks of EAEC infections in the United Kingdom in 1994, affecting a total of 133 patients. The symptoms included vomiting and diarrhea, with persistent diarrhea in a small number of patients. Each of the outbreaks was associated with consumption of a restaurant meal, but no single source was implicated.

35.13 Enteropathogenic E. coli (EPEC)

EPEC strains cause severe diarrhea in young children, especially infants.[20] The pathogen adheres to the intestinal mucosa and causes extensive rearrangement of host cell actin. As with many other *E. coli*, transmission of EPEC is by fecal-oral route, with contaminated hands or contaminated foods or infant formula as vehicles. EPEC once caused frequent outbreaks of infantile diarrhea in the United States, but is no longer as important a cause of diarrhea in developed countries as they were in the 1940s and 1950s. However, several outbreaks of EPEC infection have been reported in the last two decades in the United States and other developed countries. These outbreaks frequently occur in day care centers and occasionally in pediatric wards.[21] Foodborne outbreaks of EPEC also have been reported. A foodborne outbreak caused by atypical EPEC was recently documented among adults who ate a gourmet buffet in Minnesota.[45] No specific food item was identified as the vehicle in this outbreak. In 1995, two outbreaks in Northern France were associated with prawn mayonnaise vol au vents, and lettuce and gherkins, respectively. A total of 59 cases were reported, and EPEC O111 was isolated from patients.[85]

35.14 Enterohemorrhagic E. coli (EHEC)

EHEC cause illnesses, including hemolytic uremic syndrome (HUS), which are similar to those caused by *Shigella dysenteriae* predominantly in the young and elderly.[23,58] EHEC also are similar to EPEC except that EHEC produce one or two Shiga toxins. Although many serotypes of *E. coli* belong to the EHEC group, serotype O157:H7 predominates as a foodborne pathogen. *E. coli* O157:H7 was first recognized as a human pathogen in 1982 when two outbreaks of hemorrhagic colitis were associated with consumption of undercooked ground beef that had been contaminated with this organism.[76] Since then, this pathogen has caused many foodborne outbreaks of hemorrhagic colitis and HUS worldwide.[23] Large outbreaks of *E. coli* O157:H7 infections include an outbreak in 1992–1993 that affected more than 700 individuals with 4 deaths in the western United States,[5] and several outbreaks in 1996 in Japan in which more than 9,000 cases were reported.[84] The CDC estimates there are 20,000 cases of *E. coli* O157:H7 infection annually in the U.S. and 500 associated deaths.

Cattle[3,43,90] and to a less extent other ruminants[52,55] are considered to be the major reservoirs of *E. coli* O157:H7, although the organism has been isolated from other animals such as dogs, horses, and birds.[42] Many outbreaks of *E. coli* O157:H7 infection have been associated with the consumption of undercooked ground beef. Other foods also have been associated with *E. coli* O157:H7 outbreaks worldwide, including roast beef, venison jerky, salami, raw milk, pasteurized milk, yogurt, lettuce, unpasteurized apple cider/juice, cantaloupe, radish sprouts, and alfalfa sprouts.[62] An outbreak associated with handling potatoes was reported in the United Kingdom.[66] It is noteworthy that certain foods such as apple cider/juice and dry-cured salami that previously were considered safe and ready to eat because of their high acidity, and are usually not heated before consumption, have been vehicles of outbreaks. The pathogen has been shown experimentally to survive for several weeks to months in a variety of acidic foods, including mayonnaise,[88] sausages,[35] apple cider,[89] and cheddar cheese.[74] Sprouts were recently added to the spectrum of foods serving as vehicles of *E. coli* O157:H7 infection. Radish sprouts were implicated as the vehicle outbreaks in Japan that occurred in 1996 and 1997.[84] In addition, two outbreaks of EHEC infection in Michigan and Virginia were associated with consumption of alfalfa sprouts.[12] Reports of person-to-person and waterborne (particularly recreational lake water) transmission have been increasing.[13]

35.15 Enteroinvasive E. coli (EIEC)

EIEC strains are biochemically, genetically, and pathogenically related closely to *Shigella* spp., but do not produce Shiga toxin. EIEC infection presents most commonly as watery diarrhea, which can be indistinguishable from the secretory diarrhea associated with ETEC infection.[1] Only a minority of patients experience the dysentery syndrome. HUS, however, is not a complication of EIEC infection. Documented EIEC outbreaks are usually foodborne or waterborne, although person-to-person transmission has been reported.[21] The incidence of EIEC in developed countries is reportedly low, but occasional foodborne outbreaks, such as a French camembert cheese associated outbreak in 1973[91] and a restaurant-associated outbreak involving 370 people in Texas in 1994, have been reported.[37] A large outbreak of EIEC infection in Japan with 670 cases occurred in 1988.[86] High fever and watery diarrhea were characteristic of the infection. "Godofu (Sasayuki tofu)" was identified as the vehicle food, and EIEC O164:NM was isolated from patient stools and Godofu.

35.16 Diffusely adherent E. coli (DAEC)

DAEC strains have been associated with diarrhea in children, and are defined by a characteristic diffuse-adherent pattern of adherence to Hep-2 or HeLa cell lines.[67] The pathogenesis of DAEC, however, is poorly understood. These organisms generally do not elaborate heat-labile or heat-stable toxins or elevated levels of Shiga toxins, nor do they possess EPEC adherence factor plasmids or invade epithelial cells. No outbreaks associated with food to date have been reported.

35.2 CONVENTIONAL ISOLATION PROCEDURES

35.21 General Considerations

Pathogenic *E. coli* are phenotypically diverse, hence, there are no standard microbiological methods for isolating these pathogens. Unlike typical *E. coli*, some pathogenic strains such as EIEC do not ferment lactose[57]; hence, coliform methods that are based on lactose fermentation are not suitable for the detection of EIEC. Also, elevated incubation temperatures that are applied in fecal

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coliform confirmation or enrichment procedures can be inhibitory to the growth of EHEC O157:H7.[22] Elevated temperatures and sodium lauryl sulfate used in lauryl tryptose broth (LTB) in Most Probable Number (MPN) analysis can cause the loss of plasmids, which often encode virulence-associated factors in *E. coli*.[47] Hence, no standard methods are available for detecting all pathogenic *E. coli* and existing methods are either adapted from general methods for isolating *E. coli* or developed for a specific group of pathogenic *E. coli*. Regardless of methods, however, when testing for pathogenic *E. coli*, it is important that isolates are first identified biochemically as *E. coli* before they are serotyped and assayed for virulence factors associated with a respective pathogenic group.

35.22 Media and reagents

Levine's eosin-methylene blue agar (EMB)
Tryptone phosphate broth (TP)
Sorbitol MacConkey agar (SMAC)
Hemorrhagic colitis agar (HC)
Hektoen enteric agar (HE)
E. coli broth (EC)
Brain heart infusion broth (BHI)
Modified EC broth (mEC)
Trypticase soy broth (TSB)
Modified trypticase soy broth (mTSB)
EHEC enrichment broth (EEB)
Tellurite cefixime sorbitol MacConkey agar (TC SMAC)
Phenol red sorbitol-MUG agar (PSA-MUG)
Novobiocin solution (100 mg/mL)
Kovac's reagent
Butterfield Phospate Buffer
MacConkey Sorbitol 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide sodium salt (MSA-BCIG)
MacConkey Agar (MAC)

35.23 Pathogenic E. coli (EIEC, ETEC, EPEC, EAEC)

Pathogenic *E. coli* strains that ferment lactose and are not affected by elevated temperatures can be distinguished from other enterics using the standard MPN method for *E. coli*. However, fairly extensive serotyping and virulence analysis of colonies from Levine's eosin-methylene blue (EMB) agar plate may be required to identify a specific pathogenic group.

Alternatively, the following method has been described in Bacteriological Analytical Manual (BAM) of Food and Drug Administration (FDA), for isolating pathogenic *E. coli* from foods.[48] Aseptically weigh a 25-g sample into 225 mL of brain heart infusion broth (BHI) and pre-enrich for 3 hr at 35°C to resuscitate damaged cells. Transfer the entire pre-enrichment to 250 mL of tryptone phosphate (TP) broth and selectively enrich at 44°C for 20 hr. From the TP enrichment, streak for isolation on EMB and MacConkey (MAC) agar plates. If high numbers of *E. coli* (>2.5 × 10^4/g) are suspected, samples from BHI may be directly streaked onto EMB and MAC for differentiation and isolation. Some pathogenic *E. coli* strains may not produce colonies with the typical green sheen or may grow poorly on EMB; hence, pick 10 typical and 10 atypical colonies from EMB and MAC, and identify them biochemically as *E. coli* before serological typing and virulence analysis. For EIEC, Hektoen enteric (HE), MAC and Salmonella-Shigella agars may be used for selective plating; however, HE and MAC are least inhibitory and best suited for the isolation of EIEC.[21]

35.24 EHEC O157:H7 and other serotypes

35.241 General Method

EHEC serotypes other than O157:H7 are phenotypically diverse; hence, may be isolated from foods using the same procedure outlined above for the other pathogenic groups of *E. coli*. Serotype O157:H7 is unique in that most strains do not ferment sorbitol within 24 hr[24] and do not express β-glucuronidase which most *E. coli* posses. Hence, *E. coli* O157:H7 is typically negative in the fluorogenic, 4-methyl-umbelliferyl β-D-glucuronide (MUG) assay.[22,28] These phenotypes, especially the inability to ferment sorbitol (SOR), are used extensively to isolate O157:H7 from foods. However, relying on only a few phenotypic characteristics as the bases for selecting isolates for further characterization can yield an undesirable level of false-positive colonies. Enteric bacteria such as *Enterobacter hermanii* and *Hafnia* spp., have similar sorbitol and MUG reactions, hence, they resemble O157:H7 colonies on media that use sorbitol and MUG reagents for differentiation. Disease-causing, phenotypic variants of O157:H$^-$ strains that produce Stx2 have been isolated from HUS patients in Germany,[40] and are fairly prevalent in Central Europe.[2] These strains genetically belong to the O157:H7 clonal group,[27] but they ferment sorbitol and are positive in the MUG assay. In the U.S. only MUG positive variants of O157:H7 have been isolated.[44] However, culturing sorbitol-negative O157:H7 strains in sorbitol-containing media has resulted in strains capable of fermenting sorbitol,[33] hence, it is possible that sorbitol- fermenting variants will be encountered in the U.S. in the future. However, presently, sorbitol-containing media are effective and economical for the preliminary differentiation of EHEC serotype O157:H7 from other *E. coli*.

A number of sorbitol-containing media are used for isolating O157:H7; some are commercially available or can be prepared from individual ingredients. Also, several specialty media that use chromogenic substrates to detect enzymatic activities unique to O157:H7 and other EHEC have become available (Table 1). These, however, can be expensive and few have been tested by collaborative studies or evaluated for analysis using food samples.

35.2411 Sorbitol MacConkey Agar (SMAC). SMAC is frequently used in clinical analysis for isolating O157:H7.[60] Prompt plating of bloody stool specimens onto SMAC has been effective in isolating O157:H7.[39] For food analysis, homogenize a 10-g sample in 90 mL of 0.01% peptone water, make ten-fold serial dilutions and plate 0.1 mL, in duplicate, onto SMAC. Incubate plates overnight at 35°C. Disregard the pink, sorbitol-fermenting colonies; pick 5 to 10 non-fermenting colonies (pale to neutral/grey) to verify a negative MUG reaction, then serotype for the O157 antigen (see Section 35.4). If O157 positive, type for the H7 antigen and examine for EHEC virulence factors (see Section 35.5). Since SMAC is not very selective, high levels of normal flora in foods may mask the presence of O157:H7 on this medium.[41]

35.2412 Hemorrhagic Colitis Agar (HC). HC is another sorbitol-containing, direct plating medium, but, was developed specifically for foods.[53] HC agar is incubated at 43°C to provide greater selectivity and it also contains MUG for additional differentiation. O157:H7 on HC appear as clear colonies that do not fluoresce under long wave UV light due to the absence of GUD activity. Like SMAC, however, high levels of normal flora in foods can overgrow and mask the presence of O157:H7.[41] To provide greater selectivity as is needed in food analysis, a 25-g sample of food is blended in 225 mL of pre-enrichment broth and incubated at 35°C overnight, before plating on SMAC and HC agars. Enrichment broths often used are modified EC (mEC) or modified

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Table 1. Partial list of commercially available chromogenic media for differentiation of *Escherichia coli* strains

Target Bacteria	Commercial Name	Differentiating Characteristics	Manufacturer
O157:H7 & *E. coli*	Rainbow agar	β-galactosidase β-glucuronidase	Biolog, Hayward, CA
O157:H7 & enterics	BCMO157:H7(+)	β-glucuronidase sorbitol fermentation	BIOSYNTH, Naperville, IL
E. coli	CHROMagar	β-glucuronidase	CHROMagar, Montecino, CA

TSB (mTSB), supplemented with novobiocin. However, even with the pre-enrichment, foods having very high levels of normal microflora can be problematic with overgrowth problems on SMAC and HC agars.

35.2413 Tellurite-Cefixime Sorbitol MacConkey Agar (TC-SMAC). Due to the difficulties in isolating O157:H7 from foods with high levels of normal microflora, more selective enrichment and plating media have been developed. One enrichment broth, developed for analyzing carcass samples, uses the antibiotics vancomycin, cefixime and cefsulodin to inhibit gram-positive bacteria, aeromonads and *Proteus* spp., respectively.[14] The selectivity of SMAC plating medium also has been improved with the inclusion of tellurite-cefixime (TC), which effectively inhibits growth of other *E. coli* and other non-sorbitol fermenting bacteria. TC-SMAC agar appears to be very selective for O157:H7.[87]

Although no standardized methods are available for isolating O157:H7 from foods, the enrichment and plating media described above have been incorporated into procedures used by FDA for testing foods and methods used by the US Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) for meat analysis.

35.242 FDA Method—Bacteriological Analytical Manual (BAM)

The following is a summary of the method outlined in Chapter 4 of the BAM, 8th (rev.A), 1998, for isolating O157:H7 from foods.

1. **Enrichment.** Homogenize 25 g of sample in 225 mL of EHEC enrichment broth (EEB) and incubate at 37°C with agitation for 6 hr. Perform the "isolation" procedure outlined below, then re-incubate the enrichment broth overnight at 37°C.
2. **Isolation.** Spread-plate 0.1 mL of 6-hr enrichment culture onto a TC-SMAC plate and streak one loopful onto a second TC-SMAC plate and incubate both plates overnight at 37°C. Disregard the pink, sorbitol-fermenting colonies, and select the non-sorbitol-fermenting, neutral/grey colonies with smoky centers (1-2 mm diameter) that are typical of O157:H7. Pick 5 to 10, transfer onto slants of TSA with 0.6% yeast extract, and incubate the cultures overnight at 37°C. If no colonies typical of O157:H7 colonies grow on TC-SMAC plates, plate the overnight enrichment culture on TC-SMAC plates according to the procedure described above.

   Both EEB and TC-SMAC are highly selective and preliminary data indicate that this procedure may inhibit O157:H7 strains in foods that do not have high levels of normal microflora (S. Weagant, FDA, Bothell, WA, personal communication).
3. **Confirmation.** Use sterile toothpicks to obtain small amounts of colonies from TSA and test for indole reaction using a paper filter wetted with Kovac's reagent. If the isolate is indole-negative, it is most likely not *E. coli* and can be discarded. If isolates are indole-positive, test for the O157 antigen using commercial O157 antiserum (see Section 35.4). Latex agglutination kits such as Prolex *E. coli* O157 (PROLAB) and RIM *E. coli* O157:H7 (REMEL) give satisfactory results. Isolates that are agglutination positive with anti-O157 reagent are presumptively O157:H7. Those isolates presumptively identified as O157:H7 should be confirmed as *E. coli* by biochemical tests using miniaturized kits or conventional prodecures before performing PCR analysis for Stx1 and Stx2 (see Section 35.5).

35.243 USDA-FSIS Microbiology Laboratory Guidebook (MLG)

The following is a summary of the procedure outlined in the USFA-FSIS MLG, 3rd ed., 1998, for testing raw ground beef for O157:H7.

1. **Enrichment.** Blend 65 g of ground beef (usually 5 samples are analyzed as a composite) with 300 mL of mEC with novobiocin; add more media (about 285 mL) to bring the total volume to 650 mL. Incubate at 35°C for 18 to 24 hr.
2. **Screening.** Apply the enrichment culture to a commercial O157:H7/NM (non-motile) antibody-based test kit (Table 2) according to the manufacturer's instructions. The test kit must conform to the following performance characteristics: sensitivity ≥98%; specificity ≥90%; false-negative rate ≤2%; and false-positive rate ≤10%. Negative samples are reported as such and discarded and positive specimens are only considered presumptive positive for O157:H7, and must be confirmed by isolating the O157:H7 from the enrichment culture, and characterizing it by the biochemical and serological assays described above.
3. **Isolation.** From the presumptive-positive enrichment cultures, prepare in Butterfield's phosphate buffer ten-fold dilutions up to 10^{-7}. Spread plate 0.1 mL from each of the four highest dilutions onto MacConkey Sorbitol agar with 0.1 g of 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide sodium salt (MSA-BCIG) and incubate at 42 ± 0.5°C.[70] This medium is same as SMAC, but contains BCIG, a chromogenic substrate to detect GUD activity. SOR fermenting and/or GUD positive colonies appear as red to purple. Typical O157:H7 colonies are SOR and GUD-negative and appear white. These colonies are selected and streaked onto a plate of EMB and also onto a plate of phenol red sorbitol-MUG agar (PSA-MUG) and incubated overnight at 35°C to verify the SOR and GUD reactions.[69] Disregard the yellow SOR-positive colonies on PSA-MUG that also fluoresce under long-wave UV light. Select from PSA-MUG the clear, non-fluorescent colonies and from EMB the purple colonies with a metallic green sheen and serotype the colonies for O157 antigen using a latex agglutination kit (RIM *E. coli* O157:H7 Latex Test kit or equivalent) (see Section 35.4).
4. **Confirmation.** Confirm as *E. coli* the colonies that agglutinated with anti-O157 sera, using the biochemical tests listed in Table 3. Isolates that have the biochemical profile of *E. coli* O157:H7 should be serotyped for the H7 antigen by a tube agglutination test or latex agglutination kit (RIM *E. coli*

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Table 2. Partial list[a] of commercially available assays for detecting EHEC serotypes in foods.

Target	Trade name	Assay Format[a]	Manufacturer
O157	PetrifilmHEC	blot-ELISA	3M, St. Paul, MN
	EZ COLI	Tube-ELISA	Difco, Detroit, MI
	Dynabeads	Ab-beads	Dynal, Lake Success, NY
	Assurance[b]	ELISA	BioControl, Bellview, WA
	TECRA	ELISA	TECRA, Redmond, WA
	E. coli O157	ELISA	LMD Lab, Carlsbad, CA
	E. coli O157	ELISA	Meridian, Cincinnati, OH
	VIP[b]	Ab-ppt	BioControl, Bellview, CA
	Reveal	Ab-ppt	Neogen, Lansing, MI
	NOW	Ab-ppt	Binax, Portland, ME
	Immunocard	Ab-ppt	Meridian, Cincinnati, OH
	QUIX	Ab-ppt	UniversalHealthWatch, Columbia, MD
	VIDAS	ELISA[c]	bioMerieaux, St.Louis , MO
O157:H7	BAX	PCR	Qualicon, Wilmington, DE
O157:H7 & O26:H11	EHEC-TEK	ELISA	Organon-Teknika, Durham, NC

[a] Table modified from: Feng, P. 1998. U.S. FDA Bacteriological Analytical Manual, 8th ed. (Rev.A).

[a] ELISA, enzyme-linked immunosorbent assay; Ab-ppt, immunoprecipitation or immunochromatography; PCR, polymerase chain reaction.

[b] Approved Official first action—AOAC International.

[c] Automated ELISA

O157:H7 Latex Test kit or equivalent), using the bacterial culture grown on a blood agar plate. If the H7 test is negative, attempt to induce the motility of the isolates by growing them in motility medium and test again with anti-H7 antibody.

35.3 RAPID METHODS

Many innovative technologies have been introduced that currently are being used for the microbiological analysis of foods. Unlike conventional methods that rely on specific media to select and grow pathogens, newly developed "rapid methods" use procedures such as molecular biology techniques or immunoassay to detect in foods, gene sequences or antigens that are specific for the bacterial pathogen of interest.[26] Compared to conventional procedures, rapid methods are generally easier and more rapid to perform, with some requiring only minutes to complete. However, today's rapid methods still require that prior to analysis food samples undergo culture enrichment to grow the pathogen of interest to detectable populations. Rapid methods usually shorten analysis time by 1 to 2 days compared to conventional assays, hence they are useful for rapid screening of foods for bacterial pathogens. For screening purposes, negative results are regarded as definitive, whereas positive results are considered only as presumptive and must be confirmed by standard methods. In addition, since rapid methods use different technologies, the detection efficiencies of these assays can be dependent on the composition and microflora of the food being assayed. Hence, it is important that these methods are evaluated by collaborative studies prior to routine use in food analysis.[25] In the U.S., only methods that have been collaboratively studied and approved by the Association of Official Analytical Chemists International (AOAC) are included in the Official Methods of Analysis (OMA) and regarded as standard methods. Therefore, it is recommended to use rapid methods only for those food types specified by the OMA. Perform all rapid methods according to manufacturers' instructions provided with the kit, including the use of specified enrichment medium recommended for the assay.

Table 3. Biochemical reactions for *E. coli* O157:H7/NM

Medium	Reaction
Triple sugar iron agar	yellow butt/yellow slant, no H_2S
Sorbitol fermentation	negative
Cellobiose fermentation	negative
Tryptone broth	indole positive
MR-VP broth	MR positive; VP negative
Simmons citrate	negative (no growth)
Lysine decarboxylase	positive (purple)
Ornithine decarboxylase	positive (purple)
Decarboxylase broth base	negative (yellow)
Motility test medium	Motile (H7) or non-motile (NM)

35.31 *Pathogenic* E. coli *(EIEC, ETEC, EPEC, EAEC)*

Very few commercially available rapid methods have been developed for detecting in foods pathogenic *E. coli* other than EHEC, because these groups of pathogenic *E. coli* are not as frequently implicated in foodborne illness as EHEC. However, there are many antibody-, DNA probe-, and PCR-based assays reported for detecting characteristic virulence factors associated with these pathogens, including the toxins of ETEC, the invasion gene of EIEC, and the attachment and effacing gene of EPEC. Some of these assays are described in Section 35.5.

35.32 *EHEC O157:H7 and other serotypes*

Although EHEC serotypes O111:NM, O104:H21, O48:H21 and O26:H11 have been associated with outbreaks of foodborne disease, O157:H7 is the predominant serotype implicated in outbreaks worldwide. Hence, most commercially available assays are designed to detect the O157:H7 serotype (Table 2). With the exception of the BAX PCR assay that targets genetic sequences of O157:H7 and EHEC-TEK which detects O157:H7 and O26:H11 serotypes,[27] most of the assays target the O157 antigen and are not specific for the O157:H7 serotype. Assays that target the O157 antigen also detect O157, non-H7 serotypes that may be present in foods. Most assays use a sandwich enzyme-linked immunosorbent assay (ELISA) format or variants of ELISA that are automated or done on membranes, tubes, dipsticks, or other solid matrixes. Several, however, use immunochromatography or

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immunoprecipitation technology that was developed for clinical diagnostics and home pregnancy tests, and can provide a result with minimal manipulations and in less than 10 minutes after cultured enrichment. Regardless of the format, all of the assays require prior to analysis cultural enrichment in broth media such as mEC or mTSB with novobiocin or a medium specified with the kit. Also, only a few assays to date have been evaluated by AOAC collaborative studies and approved as official methods for analysis of O157:H7 in selected foods (Table 2).

35.4 SEROTYPING

Since pathogenic *E. coli* are classified based on virulence factors, serotyping does not provide a definitive identification of its pathogenic group, because some strains with the same serotype may belong to more than one pathogenic group. However, one exception, is O157:H7, which to date is recognized exclusively as an EHEC serotype. Hence, typing for the O157 and H7 antigens is useful for identification of this pathogen. A listing of the major O serogroups with their associated H antigen types for each pathogenic *E. coli* group has been published recently in a review by Nataro and Kaper.[68]

35.41 Pathogenic E. coli groups (EIEC, ETEC, EPEC, EAEC)

There are no assay kits available for serotyping these groups; however, sera for typing various O and H serotypes of these pathogenic groups are commercially available from several sources (Table 4). Slide agglutination of suspected colonies using commercial polyvalent antisera recognizing O antigens considered to represent EPEC has been a common procedure used for the diagnosis of EPEC . The advantage of this method is that it is extremely easy to perform. The major disadvantage is lack of accuracy.

35.42 EHEC and O157:H7

Sera for O157 and other EHEC serotypes are also available from several sources (Table 4). In addition, many latex agglutination kits are available for typing the O157 antigen of pure culture isolates (Table 4). These assays are rapid and easy to use, and simply involve making a cell suspension from growth on an agar plate, then mixing this suspension with a drop of O157 antibody bound to colored latex beads. In the presence of O157 bacteria, the antibody-latex agglutinates with the cells to form within a few minutes visible clumps. It is important that latex agglutination assays are performed according to manufacturers' instructions. With some kits, when a positive reaction is obtained, the isolate needs to be retested using the control latex reagent provided to rule out cases of autoagglutination.[8] Also, a single colony should be used when making the cell suspensions, because the use of heavy inoculum with more than one colony can result in false-positive reactions in latex agglutination assays.[82]

A positive reaction obtained with anti-O157 reagents is only a presumptive indication that the isolate may be of serotype O157:H7. Many *E. coli* of serogroup O157, but not H7, have been isolated from clinical and food samples.[9] These O157, non-H7, strains do not produce Shiga toxin(s) and are generally regarded as non-pathogenic for humans. Furthermore, sera to O157 can cross-react with antigens of *E. hermanii*,[58] *Salmonella* group N,[75] a few species of *Citrobacter*,[6,72] and other bacteria. Many of these cross-reacting genera, however, ferment sorbitol and can be distinguished from O157:H7 phenotypically. Because of the potential for false-positive reactions, it is important that presumptively positive O157 strains be further tested for the H7 antigen or for the characteristic EHEC virulence markers to obtain a definitive identification.

Some kits listed in Table 4 also provide latex reagents for typing the H7 antigen. However, expression of the H7 antigen can be variable, and often, O157:H7 strains isolated from clinical and environmental samples are non-motile and need to be induced for motility before H typing.[29,82] Sowers et al.[82] reported that a single colony from an agar plate is not sufficient to agglutinate the H7 latex reagent, hence, a sweep of growth must be used. They also recommended that the H7 latex reagent not be used independently of the O157 latex reagent, as a few false-positive H7 reactions were observed. The use of the H7 latex reagent alone will also detect other non-O157 *E. coli* serotypes that posses the H7 antigen.

35.5 Pathogenicity Testing

Pathogenic *E. coli* strains are defined on the basis of virulence properties, hence, pathogenicity tests are essential for the identification of pathogenic *E. coli* strains. Two approaches can be taken in the laboratory to detect pathogenic *E. coli*. These are based on phenotypic and genotypic characteristics of the pathogen. The phenotypic approach includes bioassays, tissue cultures, and

Table 4. Partial list* of commercially available sera for serotyping of pathogenic *E. coli* strains

Target	Trade Name	Assay Format[a]	Manufacturer
O (various)		sera	Denka Seiken, Tokyo, Japan
		sera	Difco, Detroit, MI
O157	RIM	LA[a]	REMEL, Lenexa, KS
	E. coli O157	LA	Oxoid, Hampshire, England
	Prolex	LA	PRO-LAB, Austin, TX
	Ecolex O157	LA	Orion Diagnostica, Somerset, NJ
	Wellcolex	LA	Murex, Dartford, United Kingdom
	Bactrace	Serum[b]	Kirkegaad & Perry Lab, Gaithersburg, MD
		Serum	Difco
		Serum	Denka Seiken
H (various)		Sera	Denka Seiken
H7	RIM	LA	REMEL
	Wellcolex	LA	Murex
		Serum	Difco

* Table modified from: Feng, P. 1998. U.S. FDA Bacteriological Analytical Manual, 8th ed. (Rev.A).

[a] LA , latex agglutination

[b] available as unlabeled, fluorescein-labeled for fluorescent antibody assays or enzyme conjugate for ELISA.

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immunoassays to detect production of toxins or activity of virulence factors. The genotypic methods include DNA hybridization, PCR assay, and other DNA amplification methods such as isothermal amplifications, to determine presence of virulence genes. Bioassays for identifying pathogenic *E. coli* have been described in detail in the 3rd edition of the *Compendium of Methods for the Microbiological Examination of Foods*.[49] The following section addresses immunoassays and DNA-based methods for detecting foodborne pathogenic *E. coli*, with an emphasis on EHEC. For more information on pathogenicity testing of diarrheagic *E. coli*, the reader is referred to a recent review by Nataro and Kaper.[58]

35.51 ETEC

Enterotoxins (LT and ST) and fimbrial attachment structures (colonization factor antigens) are characteristic virulence factors of ETEC. Detection of ETEC in foods has mainly relied on identifying the presence of LT and/or ST. The LTs of *E. coli* are oligomeric toxins that are closely related in structure and function to the cholera enterotoxin expressed by *Vibrio cholerae*. The LTs can be divided into two major serogroups, LT-I and LT-II, which do not cross-react immunologically. LT-I is expressed by ETEC that are pathogenic for both humans and animals, whereas LT-II is found primarily in animals and has not been associated with human or animal disease. The STs are small monomeric toxins that contain multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins. The STs can be divided into two unrelated categories, STa (ST-I) and STb (ST-II), which differ in structure and mechanism of action. STa can also be abbreviated STp or STh to indicate strains of porcine or human origin. STb is associated primarily with ETEC strains isolated from pigs.

A rabbit ligated ileal loop assay used to be performed to detect ST, but the expense and lack of standardization resulted in this test being replaced by the suckling-mouse assay, which became the standard bioassay for the presence of ST for many years.[34] The suckling-mouse assay involves the measurement of intestinal fluid in CD4 infant mice after percutaneous injection of culture supernatants. Several immunoassays also have been developed for detection of ST, including radioimmunoassay and an ELISA.[18] A competitive enzyme immunoassay is commercially available (Denka Seiken, Co Ltd., Tokyo, Japan) for detecting STa in culture fluid (Table 5). This test uses a synthetic peptide that is an analogue of STa to coat the wells and an anti-STa monoclonal antibody-enzyme conjugate. The antibody binds specifically to the solid-phase peptide. If the toxin is present in the culture filtrate, the toxin reduces binding of the conjugate to the solid phase, resulting in a reduced color intensity when compared to the negative control. These tests correlate well with results of the suckling-mouse assay.

The two standard assays for LT are the Biken test and the Y1 adrenal cell culture test. In the Biken test, LT is detected by the appearance of a precipiting line between growth of the organism and antisera against LT. In the Y1 assay, ETEC culture supernatants are added to Y1 cells and the cells are examined for rounding. Immunologic methods such ELISA, latex agglutination, and reversed passive latex agglutination test also have been developed for detecting LT (Table 5). In the reversed passive latex agglutination test, latex particles are sensitized with purified polyclonal antiserum raised in rabbits immunized with purified cholera toxin. These latex particles agglutinate in the presence of LT or cholera toxins. DNA probes and PCR assay targeting ST and LT also have been developed.[17,19] Three DNA probes are introduced in FDA BAM: the LT probe, eltA11, is a 22-mer oligonucleotide targeting the DNA sequence encoding the A subunit of LT; the STP probe is a 22-mer oligonucleotide for ETEC first isolated from pigs; and the STH probe is also a 22-mer oligonucliotide for ETEC isolated from a human patient.[46] Nataro and Kaper[58] have listed the sequences of oligonuclotides used for probing and PCR amplification of diarrheagenic *E. coli* strains. These DNA-based methods have been determined to be quite sensitive and specific when used directly on clinical samples or on isolated bacterial colonies.

35.52 EPEC

The most important virulence characteristics of EPEC strains are the attaching and effacing (A/E) histopathology, the possession of the EPEC adherence factor (EAF) plasmid, and the absence of Shiga toxins. Many EHEC strains also produce the A/E lesion, hence the determining the presence or absence of Stx is essential to differentiate EPEC from EHEC. Several assays have been developed for determining A/E activities or genes, including tissue culture tests for adherence phenotypes, fluorescence microscopy with actin probes for cytoskeletal disruption, radioactive and nonradioactive DNA probes, and PCR assays. The localized adherence phenotype is highly conserved among EPEC from the most common serotypes[20] and easily tested using cultured Hep-2 or HeLa cells and a light microscope. Transformed cell lines are incubated with bacteria, fixed, strained, and examined for adherent microcolonies of bacteria. A fluorescence actin staining (FAS) test also has been developed to identify the concentrated filamentous actin in epithelial cells beneath the sites of EPEC attachment, which serves as a surrogate marker for the A/E effect.[53] This latter test appears to correlate well with electron microscopy for attaching and effacing. The EAF probe and the *bfpA* gene probe targeting the EAF plasmid[30] and the gene probe targeting the *eae* gene that is associated with the A/E lesion,[50] are useful for the diagnosis of EPEC. Since many EHEC strains also carry an *eae* gene that is highly homologous to the EPEC *eae*, DNA probes and PCR primers derived from within the *eae* coding sequences often react with both EPEC and EHEC. Therefore, additional tests that determine the lack of Stx expression are necessary for identifying EPEC. Louie et al.[59] and Meng et. al.[64] exploited the heterogeneity of the 3′ end of oligonucleotide primers derived from the

Table 5. Partial list* of commercially available assays for detecting toxins produced by pathogenic *Escherichia coli* strains

Organism	Target	Trade Name	Assay Format*	Manufacturer
EHEC	Shiga toxins 1 & 2	VEROTEST	ELISA	MicroCarb, Gaithersburg, MD
		Premier EHEC	ELISA	Meridian, Cincinnati, OH
		Verotox-F	RPLA	Denka Seiken, Tokyo, Japan
		VTEC	RPLA	Oxoid, Hampshire, England
ETEC	Heat labile toxin	VET	RPLA	Denka Seiken
	Heat stable toxin	COLIST	ELISA	Denka Seiken
	Heat stable toxin	E.coli ST	ELISA	Oxoid

* Table modified from: Feng, P. 1998. U.S. FDA Bacteriological Analytical Manual, 8th ed. (rev.A).

* ELISA = enzyme-linked immunosorbent assay; RPLA = reverse passive latex agglutination

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eae gene region to design PCR assays specific for EHEC O157:H7. The assays were quite specific, but failed to exclude EPEC O55:H7 and O55:NM strains as they are reported to be the progenitor of O157:H7.[37]

35.53 EIEC

As EIEC can be difficult to differentiate from *Shigella* spp., identification of EIEC involves demonstrating that the organism possesses the biochemical profile of *E. coli*, but has the genotypic or phenotypic characteristics of *Shigella* spp. (except the production of Shiga toxin). EIEC strains can be presumptively identified by determining their ability to invade HeLa cells and confirmed for invasive potential by the Sereny test (keratoconjunctivitis) using the eye of a guinea pig.[58] DNA probes and PCR methods have been developed to detect invasion genes, encoded on a large (220 kb) plasmid of EIEC.[31,36] Probes pMR17 (17 kb) derived from the pInv plasmid of a *Shigella flexneri* strain, and *ial* (2.5 kb) derived from the pInv plasmid of a EIEC strain have shown 100% sensitive and specific for EIEC strains that have retained their virulence. A PCR assay amplifying *ial* was also developed.[31] Several DNA probes for detecting EIEC are listed in FDA BAM.[46]

35.54 EHEC

Some of the characteristic virulence factors of EHEC include: Shiga toxins or verotoxins; the *eae* gene-encoded intimin protein that is associated with A/E lesions; and enterohemolysin encoded by a 60-mDal plasmid.[68] Of these, the Shiga toxins, i.e., Stx1 and Stx2, are closely correlated with human illness. The original assay for Stx was based on cytotoxic effect of Stx on Vero cells. The assay played an important role historically in establishing a diagnosis of EHEC infection in clinical laboratories. However, easier assays that test bacterial isolates for *stx* genes or the production of Stx have been developed. With few exceptions, most assays identify all *E. coli* serotypes that produce Stx, hence, they are not specific for serotype O157:H7. There are several commercially available antibody assays (Table 5) for detecting both Stx1 and Stx2, but only Verotox-F differentiates the two toxins. These assays should be performed according to the instructions provided by the manufacturer.

DNA probes also have been developed for detecting EHEC[46]; and most are targeted to the *eae* or *stx* genes. Samadpour et al.[79] used *stx* gene probes to detect STEC in calf fecal samples and foods, including ground beef, raw goat milk, blueberries, and surimi-based delicatessen salad. Two procedures were used: colony hybridization (colonies formed by plating of enrichment cultures were probed for STEC) and dot blotting (enrichment cultures were analyzed for STEC). A DNA probe (CVD419) based on the 60 MDa plasmid that is common among EHEC was developed by Levine et al.[56] The probe hybridized with most EHEC serotypes, isolated from patients with hemorrhagic colitis and HUS, including O157:H7, O26:H11. Using the same probe and an *eae* gene probe derived from EPEC, Barrett et al.[3] determined that both the *eae* and CVD419 sequences were significantly more common among human isolates than among cattle isolates.

There are many PCR assays for detecting *eae*, EHEC-*hlyA* encoded on the 60MDa plasmid, and *stx1* and *stx2* genes in EHEC isolates in pure culture,[32,51,63,73] but none of these assays is commercially available. Two multiplex PCR methods for *stx* genes for testing the toxigenic potential of isolates are listed in the FDA BAM. The PCR procedure detects only *stx1* and *stx2* genes, whereas the mismatch amplification mutation assay (MAMA) can simultaneously identify both toxin genes as well as the O157:H7 serotype based on a unique base mutation in the *uid*A gene.[10] The primers for these methods are shown in Table 6. The PCR parameters and conditions are described in the specific references.

35.55 EAEC and DAEC

EAEC can be identified using HEp-2 assay or DNA probe or PCR assays. The characteristic aggregative adherence of EAEC on HEp-2 cells can be distinguished by prominent autoagglutination of the bacterial cells to each other, which often occurs on the surfaces of the cells, as well as on the glass coverslip free from the HEp-2 cells.[16] A DNA probe derived from a large plasmid of EAEC hybridized 56 (89%) of 63 EAEC strains examined (by HEp-2), compared to only 2 of 376 strains representing the normal flora and other diarrheagenic groups.[6] A PCR assay with primers derived from this EAEC probe also gave similar sensitivity and specificity.[80]

Methods used for identification of DAEC are similar to those used for EAEC. Presence of the diffusely adherent pattern in the HE-2 assay defines DAEC.[67] A DNA probe derived from the daaC gene necessary for expression of the F1845 fimbriae also has been used for the diagnosis of DAEC.[7]

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Table 6. Primer sequences and expected size of products of PCR assays used in the FDA BAM for detecting Shiga-toxin genes in isolates

Assay	Target	Expected Size	Primer	Sequence
PCR	Stx1	274 bp	807F	5′-GCAATTCTGGGAAGCGTGGC-3′
			1095R	5′-CACAATCAGGCGTCGCCAGC-3′
	Stx2	364 bp	722F	5′-CTGGGGGCGAATCAGCAATG-3′
			1089R	5′-CCGCCGCCATTGCATTAACA-3′
MAMA	Stx1	348 bp	LP30	5′-CAGTTAATGTGGTGGCGAAGG-3′
			LP31	5′-CACCAGACAATGTAACCGCTG-3′
	Stx2	584 bp	LP43	5′-ATCCTATTCCCGGGAGTTTACG-3′
			LP44	5′-GCGTCATCGTATACACAGGAGC-3′
	UidA	252 bp	PT-2	5′-GCGAAAACTGTGGAATTGGG-3′
	(O157:H7)	252 bp	PT-2	5′-GCGAAAACTGTGGAATTGGG-3′
			PT-3	5′-TGATGCTCCATAACTTCCTG-3′

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Exhibit N

COMPENDIUM OF METHODS FOR THE

MICROBIOLOGICAL EXAMINATION OF FOODS

FOURTH EDITION

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Chapter 37

Salmonella

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37.1 GENERAL BASIS OF METHODS

37.11 *Introduction*

The incidence of salmonellosis appears to be increasing. Most recent summary data[13] from the Centers for Disease Control and Prevention (CDC) are shown in Figure 1. Large outbreaks caused by raw and processed foods continue to occur. For example, an estimated 224,000 people nationwide were estimated to be made ill by ice cream produced in a Minnesota factory.[66] New vehicles of transmission have emerged, most notably shell eggs contaminated internally with *S. enteritidis*. In fact, egg-associated illness caused by this serotype now accounts for 25% of all salmonellosis reported in humans in the United States, and *S. enteritidis* is now the most common serotype associated with illness, replacing *S. typhimurium*. Fresh produce is of increasing concern.[83] Two major outbreaks were attributed to cantaloupe. In one, due to *S. chester*, cases were reported in 30 states, and it was estimated that more than 25,000 individuals were eventually infected.[107] The second was due to *S. poona* involving 185 confirmed cases in the United States and 56 in Canada.[12] Cantaloupe on salad bars was implicated in both outbreaks. A substantial volume of produce is imported, often from under-developed areas with poor hygiene practices. Increased surveillance for enteric pathogens in imported produce is essential.

The data in Figure 1 likely underestimates the extent of the *Salmonella* problem. The U.S. Department of Agriculture (USDA) estimates 696,000–3,840,000 cases of foodborne illness due to nontyphoid *Salmonella* with 870–1,920 deaths annually.[11] The estimated costs of illness range from 0.9 to 12.2 billion dollars.

Control of salmonellosis rests upon consumer education and implementation of adequate laboratory quality control programs in the food industry. Though the application of the Hazard Analysis and Critical Control Point (HACCP) system purports to reduce the need for testing, verification that HACCP is working properly will pose a continuing need for microbiological analysis. Greater surveillance of imported products is recognized because of the poor hygienic standards in many exporting countries.

The "official" method for analysis of foods for *Salmonella* detailed in this chapter is that of the Association of Official Analytical Chemists International (AOAC).[8] This method is tedious and labor-intensive. From the pioneering fluorescent antibody (FA) technique,[5] a variety of rapid screen techniques has been developed and will be discussed elsewhere in this chapter. These procedures give presumptively positive results one to 2 days earlier than the conventional culture method. However, these techniques still require pre-enrichment of food samples, and presumptively positive results must be confirmed by the conventional method.

37.12 *General Description of Methods for the Isolation of* Salmonella *from Foods*

37.121 *Introduction*

The examination of foods for *Salmonella* requires methods different from those used in clinical laboratories. Generally low numbers are present in foods, necessitating the analysis of larger samples than are used with clinical materials. The introduction of large amounts of food directly into selective media tends to reduce the selectivity of the media for *Salmonella* organisms.[112] Further, the *Salmonella* organisms in foods are often in a poor physiological condition as a result of food processing and storage. These two problems are simultaneously circumvented by pre-enrichment of the sample in a non-selective medium which permits resuscitation of debilitated organisms. Inoculation of selective media from the pre-enrichment medium obviates introduction of food into the selective medium. The analysis is a qualitative test and determines the presence or absence of *Salmonella* in a given sample. However, by analyzing replicate samples at a series of dilutions, it is possible to obtain a quantitative estimate of numbers, i.e., a most probable number.

The reference procedure described hereafter for the isolation and identification of *Salmonella* (Section 37.5) has been adapted from the *Official Methods of Analysis of the AOAC International*.[8] The biochemical and serological tests used to identify *Salmonella* are described in the reference procedure and are listed in Table 1.

The classification of the *Salmonella-Arizona* group of *Enterobacteriaceae* has been a source of confusion because of different systems of nomenclature.[61,75,83] The Enteric Bacteriology Laboratories at the CDC recently introduced changes in their nomenclature of the *Salmonella-Arizona* group by incorporating the *Arizona* group[41] into the *Salmonella* genus.[19,43a,84,85] Table 2 summarizes the properties of the various subspecies within the genus *Salmonella* defined on the basis of DNA hybridization studies.[19,84,85]

Many scientific journals require the nomenclature of microorganisms to comply with *Bergey's Manual of Systematic Bacteriology*. *Bergey's Manual* has taken a different approach from CDC and has adopted the position that the use of "species" names for *Salmo-*

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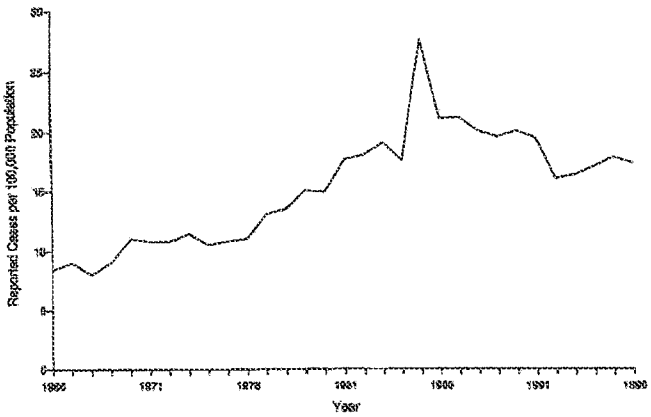


Figure 1. *Reported cases of human Salmonellosis (1966–1996), excluding typhoid fever. (Source: Centers for Disease Control and Prevention. 1997. Summary of notifiable diseases, United States. 1996. Morbid Mortal Wkly Rep. 45:54.)*

nella serovars is useful in many fields, so long as serovars' names are not taxonomically equated with species. The nomenclature in this chapter and the classic definition of the genus *Salmonella* are as described in *Bergey's Manual of Systematic Bacteriology*.[68,83]

Not all serovars within the genus *Salmonella* are equally pathogenic to humans and animals, but all are important to public health, and isolation procedures should recover all serovars of *Salmonella*. Analysts should be thoroughly familiar with the various biochemical and serological characteristics involved in differentiating *Salmonella* from the other foodborne microorganisms.

37.122 Sampling

General directions for sample collection and handling are discussed in Chapter 2. Various organizations have suggested sampling plans that can be used specifically for *Salmonella*. One of these plans has been proposed by the National Academy of Sciences (NAS)[84] through the Committee on *Salmonella* of the National Research Council (NRC). Foods are categorized on the basis of hazards presented by the particular food: 1) the food or food ingredient is a significant potential source of *Salmonella;* 2) the manufacturing process does not include a controlled step that should kill *Salmonella* microorganisms; and 3) a potential exists for microbiological growth if the food is mishandled in distribution or by consumers. Considering these hazards, foods are placed in one of five categories:

Category I—foods intended for use by infants, the aged, and the infirmed (the restricted population of high risk);

Category II—foods that present the three hazards;

Category III—food with two hazards;

Category IV—foods with one hazard;

Category V—foods with none of the hazards.

Criteria for acceptance of any particular lot of food are based on the results of analyses of a required number of 25-g analytical units, the actual portions of food analyzed. Each analytical unit is taken from a larger sample unit, usually a minimum of 100 g, the balance being set aside for possible testing. A series of random sample units make up the sample, and a sample that is representative of the lot is used to determine the acceptability of the entire lot of food. A food lot is defined as an identifiable unit of food produced and handled under similar conditions, usually determined by a limited period of time. This sampling plan is based on the premise that the distribution of the *Salmonella* organisms within the lot will be homogeneous. This premise implies that any analytical unit is as likely as any other analytical unit to contain *Salmonella*. The NAS/NRC Committee proposed criteria for the acceptance of any particular food lot in the various categories:

Category I—60 analytical units tested and found negative, indicating a 95% probability of ≤1 *Salmonella* organism per 500 g of food in the lot tested;

Table 1. Typical Biochemical and Serological Reactions of *Salmonella*

Test or Substrate	Positive	Negative	*Salmonella* Species Reaction[a]
1. Glucose (TSI)	Yellow butt	Red Butt	+
2. Lysine decarboxylase (LIA)	Purple butt	Yellow butt	+
3. H_2S (TSI and LIA)	Blackening	No blackening	+
4. Urease	Purple-red color	No color change	–
5. Lysine decarboxylase broth	Purple color	Yellow color	+
6. Phenol red dulcitol broth	Yellow color and/or gas	No gas; no color change	+[b]
7. KCN broth	Growth	No growth	–[c]
8. Malonate broth	Blue color	No color change	–[d]
9. Indole text	Red color at surface	Yellow color at surface	–
10. Polyvalent flagellar test	Agglutination	No agglutination	+
11. Polyvalent somatic test	Agglutination	No agglutination	+
12. Phenol red lactose broth	Yellow color and/or gas	No gas; no color change	–[e]
13. Phenol red sucrose broth	Yellow color and/or gas	No gas; no color change	–
14. Voges-Proskauer test	Pink-to-red color	No color change	–
15. Methyl red test	Diffuse red color	Diffuse yellow color	+
16. Simmons citrate	Growth; blue color	No growth; no color change	V

[a] +, 90% or more positive in 1 or 2 days; –, 90% or more negative in 1 or 2 days; V, variable.

[b] Majority of *Salmonella* subspecies 3a, 3b, 4, and 6 are negative (see Table 2).

[c] Majority of *Salmonella* subspecies 4 and 5 grow in KCN broth (see Table 2).

[d] Majority of *Salmonella* subspecies 2, 3a, and 3b, are positive (see Table 2).

[e] Majority of *Salmonella* subspecies 3b are positive (see Table 2).

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Category II—29 analytical units found negative, indicating ≤1 *Salmonella* organism per 250 g of food;

Categories III, IV, V—13 analytical units found negative, indicating ≤ 1 *Salmonella* organism per 125 g food.

The International Commission on Microbiological Specifications for Foods (ICMSF)[71] proposed a general microbial sampling plan that also includes a sampling plan specific for *Salmonella*. Basically, any particular food is sampled according to its placement in 1 of 15 categories or "cases." Such placement depends on two factors: 1) type of microbial hazard involved and 2) anticipated conditions of handling and use of the food after sampling. The microbial hazard can range from no direct health hazard (e.g., spoilage or reduced shelf life) to severe hazard (e.g., suspected presence of *Clostridium botulinum*). Anticipated conditions of handling and use of the food can be reduced hazard (e.g., cooking prior to consumption), unchanged hazard (e.g., direct consumption of dried foods), or increased hazard (e.g., storage of thawed frozen food at ambient or high temperatures). The stringency of sampling varies directly with the case number assigned to the food.[71]

Under the ICMSF plan, food sampling for most *Salmonella* corresponds to Cases 10, 11, and 12 (decreased, unchanged, and increased hazard in use, respectively). The case number increases as the stringency of the criteria for acceptability of any particular food lot increases. However, foods suspected of containing *S. typhi*, *S. paratyphi*, or *S. cholerae suis* are assigned higher risk levels and are sampled with greater stringency (Cases 13, 14, or 15), as are any foods associated with outbreaks ("investigational sampling"). In contrast, the NAS/NRC sampling plan recognizes an equally high degree of risk for all serovars and thus calls for greater sampling stringency than the ICMSF plan under most conditions. An added feature of the ICMSF plan, not found in the NAS/NRC plan, however, is the distinction made between sampling under normal (routine) and special (investigational) conditions, such as investigation of a foodborne outbreak. The ICMSF plan compares in stringency to the NAS/NRC plan only in the investigational mode.

The two sampling plans are nevertheless similar in that both recommend increasing the number of analytical units with increased health risk. In general, the assignment of a food to a particular category or case depends on the sensitivity of the consumer group, the history of the food, whether there is a step lethal to *Salmonella* microorganisms during processing or in the home, and

Table 2. Properties of the Seven Subspecies within *Salmonella enterica* ("the *Salmonella-Arizona* Group")[a]

	Designation						
	Salmonella subspecies 1	*Salmonella* subspecies 2	*Salmonella* subspecies 3a	*Salmonella* subspecies 3b	*Salmonella* subspecies 4	*Salmonella* subspecies 5	*Salmonella* subspecies 6
DNA-hybridization group of Crosa et al.[19]	1	2	3	4	5	Not studied	
Salmonella subgenus names formerly used	I	II	III	III	IV	V	
Genus according to Ewing[46]	*Salmonella*	*Salmonella*	*Arizona*	*Arizona*	*Salmonella*	*Salmonella*	*Salmonella*
Subspecies according to Le Minor et al.[82]	*enterica*	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*bongori*[d]	*indica*
Usually monophasic (Mono) or diphasic (Di) flagella	Di	Di	Mono	Di	Mono	Mono	Di
Usually isolated from humans and warm-blooded animals	+	–	–	–	–	+	+
Usually isolated from cold-blooded animals and the environment	–	+	+	+	+	+	+
Pathogenic for humans	+	+	+	+	+	+	+
TESTS TO DIFFERENTIATE THE SUBSPECIES							
Dulcitol fermentation	96[b]	90	0	1	0	100	variable
Lactose fermentation	1	1	15	85	0	0	? not given
ONPG Test	2	15	100	100	0	100	variable
Malonate utilization	1	95	95	95	0	0	0
Growth in KCN medium	1	1	1	1	95	100	0
Mucate fermentation	90	96	90	30	0	100	+
Gelatin hydrolysis[c]	–	+	+	+	+	–	+
D-Galacturonate fermentation[c]	–	+	–	+	+	–	+
Analysis by 01 bacteriophage[c]	+	+	–	+	–	v	+

[a] Adapted from Le Minor et al.[81,82,83]

[b] The numbers give the % positive for the tests after 2 days incubation at 35%C and are based on CDC data. The vast majority of the positive tests occur within 24 hr; reactions positive after 2 days are not considered.

[c] Based on the data of Le Minor et al.[81,82,83] + = 90% or more positive; – = 10% or less positive. The test for gelatin hydrolysis is the rapid "film" method at 35%C (almost all strains are negative by the tube method at 22%C within 2 days).

[d] Currently recognized as the second species of *Salmonella*, i.e., *S. enterica* and *S. bongori*.

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the abuse potential of the product. The sensitivity of the consumer group and whether the food undergoes a step lethal to *Salmonella* during processing or in the home were the most important to the U.S. Food and Drug Administration (FDA) in selecting a sampling plan for *Salmonella*. Accordingly, a sampling plan has been implemented, placing foods into one of three categories:

Category I—foods that would normally be placed in Category II, except that they are intended for consumption by the aged, the infirmed, and infants (60 analytical units);

Category II—foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption (30 analytical units);

Category III—foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption (15 analytical units).

Various studies have demonstrated that dry compositing (combining analytical units into a single test)[69,110] or wet compositing (combining pre-enrichment cultures of individual analytical units)[100,109,110] substantially reduces the analytical workload without compromising method sensitivity. In the FDA sampling plan, up to 1.5 × 25-g analytical units may be combined and tested as a single 375-g composite unit. The presence of *Salmonella* in foods assigned to Categories I, II, or III may be determined by the analysis of 4, 2, 1 composite unit(s), respectively.

Whether the FDA or the ICMSF sampling plan is used, the assumption is often made that negative results indicate that the product is deemed free of *Salmonella* (95% confidence level), ergo the zero tolerance connotation. In applying the NRC Category I sampling plan, negative results indicate that the sample contains no more than one *Salmonella* per 500 grams, not that it is completely free of the organism. Further, this limit assumes random distribution of the contaminant, which is certainly not always the case. To reach the same degree of confidence with non-random distribution would require negative results of a much larger number of randomly drawn samples.

37.123 Pre-enrichment

Salmonella organisms in foods are often present in low numbers and in a debilitated condition. As indicated previously, pre-enrichment in a non-selective medium obviates this problem as well as that caused by adding large amounts of food into the selective enrichment broth. North[96] first noted enhanced recovery of *Salmonella* from egg albumen as a result of pre-enrichment in lactose broth. Even though lactose is not fermented by *Salmonella* organisms and is fermented by competing coliforms, attempts to replace lactose with sugars fermented by *Salmonella* organisms proved no more effective than lactose broth. Lactose broth is commonly used in the prescribed methods for a wide variety of foods. A number of other pre-enrichment media is used in the standard analysis of specific foods (Table 3).

In connection with *Salmonella* control programs, a variety of environmental samples may be analyzed, e.g., air filters, contents of dust collectors, static material collecting on processing equipment, swabs from floor drains, etc. The analysis of these samples does not differ from that of the finished product, except that the pre-enrichment procedure must be consistent with the nature of the environmental sample. For example, an entire air filter may be examined. Pre-enrichment should take into account the mass of the filter examined. Beyond pre-enrichment, the analytical method is the same as for food samples.

37.124 Selective Enrichment

Enrichment media, which are inoculated with portions of pre-enrichment cultures, favor the growth of *Salmonella* organisms. In the past, direct enrichment of highly contaminated raw products, such as meats and sewage, was advocated. However, it has been concluded that such samples should be pre-enriched for greater recovery of the target organism.[25]

In the third edition of this *Compendium*, only selenite cystine (SC) and tetrathionate brilliant green (TBG) broths were used for selective enrichment. However, the 8th edition of the *Bacteriological Analytical Manual* (BAM)[129] specifies that a modified Rappaport medium, Rappaport-Vassiladis (RV) medium[130] be used for raw flesh foods, highly contaminated foods and animal feeds. It is the intent of the FDA to eventually replace SC broth with RV medium for the analysis of all foods because of the known toxicity of selenium which is formed from the reduction of sodium acid selenite during incubation. Moreover, disposal of the medium is expensive according to the guidelines of the U.S. Environmental Protection Agency. The use of two or more selective media provides greater method sensitivity than a single enrichment medium.

A number of studies has shown increased recovery of *Salmonella* organisms when incubating selective broths at 43°C, instead of the more frequently employed 35–37°C.[24,40,93,131] However, McCoy[89] and Aleksic et al.[1] reported that, in some instances, incubation at 43°C prevented recovery of *Salmonella*. For foods with a high microbial load, the reference method described hereafter (Section 37.2) prescribes incubation of TBG broth at 43°C and RV medium at 42°C. For foods with a low microbial load, the SC and TBG broths are incubated at 35°C.

In an attempt to provide greater analytical flexibility, D'Aoust et al.[36] investigated the potential of refrigerating pre-enrichment and selective enrichment cultures for 72 hr, thereby avoiding weekend work. Collaborative studies showed that the refrigeration (4°C) of pre-enrichment and enrichment cultures did not adversely affect the detection of *Salmonella* in a variety of low- and high-moisture foods, thereby increasing the number of weekdays on which *Salmonella* analyses could be initiated.[24]

37.125 Selective Plating Media

Selective plating media are formulated so that growth of *Salmonella* bacteria results in the formation of discrete colonies with concomitant repression of competing microflora. Separation of *Salmonella* from non-*Salmonella* bacteria is obtained through the incorporation of various dyes, bile salts, and other selective agents into the agar media. When non-*Salmonella* colonies appear on these media, they are generally distinguished by their ability or inability to produce hydrogen sulfide and metabolism of one or more discriminating carbohydrates in the media.[26] Selective plating media that have been used for the isolation of *Salmonella* include brilliant green (BG),[81] BG sulfa,[97] bismuth sulfite (BS),[133] *Salmonella Shigella*,[37] MacConkey's,[86] desoxycholate citrate,[82] Hektoen enteric (HE),[78] xylose lysine desoxycholate citrate (XLD),[109] Shanson's,[108] Rappold-Bolderdijk modified lysine iron,[102] Rambach[101] lysine-iron-cystine,[105] modified semi-solid RV (MSRV) medium[55] and novobiocin-brilliant green-glucose.[37] None of these agars are ideal for all situations, justifying the recommendation in many reference methods for the use of two or more agar media.[8,63,74,95,129]

Since their original formulation, some of these agars have been modified by the addition of antibiotics, sulfa drugs, surfactants,

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Table 3. Analysis of Foods by Methods of the *Bacteriological Analytical Manual* and the Association of Official Analytical Chemists International

Food	Pre-enrichment[a]	Preparation[b]
Dried egg yolk, egg white, and whole egg, liquid milk; powdered bread and pastry mixes; infant formulas; oral or tube feedings containing egg; coconut[c]; food dyes with pH ≥ 6.0 (10% aqueous solution); gelatin[d]; guar gum[e]	Lactose broth	Mix
Pasta (noodles, macaroni, spaghetti); egg rolls; cheese; dough; prepared salads; fresh, frozen, or dried fruits and vegetables; nut meats; crustaceans; fish; meats, meat substitutes, meat by-products, animal substances, glandular products, meals (fish, meat, and bone); casein	Lactose broth	Blend
Shell eggs, liquid whole eggs (homogenized), and hard-boiled eggs	Trypticase (tryptic) soy broth[f]	Mix[f]
Food dyes with pH<6.0 (10% aqueous solution)	None[g]	Mix 25 g/225 mL TBG
Nonfat dry milk (instant and noninstant)	Brilliant green water (add 2 mL 1% brilliant green dye solution per 1,000 mL distilled water)	Soak (gently layer powder on broth; do not shake, do not adjust pH
Dry whole milk	Distilled water and add 0.45 mL 1% brilliant green solution	Mix
Dried active[h] and inactive yeast; black pepper, white pepper, celery seed and flakes, chili powder, cumin paprika, parsley flakes, rosemary, sesame seed, thyme and vegetable flakes	Trypticase (tryptic) soy broth	Mix
Onion flakes and powder, garlic flakes and powder	Trypticase (tryptic) soy broth containing 0.5% K_2SO_3 final concentration	Mix
Allspice, cinnamon, oregano	Trypticase (tryptic) soy broth	Mix, using 1:100 sample/broth ratio
Clove	Trypticase (tryptic) soy broth	Mix, using 1:1,000 sample/broth ratio
Leafy condiments[i]	Trypticase (tryptic) soy broth	Mix, using >1:10 sample/broth ratio
Candy and candy coating; chocolate	Reconstituted nonfat dry milk[j] add 0.45 mL 1% brilliant green solution	Blend
Frosting and topping mixes	Nutrient broth	Mix
Frog legs	Lactose Broth	Immerse/rinse let pairs, (individual leg if ≥ 25 g) and examine rinsings

[a] Unless specified otherwise, 25-g samples are pre-enriched in 225 mL of indicated medium.
[b] Unless specified otherwise, 1-mL volumes of incubated pre-enriched cultures of foods are subcultured to 10-mL volumes of selenite cystine and tetrathionate brilliant green (TBG) broth. For RV enrichment, 0.1 mL of pre-enriched cultures are subcultured to 10 mL RV medium.
[c] Add up to 2.2 mL of Tergitol 7 or Trition X-100 to initiate foaming.
[d] Add 5 mL of 5% papain solution.
[e] Add 2.25 mL of filter-sterilized 1% cellulase solution.
[f] For shell eggs and hard-boiled eggs, examine egg yolk only.
[g] Pre-enrichment in lactose broth may provide improved recovery.
[h] One-mL volumes of pre-enrichment cultures of dried active yeast are transferred to 10-mL volumes lauryl tryptose broth and TBG broth.
[i] It may be necessary to examine leafy condiments at a ratio of broth-to-sample that is greater than 1:10 because of the physical difficulties encountered by absorption of broth by the dehydrated product.
[j] Reconstituted nonfat dry milk is prepared by adding 100 g of nonfat dry milk to 1 L distilled water.

and other chemical substances in an attempt to improve media selectivity and/or sensitivity. Gadberry and Highby[62] incorporated dimethylchlortetracycline hydrochloride into BG agar and reported greater inhibition of species of *Proteus, Providencia,* and *Citrobacter.* Sulfadiazine, sulfapyridine, or sulfanilamide reportedly improves BG agar selectivity.[63,91,97,138] Moats and Kinner[92] subsequently found that addition of hydrogen sulfide detection system to BG agar aided in the identification of *Salmonella* colonies, particularly in the presence of large populations of competing microorganisms. Addition of novobiocin to XLD agar or to HE agar resulted in improved media selectivity[67,106] and increased rates of *Salmonella* recovery.[106]

Variations in the preparation of the selective agars are common and can adversely affect media performance. Although Difco[38] recommends using freshly prepared plates of BS agar, Cook[15] reported that such plates were inhibitory to several *Salmonella*

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serovars; such inhibition decreased upon storage of prepared plates under refrigeration for up to 5 days before use. McCoy[89] found that freshly poured BS plates were too inhibitory for serovars other than *S. typhi*. D'Aoust[24] also reported that inoculated plates of freshly poured BS agar required incubation for 48 hr to yield *Salmonella* recoveries similar to those obtained on homologous refrigerated plates. When a manufacturer's directives for medium preparation differ from those described in a reference method, the reference method should be followed. Where such directives are not specified in the analytical method, the media should be prepared according to the manufacturer's instructions.

The selective plating media are inoculated by streaking a loopful of the enrichment culture onto the dry surface of prepared plates to obtain well-separated colonies. Plates are usually incubated at 35° or 37°C. Incubation at an elevated temperature (41.5°C) was reportedly advantageous in recovering different *Salmonella* serovars on BG agar and *S. typhi* on XLD and HE agars.[104,134] Wilson et al.,[132] however, reported that incubation of BS agar plates at 43°C resulted in the appearance of small atypical *Salmonella* colonies and, in several instances, reduced sensitivity. Earlier results had also shown significantly lower recoveries on BS plates incubated at 43°C than at 35°C.[61] DeSmedt and Bolderdijk[35] introduced a motility enrichment on MSRV medium incubated at 42°C. With this technique, a serologically confirmed *Salmonella* result can be obtained 48 hr after the beginning of pre-enrichment.

Inoculated plates are usually incubated 18 to 24 hr. BS agar plates should be observed after 24 hr of incubation, and re-incubated for additional 24 hr before making a final determination on the presence or absence of *Salmonella* in the test material.

37.126 Biochemical Media

Biochemical screening media are nonselective and generally establish different biochemical traits of the test culture through color change(s) in the medium. Such data generally pertain to the production of hydrogen sulfide or to the utilization of one or more fermentable carbohydrates in the medium. Additionally, inoculated differential media also provide an inoculum for subsequent biochemical and serological testing.

Two differential agar tube media are widely used. Triple sugar iron agar (TSI) measures production of hydrogen sulfide and utilization of glucose, lactose, and sucrose,[114] whereas lysine iron agar (LIA) monitors production of hydrogen sulfide and decarboxylation of lysine.[42] These two agars are usually used in combination and provide a preliminary biochemical screening of suspect cultures. It is important to recognize that some of the TSI reactions may be redundant with those observed on plating media. Cultures that appear to be contaminated on these agar tube media should be restreaked on an appropriate selective plating agar and a small portion of a single colony exhibiting typical characteristics transferred to the differential media for repeat testing. Other *Salmonella* differential media include dulcitol lactose iron agar[118]; malonate dulcitol lysine iron agar[113]; DMS agar[10]; dulcitol-malonate-phenylalanine agar[45]; and selective Padron-Dockstader agar.[98]

37.13 General Description for the Identification of Salmonella

37.131 Biochemical Tests

Many biochemical tests are available for the characterization of foodborne isolates, obtained from food products. A comprehensive list of biochemical and nutritional characters of the family *Enterobacteriaceae* is found in *Bergey's Manual of Systematic Bacteriology*,[83] and many of these tests have been described in detail by Ewing.[46] Presumptive identification of typical *Salmonella* can be obtained from a minimal number of biochemical tests that may include TSI/LIA reactions, urease, KCN, and indole. The urease, lysine decarboxylase, growth in KCN, and indole tests are generally sufficient for presumptive identification of salmonellae.[8,128] Note: Final identification rests with serological tests, not biochemical tests, because *Salmonella* strains do not always produce typical biochemical reactions.

37.132 Commercial Biochemical Multitest Systems or Kits

Many biochemical tests are routinely used to differentiate members of the family *Enterobacteriaceae* and to characterize presumptive *Salmonella* isolates. Commercially available multitest systems or diagnostic kits are more convenient than conventional tube systems and generally provide reliable test results.[16,17] The API 20E, Minitek, and Enterotube systems received AOAC approval in 1978,[99] the Micro-ID Kit in 1989,[76] and the Vitek GNI + system in 1991.[79] Other miniaturized systems have shown a high degree of correlation with conventional tube tests but have not been subjected to AOAC collaborative studies. These and other diagnostic systems and kits are thoroughly reviewed in Chapter 10. Note: Complete identification of *Salmonella* should not be based solely on biochemical tests because strains do not always produce typical biochemical reactions (Tables 1 and 2).

37.133 Confirmatory Serological Tests

The genus *Salmonella* is characterized serologically by specific antigenic components.[66] The antigens are divided into somatic (O), flagellar (H), and capsular (K) antigens. The somatic (O) antigens are composed of lipopolysaccharide complexes that are heat stable and resistant to alcohol and dilute acid. The proteinaceous flagellar (H) antigens are heat labile, whereas the surface K antigens consist of heat-sensitive polysaccharides that occur in the capsule or in the outer membrane of the bacterium. K antigens such as the Vi antigen tend to inhibit somatic (O) agglutination reactions and must be thermally denatured before undertaking somatic agglutination reactions. For further information concerning *Salmonella* antigens and antisera, see Edwards and Ewing,[41] Kauffmann,[75] and ICMSF.[72]

Since other members of the *Enterobacteriaceae* e.g., *Citrobacter* and *Providencia*, have related somatic (O) antigens, false-positive serological tests may be encountered, on occasion, upon testing unknown isolates with *Salmonella* antisera. This problem is diminished when more specific single factor or adsorbed antisera are used. Definitive serotyping of a culture should be performed by specially trained personnel working in a reference laboratory.

Concurrent serological confirmation and biochemical characterization of a single colony forming unit by some biochemical kits can be completed within 8 to 24 hr.[16,17]

37.14 Rapid Cultural Methods

Early attempts to reduce the time required for *Salmonella* analysis involved the use of short incubation times. Abbreviated incubation of pre-enrichment (≤6 hr) is desirable because it would allow presumptive identification of salmonellae one day earlier compared to standard culture methods employing 18 to 24-hr pre-enrichment. However, attempts to reduce pre-enrichment incubation to 6 hr resulted in an unacceptable number of false-negative results compared to 18 to 24-hr pre-enrichment.[25,29]

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Combining pre-enrichment and enrichment steps into a single analytical procedure also has been proposed to reduce analytical time. In this approach, selective agents were added to the pre-enrichment medium, either gradually through the use of time-release capsules[115] or by direct addition after 4-hr incubation.[116] The time-release capsule method was not effective in reducing analytical time required for analysis, presumably because of the inconsistency in the dissolution of the capsules from lot to lot. In a subsequent study, Sveum and Kraft[116] added selective agents to a nonselective basal medium after 4 hr of incubation and continued incubation for a total of 24 hr at 35°C. Results with pure cultures of heat-injured and freeze-injured salmonellae and with artificially contaminated foods compared favorably with the standard culture method. However, this novel approach has not found wide application, possibly because addition of selective agents after 4-hr incubation is inconvenient and could result in a longer workday (>8 hr). The need for large quantities of selective agents to convert pre-enrichment cultures into selective enrichment broths would significantly increase analytical costs.

Reduced incubation of selective enrichment broths also has been investigated. One study utilizing a 6-hr selective step following overnight pre-enrichment yielded results similar to that obtained with a 24-hr selective step for feeds and feed ingredients.[29] However, use of a 6-hr enrichment with raw and processed foods resulted in a high percentage of false-negative results compared to that obtained after 24 hr of incubation.[34] The influence of reduced incubation time (≤8 hr) on productivity of tetrathionate and Rappaport broths for detection of *Salmonella* in naturally- and artificially-contaminated foods was examined by Rappold et al.[103] Modified Rappaport broth identified a similar number of *Salmonella*-positive samples after 8 hr and 24 hr of incubation. With tetrathionate broth, all but 3 of 27 contaminated samples were positive after 8 hr of incubation. Thus, the use of reduced incubation of selective enrichment periods may provide adequate detection under certain conditions, but is not consistently reliable and is not recommended. Some evidence suggests that extended incubation (> 24 hr) of selective enrichment may improve detection,[40,65,130] but other data indicate no benefit and reduced recovery from extended (>24 hr) selective enrichment.[25,26,28]

Although reduced incubation of enrichments may not always provide optimal conditions for isolation of salmonellae, some evidence shows that reduced enrichment can be productive when used in combination with rapid screening methods. For example, 6-hr selective enrichment procedures for dry foods are employed in three rapid methods approved by the AOAC: hydrophobic grid membrane filtration (HGMF),[43] enzyme immunoassay (EIA),[58,59] and DNA-DNA hybridization.[57]

Most of the methods proposed for the rapid detection of *Salmonella* in foods are screening tests and designed to provide rapid negative results, but requiring cultural confirmation of presumptive *Salmonella* isolates. Ideally, a rapid screening method would be performed directly on the food product without the need for enrichment of the sample. However, none of the methods currently available possesses the required sensitivity to detect the low numbers of salmonellae usually encountered in foods. Most employ one or more enrichment steps whose purpose is the same as described in standard cultural methods; i.e., pre-enrichment allows for the recovery and growth of injured cells whereas selective enrichment favors the growth of salmonellae and inhibits the growth of competitive bacteria. Several methods also employ a third nonselective enrichment broth (post-enrichment) which results in a modest increase in cell numbers.

37.15 Association, Agency, and International Methods

37.151 Association of Official Analytical Chemists International (AOAC)

The primary objective of the AOAC is ". . .to obtain, improve, develop, test, and adopt uniform, precise, and accurate methods for the analysis of foods, drugs, feeds, fertilizers, pesticides, water, or any other substances affecting public health and safety, economic protection of the consumer, or quality of the environment. . . .".[8] To be adopted as official, a method must be 1) reliable, 2) practical, 3) available to all analysts, and 4) substantiated. The proposed method of analysis must be subjected to a collaborative study and the results from that study reviewed by an AOAC committee. If approved, the study is published in the AOAC's journal and the method is adopted as an official first action method, the first level of sanction granted by AOAC. After a minimum period of two years, the first action method is adopted as a final action method, the highest level of AOAC sanction, after all adverse comments, if any, have been satisfactorily addressed. The recommended procedure for the analysis of various foods delineated in Section 37.5 is based on AOAC methods.

37.152 U.S. Food and Drug Administration (FDA)—Bacteriological Analytical Manual

This book[129] contains the microbiological methods used in FDA laboratories to analyze foods. These methods are currently considered to be the most useful to the FDA in enforcing the provisions of the Federal Food, Drug, and Cosmetics Act.[126] Methods having official AOAC status are included in this manual. The *Bacteriological Analytical Manual* (BAM) and AOAC methods of preparing various foods for the isolation of *Salmonella* are listed in Table 3. Isolation and confirmation procedures are described in Sections 37.52 and 37.53.

37.153 Centers for Disease Control and Prevention

A manual[77] used in the CDC's course on *Salmonella* isolation procedures is available. Directions for sampling, isolation, and identification of *Salmonella* are provided.

37.154 United States Department of Agriculture

The Agricultural Research Service publishes manuals for *Salmonella* analysis of poultry[18] and animal feeds.[123] Recommended test procedures are presented, followed by descriptions of serological and biochemical confirmatory test procedures.

The Poultry Division of the Agricultural Marketing Service publishes a laboratory handbook[125] that includes directions for the analysis of eggs and egg products.

The Food Safety and Inspection Service publishes a laboratory guidebook[124] that contains a comprehensive discussion of isolation and identification procedures for *Salmonella* in foods.

37.155 International Organizations

Many organizations are concerned with microbiological criteria and safety of foods involved in international trade. Specifically, these agencies are involved in the collection and assessment of microbiological criteria for foods, and the development, study, and standardization of methods for the microbiological examination of foods. Figuring prominently in this area of endeavor are the ICMSF (of the International Association of Microbiological Societies) and the Codex Alimentarius Commission, as well as

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temporary Expert Consultations of the Joint Food Standards Programme of the United Nations' Food and Agriculture Organization and World Health Organization, and Technical Committee 34 of the International Organization for Standardization (ISO).

37.2 TREATMENT OF SAMPLE

37.21 Collection (see Chapter 2 and Section 37.122)

37.22 Holding (see Chapter 2)

37.23 Mixing and Homogenization

If the food product is frozen, thaw a suitable portion at 2° to 5°C for 18 hr before analysis, or, if rapid thawing is desired, thaw at <45°C for ≤15 min. If the food product is powdered, ground, or comminuted, mix it with a sterile spoon or other sterile equipment before withdrawing an analytical unit. A homogeneous suspension of most powdered products can be obtained by mixing the analytical unit and broth with a sterile glass rod or other appropriate sterile instrument. Homogeneous suspensions are obtained in some cases by shaking the food-broth mixture by hand or by using a mechanical shaker. Mechanical blending may be required if the food consists of large pieces. A blending time of 2 min at 8,000 rpm is usually satisfactory for most foods.

37.3 EQUIPMENT AND SUPPLIES

37.31 Equipment and Materials

1. Blender and sterile blender jars
2. Sterile 16-oz (500-mL) wide mouthed screw-cap jars, sterile 500-mL Erlenmeyer flasks, sterile 250-mL beakers, sterile glass or paper funnels of appropriate size, and containers of appropriate capacity to accommodate composited samples
3. Sterile bent-glass spreader rods
4. Balance, with weights; 2,000-g capacity, sensitivity of 0.1 g
5. Balance, with weights; 120-g capacity, sensitivity of 5 mg
6. Incubator, 35°C
7. Waterbaths, 48°C to 50°C (polyvalent flagellar and Spicer-Edwards testing; 42 ± 0.2° C (RV medium); and 43 ± 0.2° (TBG broth); waterbaths for RV medium and TBG broth should be circulating and thermostatically-controlled
8. Sterile spoons or other appropriate instruments for transferring food specimens
9. Sterile culture dishes, 15 × 100 mm, glass or plastic
10. Sterile pipettes, 1 mL, with 0.01 mL graduations; 5 and 10 mL, with 0.1 mL graduations
11. Inoculating needle and inoculating loop (about 3 mm inner diameter), nichrome, platinum-iridium, or chromel wire
12. Sterile test or culture tubes, 16 × 150 mm and 20 × 150 mm; serological tubes, 10 × 75 mm or 13 × 100 mm
13. Test or culture tube racks
14. Vortex mixer
15. Sterile shears, large scissors, scalpel, and forceps
16. Lamp (for observing serological reactions)
17. Fisher or Bunsen burner
18. pH test paper (pH range 6 to 8) with maximum graduations of 0.4 pH units per color change
19. pH meter
20. Plastic bags, 28 × 37 cm, sterile, with resealable tape. (Items 20 to 22 are needed in the analysis of frog legs and rabbit carcasses.)
21. Plastic beakers, 4 L, autoclavable, for holding plastic bag during shaking and incubation
22. Mechanical shaker, any model that can be adjusted to give 100 excursions per min with a 4-cm (1 1/2-in) stroke, such as the Eberback shaker with additional 33- and 48-cm clamp bars

37.32 Media and Reagents

For preparation of media and reagents, refer to Section 967.25 in *Official Methods of Analysis*.[8]

1. Nonselective media for pre-enrichment and propagation
   a. Lactose broth
   b. Nonfat dry milk (reconstituted)
   c. Trypticase (BBL) or tryptic (Difco) soy broth
   d. M-broth
   e. GN-broth
   f. Nutrient broth
   g. Brain heart infusion broth
2. Selective enrichment media
   a. Selenite cystine broth
   b. Tetrathionate broth (commercial formulation from BBL or Difco) with brilliant green dye
   c. Rappaport-Vassiliadis (RV) medium
3. Selective isolation media
   a. Xylose lysine desoxycholate agar
   b. Hektoen enteric agar
   c. Bismuth sulfite agar
   d. MacConkey agar
4. Media for biochemical characterization of isolates
   a. Triple sugar iron agar
   b. Tryptone (tryptophane) broth
   c. Trypticase (tryptic) soy broth
   d. Lauryl tryptose broth
   e. Trypticase soy-tryptose broth
   f. MR-VP broth
   g. Simmons citrate agar
   h. Urea broth
   i. Urea broth (rapid)
   j. Malonate broth
   k. Lysine iron agar (Edwards and Fife)
   l. Lysine decarboxylase broth
   m. Motility test medium (semi-solid)
   n. Phenol red carbohydrate broth
   o. Potassium cyanide
   p. Bromcresol purple carbohydrate broth
5. Reagents
   a. Papain solution, 5%
   b. Cellulase solution, 1%
   c. Potassium sulfite powder, anhydrous
   d. Kovacs' reagent
   e. Voges-Proskauer test reagents
   f. Creatine phosphate crystals
   g. Potassium hydroxide solution, 40%
   h. 1 N sodium hydroxide solution
   i. 1 N hydrochloric acid
   j. Brilliant green dye solution, 1%
   k. Malachite green oxalate solution, 0.4%
   l. Bromcresol purple dye solution, 0.2%
   m. Methyl red indicator
   n. Magnesium chloride solution, 40%
   o. Sterile distilled water
   p. Tergitol Anionic 7
   q. Triton X-100

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r. Physiological saline solution (sterile)
s. Formalinized physiological saline solution
t. *Salmonella* polyvalent somatic (O) antiserum
u. *Salmonella* polyvalent flagellar (H) antiserum
v. *Salmonella* somatic group (O) antisera: A, B, C_1, C_2, C_3, D_1, D_2, E_1, E_2, E_3, E_4, F, G, H, I, Vi, and other groups as appropriate
w. *Salmonella* Spicer-Edwards flagellar (H) antisera

37.33 Additional Materials and Equipment

37.331 Fluorescent Antibody Technique

1. Fluorescent microscope with exciter filter (330 to 500 nm) and barrier filter (>400 nm)
2. Multiwell slides coated with fluorocarbon material (available from Cell-Line Associates, Minotola, NJ 08341 or Clinical Sciences, Inc., 30 Troy Road, Whippany, NJ 07981); slides may also be prepared as described in the AOAC method.[8]

37.332 Hydrophobic Grid Membrane Filtration

1. HGMF filter, polysulfone membrane filter with pore size of 0.45 µm and imprinted with nontoxic hydrophobic material in a grid pattern (QA Laboratories, San Diego, CA), or equivalent
2. Filtration units for HGMF filter, equipped with 5-µm mesh prefilter to remove food particles during filtration; one unit is required for each test sample
3. Vacuum pump, water aspirator vacuum source is necessary
4. Manifold or vacuum flask

37.333 Immunodiffusion

Salmonella 1-2 TEST unit (BioControl Systems, Inc., Bellevue, WA 98005)

37.334 Enzyme Immunoassays

1. Multipipettes capable of delivering accurate amounts in ranges 50 to 250 µL
2. Waterbath, autoclave, or steamer capable of maintaining 100°C
3. Microtiter plate reader; specifications of instrument may vary with the assay and are described in the methods (Section 37.74)

37.335 DNA Hybridization

1. Micropipettes capable of accurately dispensing 0.10 mL, 0.25 mL, and 0.754 mL
2. Waterbath capable of maintaining 65°C
3. Photometer to read absorbance at 450 nm in 12 × 75-mm test tubes
4. Dipstick holders and wash basins (available from GENE TRAK Systems, Hopkington, MA 01748)

37.336 Conductance

1. Microbiological analyzer instrument operating at 35°C that measures microbial growth based on conductance changes recorded at frequency of 10 kHz (Malthus Instruments Ltd, The Manor, Manor Royal, Crawley, West Sussex, RH10 2PY, United Kingdom)
2. Automatic pipette capable of delivering 100 µL

37.4 PRECAUTIONS AND LIMITATIONS OF REFERENCE AND RAPID METHODS

37.41 Sampling

Specific sampling plans for *Salmonella* have been in place for several years.[71,94,129] To maintain the statistical significance of these plans, they should be used exactly as directed, without modification. Particular attention should be given to instructions for compositing samples. The maximal number of 25-g analytical units that may be composited is 15, resulting in composites weighing 375 g.[128]

In applying the soak method for the analysis of low-moisture, powdered foods, be aware that composited samples of certain foods such as non-instant nonfat dry milk, dry whole milk and soy flour are not readily wetted. In these cases, 25-g analytical units should not be composited but analyzed individually.

37.42 Media Preparation and Disposal

Unreliable laboratory results can arise from improper preparation of laboratory media and absence of appropriate controls that form an integral part of sound laboratory quality assurance programs.

A positive medium control ensures that no substances in the laboratory medium are inhibitory to salmonellae. This control is prepared by inoculating the medium with a suitable strain of *Salmonella* and proceeding through the entire analytical protocol used for test sampling.

A negative medium control ensures that the prepared medium is not contaminated with salmonellae. This control is prepared by carrying a flask of the uninoculated medium through the analytical procedure used for test samples.

In addition to the above, the following specific points should be considered in the preparation of media for *Salmonella* isolation:

1. Potential toxicity of various lots of brilliant green dye should be determined using appropriate laboratory strains of salmonellae.
2. Flasks of TBG broth should be shaken frequently during dispensing into culture tubes because calcium carbonate tends to settle.
3. SC broth should be dispensed in 16 × 150-mm tubes to a depth of at least 5 cm, since this medium is most efficient in an environment of reduced oxidation-reduction potential.[98]
4. Plates of BS agar should be made the day before use since freshly prepared agar may be inhibitory for *Salmonella*. Prepared plates should be stored at 4° to 5°C and tempered to room temperature before use to avoid condensation of moisture on the surface.
5. LIA slants should be prepared with a deep butt (4 cm) since lysine decarboxylation reactions are more reliable under microaerophilic conditions.
6. KCN should not be stored under refrigeration longer than 2 weeks since it may become unstable and give false-positive results.
7. TSI tubes should be capped loosely; otherwise erroneous reactions occur. By definition/convention, H_2S production by *Salmonella* is defined using TSI.
8. In many localities, the selenium formed during the incubation of SC broth is considered a toxic waste. Therefore, local environmental guidelines concerning disposal of selenite should be followed at all times.

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37.43 Conventional Culture Procedure

For the analysis of a test sample, the following specific points should be considered:

1. The actual rpm speed of a food blender should be determined by a tachometer or similar device. Use of a blending speed higher than that recommended in Section 37.23 may result in injury or death of *Salmonella* organisms. To avoid spillage, initiate blending at lowest speed for a few seconds, and then gradually increase to the recommended blending speed.
2. If it is necessary to thaw a frozen sample for analysis, waterbath temperature must not exceed 45°C.
3. Modified Rappaport medium (RV medium) has replaced SC broth as a selective enrichment broth for raw flesh and highly contaminated foods and animal feeds. Because this medium is less selective than some others, it works best at an incubation temperature of 42°C.
4. Many procedures call for incubating enrichment broths at 43°C. While this elevated temperature of incubation is a valuable tool for recovery of *Salmonella*, care should be taken to assure that incubators do not operate above 43°C. A recording thermometer should be used to monitor the temperature.
5. Freshly prepared BS agar is often too inhibitory for serovars other than *S. typhi*. Storage of BS agar plates in the refrigerator for one to 5 days before use will greatly decrease this toxicity and improve the efficiency of the medium.
6. For sample types that contain many competing organisms such as *Proteus*, addition of novobiocin to HE and XLD agars at a concentration of 15 ppm will greatly improve the efficiency of these media.
7. Analysts should be aware that atypical *Salmonella* strains, e.g., *S. arizonae* and other lactose/sucrose-positive and H_2S-negative *Salmonella* biotypes, may be encountered on differential agar media. Lactose- and/or sucrose-positive cultures may resemble coliforms on HE, XLD, and TSI agars. TSI cultures with an acid slant should not be discarded as non-*Salmonella* but further screened biochemically and serologically.
8. The center of suspect *Salmonella* colonies on plating media should be picked lightly to avoid transfer of variable non-salmonellae that may lie under or adjacent to the suspect *Salmonella* colony.
9. TSI slants should be capped loosely during incubation for maintenance of aerobic conditions to prevent erroneous acid reactions on slant and excessive H_2S production in the butt of the tube. If heavy H_2S production masks the reaction in the butt, glucose utilization should be assumed.
10. Caps of inoculated lysine decarboxylase broth should be replaced tightly. Lysine decarboxylation occurs anaerobically, and exclusion of air will eliminate false-positive reactions resulting from oxidative deamination of peptones in the medium. For LIA slant and lysine decarboxylation broth reactions, only a distinct yellow color in the butt or broth should be considered negative. Cultures giving weak, indeterminate reactions in the decarboxylase broth medium should be retested by adding a few drops of 0.2% bromcresol purple dye and reading immediately.
11. If the purity of the TSI slant culture is in doubt, streak the culture to MacConkey, HE, BG sulfa, XLD or similar agars and repeat the TSI test using a well isolated colony.
12. For each type of biochemical determination, a tube of uninoculated medium (negative medium control) should be included.
13. Only the AOAC approved diagnostic kits described in Section 37.132 should be used for the rapid biochemical characterization of *Salmonella*.

37.44 Rapid Screening Methods

37.441 Fluorescent Antibody Technique

1. This method is recommended only as a screening test for the presence of *Salmonella* because the antibodies may produce false-positive reactions by cross-reacting with other members of the *Enterobacteriaceae* and through nonspecific reactions. Consequently, presumptive FA-positive enrichment cultures must be streaked onto plating media and suspect colonies confirmed by biochemical and serological techniques described in Section 37.5. Interpretation of FA results is highly subjective and requires trained analysts.
2. The method is subject to variations in the preparation of smears, conjugate, and other reagents, and misalignment of the fluorescent microscope may lead to erroneous results.

37.442 Hydrophobic Grid Membrane Filtration Described in Section 37.72

1. In this technique, TBG broth medium (10 mL) is inoculated with 0.1 mL of pre-enrichment culture instead of the more standard 1.0 mL transfer volume; larger volumes do not provide favorable *Salmonella* to non-*Salmonella* ratios in TBG broth after a 6-hr incubation.
2. Tubes of TBG broth are inoculated with a portion of pre-enrichment culture and incubated at 35° to 37°C in a waterbath, rather than in a convection incubator, because a full 6-hr incubation period is needed for *Salmonella* organisms to reach detectable levels.
3. Growth units on HGMF filters should be picked with a fully cooled sterile needle.

37.443 Immunodiffusion: 1-2 Test™ Described in Section 37.73

1. This method is a screening test for foodborne *Salmonella*.
2. Nonmotile *Salmonella* strains will not be detected because the method is based on the migration of salmonellae in a semi-solid medium.
3. The chamber plug that seals the opening between the motility chamber and the TBG broth inoculation chamber must be removed prior to inoculation of the 1-2 Test. Failure to remove this plug will prevent *Salmonella* from migrating from the inoculation site to the motility chamber.
4. The gel former tip, an interior protrusion of the cap of the motility chamber, must be cut off to prevent displacement of the added antibody solution.

37.444 Enzyme Immunoassays (Salmonella-Tek™, TECRA™, Assurance™ Described in Section 37.74)

Although commercially available kits differ in their sensitivity and specificity, the inherent limitations of these diagnostic tools are similar.

1. These methods are screening tests for the presence of *Salmonella* in foods. The antibodies employed may produce false-

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positive reactions through cross-reactions with other *Enterobacteriaceae* or as a result of nonspecific reactions. Presumptively positive EIA samples must be confirmed by streaking homologous enrichment broths on appropriate plating media and identifying suspect colonies as described in Section 37.5.

2. Antibodies used in these diagnostic kits react with salmonellae in the "common" serological (O) groups, e.g., B, C, D, and E. Thus, a certain percentage of salmonellae reportedly produce false negative results.[26,27,32]
3. Commercially available kits contain standardized reagents. Use of other materials or procedures may yield erroneous results.
4. Positive and negative controls are included in test kits and must be included with each group of test samples. Controls must fall within acceptable ranges to validate test results.

37.445 DNA Hybridization (GENE TRAK™, Described in Section 37.75)

1. This method is a screening test for the presence of *Salmonella* in foods. Presumptively positive assays must be confirmed by streaking onto appropriate plating media and suspect colonies identified as described in Section 37.5.
2. Commercially available kits contain standardized reagents, and use of other materials or procedures may yield erroneous results.
3. Positive and negative controls are included in the test kits and must be included with each group of samples. Controls must fall within acceptable ranges to validate test results.
4. Reagents that contain sodium azide should be flushed down sinks with an abundant supply of water. Do not pipette reagents by mouth.

37.446 Conductance Method

1. This method is a screening test for the presence of *Salmonella* in foods. Presumptively positive assays must be confirmed by streaking onto appropriate plating media and suspect colonies identified as described in Section 37.5.
2. Samples are pre-enriched in buffered peptone water-lysine-glucose broth followed by two-tube conductance assay in two selenite-based media containing trimethylamine-N-oxide (TMAO) and dulcitol (*Salmonella* Medium 1) and lysine (*Salmonella* Medium 2).
3. *Salmonella* spp. typically give large conductance changes in these two media compared to those for non-salmonellae. Competitive microorganisms will occasionally give similar conductance changes which result in false-positive screening results. Moreover, some *Salmonella* organisms may not show sufficient changes in conductance, thereby resulting in false-negative readings.

37.5 REFERENCE METHOD

This section contains the approved methods of the *Bacteriological Analytical Manual*[129] and the AOAC International[8] for the isolation and identification of foodborne *Salmonella* sp. organisms.

Methods of pre-enrichment and preparation of sample for each food are listed in Table 3.

37.51 Pre-enrichment

Aseptically open a sample container, and continue as directed under appropriate food section below.

37.511 Dried Egg Yolk, Dried Egg Whites, Dried Whole Eggs, Liquid Milk (Skim Milk, 2% Fat Milk, Whole Milk, and Buttermilk), Prepared Powdered Mixes (Cake, Cookie, Doughnut, Biscuit, and Bread), Infant Formula, and Oral or Tube Feedings Containing Egg

1. For non-powdered foods, add 225 mL of sterile lactose broth to 25-g analytical unit. For powdered foods, gradually add 225 mL of sterile lactose broth in small increments to 25-g analytical unit. Add a small portion (15 mL) of sterile lactose broth and mix with a suitable sterile stirrer to obtain a homogeneous suspension. Repeat this procedure three times and then add the remainder of the lactose broth. Stir until a lump-free suspension is obtained.
2. Cap the container securely, and let stand at room temperature for 60 min.
3. Mix well by swirling, and determine the pH with appropriate test paper.
4. Adjust the pH, if necessary, to 6.8 ± 0.2 with 1 N sodium hydroxide or 1 N hydrochloric acid.
5. Loosen the container cap about one-quarter turn, and incubate 24 ± 2 hr at 35°C.
6. Continue as directed under Section 37.52.

37.512 Eggs

37.5121 Contents of shell eggs

1. Wash eggs with stiff brush and drain. Soak in 200 ppm Cl^- solution containing 0.1% sodium dodecyl sulfate (SDS) for 30 min. Prepare the 200 ppm Cl^-/0.1% SDS solution by adding 8 mL of commercial bleach (5.25% sodium hypochlorite) to 992 mL of distilled water containing 1 g of SDS. This disinfectant should be prepared immediately before analysis.
2. Crack eggs aseptically and, using a sterile egg separator, discard the whites.
3. Aseptically weigh 25 g egg yolk into a sterile 500 mL Erlenmeyer flask or other appropriate container.
4. Add 225 mL tryticase (tryptic) soy broth (TSB), and mix well by swirling.
5. Continue as directed under Section 37.511, 2 to 6.

37.5122 Liquid whole eggs (homogenized)

1. Aseptically weigh 25 g into a sterile 500 mL Erlenmeyer flask or other appropriate container.
2. Add 225 mL of TSB, and mix well by swirling.
3. Continue as directed under Section 37.511, 2 to 6.

37.5123 Hard-boiled eggs

1. Aseptically separate the egg yolk from the egg white.
2. Weigh 25 g pulverized egg yolk solid into a sterile 500 mL Erlenmeyer flask or other appropriate container.
3. Add 225 mL of TSB, and mix well by swirling.
4. Continue as directed under Section 37.511, 2 to 6.

37.513 Nonfat Dry Milk

37.5131 Instant

1. Aseptically weigh 25-g analytical unit into a sterile beaker (250 mL) or other appropriate container.
2. Using a sterile glass or paper funnel (made with tape to withstand autoclaving), pour the 25-g analytical unit gently and slowly over the surface of 225 mL of brilliant green water contained in a sterile 500 mL Erlenmeyer flask or other appropriate container. Alternatively, 25-g analytical units may be composited and poured over the surface of proportion-

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ately larger volumes of brilliant green water. Prepare brilliant green water by adding 2 mL of 1% brilliant green dye solution per 1,000 mL of sterile distilled water.
3. Allow container to stand undisturbed for 60 ± 5 min.
4. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 hr at 35°C.
5. Continue as directed under Section 37.52.

37.5132 Non-instant

Examine as described for instant nonfat dry milk, except that the 25-g analytical units may not be composited.

37.514 Dry Whole Milk

1. Aseptically weigh 25-g analytical unit into a sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container.
2. Add 225 mL of sterile distilled water, and mix well by stirring.
3. Cap the jar securely, and let stand for 60 min at room temperature.
4. Mix well by swirling, and determine the pH with test paper.
5. Adjust pH, if necessary, to 6.8 ± 0.2. Cap the jar securely, and mix well before determining the final pH.
6. Add 0.45 mL of a 1% aqueous brilliant green dye solution and mix well.
7. Loosen the jar cap 1/4 turn, and incubate the jar for 24 ± 2 hr at 35°C.
8. Continue as directed under Section 37.52.

37.515 Casein

1. Aseptically weigh 25-g analytical unit into sterile blender jar.
2. Add 225 mL of sterile lactose broth to 25-g analytical unit, and blend for 2 min.
3. Aseptically transfer blended homogenate into a sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container.
4. Continue as directed under Section 37.511, 2 to 6.

37.516 Soy Flour

1. Aseptically weigh 25-g analytical unit into a sterile beaker (250 mL) or other appropriate container.
2. Using a sterile glass or paper funnel (made with tape to withstand autoclaving), pour 25-g analytical unit gently and slowly over the surface of 225 mL of lactose broth contained in a sterile 500 mL Erlenmeyer flask or other appropriate container. Analytical units (25 g) may not be composited.
3. Allow container to stand undisturbed for 60 ± 5 min.
4. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 hr at 35°C.
5. Continue as directed under Section 37.52.

37.517 Egg-Containing Products (Noodles, Egg Rolls, Macaroni, Spaghetti, Cheese, Dough); Prepared Salads (Ham, Egg, Chicken, Tuna, Turkey); Fresh, Frozen, or Dried Fruits and Vegetables; Nut Meats; Crustaceans (Shrimp, Crab, Crayfish, Langostinos, Lobster); and Fish

1. Preferably, do not thaw frozen foods before analysis. If frozen foods must be tempered to obtain an analytical portion, thaw below 45°C for ≤ 15 min with continuous agitation in thermostatically-controlled waterbath, or thaw within 18 hr at 2–5°C.
2. Aseptically weigh 25-g analytical unit into a sterile blending container.
3. Add 225 mL of sterile lactose broth and blend for 2 min.
4. Aseptically transfer blended homogenate into sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container.
5. Continue as directed under Section 37.511, 2 to 6.

37.518 Dried Yeast

1. Aseptically weigh 25-g analytical unit into a sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container.
2. Add 225 mL of sterile TSB, and mix well to form a smooth suspension.
3. Continue as directed under Section 37.511, 2 to 6.

37.519 Spices

37.5191 Black pepper, white pepper, celery seed or flakes, chili powder, cumin, paprika, parsley flakes, rosemary, sesame seed, thyme, and vegetable flakes

1. Aseptically weigh 25-g analytical unit into a sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container.
2. Add 225 mL of TSB, and mix well by swirling.
3. Continue as directed under Section 37.511, 2 to 6.

37.5192 Onion flakes, onion powder, garlic flakes, and garlic powder

1. Aseptically weigh 25-g analytical unit into a sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container.
2. Pre-enrich spice in TSB with added K_2SO_3 (5 g K_2SO_3 per 1,000 mL of TSB, resulting in final 0.5% K_2SO_3 concentration). Add K_2SO_3 to broth before autoclaving 225-mL volumes in 500-mL Erlenmeyer flasks at 121°C for 15 min. After autoclaving, aseptically determine and, if necessary, adjust the final volume to 225 mL with sterile broth. Add 225 mL of sterile TSB with added K_2SO_3 to the 25-g analytical unit, and mix well.
3. Continue as directed under Section 37.511, 2 to 6.

37.5193 Allspice, cinnamon, cloves, and oregano

1. Examine allspice, cinnamon, and oregano at 1:100 spice/broth ratio and cloves at 1:1000 spice/broth ratio. Examine leafy condiments at a spice/broth ratio that is greater than 1:10.
2. Examine these spices as directed under Section 37.5191.

37.5194 Candy and candy coating (including chocolate)

1. Aseptically weigh 25-g analytical unit into a sterile blending container.
2. Add 225 mL of sterile, reconstituted nonfat dry milk, and blend 2 min.
3. Aseptically transfer homogenized mixture to sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container.
4. Continue as directed under Section 37.511, 2 to 4.
5. Add 0.45 mL of a 1% aqueous brilliant green dye solution, and mix well.
6. Loosen the jar caps 1/4 turn, and incubate 24 ± 2 hr at 35 C.
7. Continue as directed under Section 37.52.

37.5195 Coconut

1. Aseptically weigh 25-g analytical unit into a sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container.

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2. Add 225 mL of sterile lactose broth, and shake well.
3. Continue as directed under Section 37.511, 2 to 4.
4. Add up to 2.25 mL of steamed (15 min) Tergitol Anionic 7 (undiluted) or Triton X-100 (undiluted), and mix well.
5. Loosen the jar caps about 1/4 turn, and incubate 24 ± 2 hr at 35 C.
6. Continue as directed under Section 37.52.

37.5196 Gelatin

1. Aseptically weigh 25-g analytical unit into sterile, wide-mouth Erlenmeyer flask (500 mL) or other appropriate container.
2. Prepare a 5.0% papain solution (add 5 g of papain to 95 mL of sterile distilled water). Add 225 mL of lactose broth and 5 mL of the 5% papain solution to the 25-g analytical unit, and mix well by swirling.
3. Cap the jar securely, and incubate at 35°C for 60 min.
4. Continue as directed under Section 37.511, 3 to 6.

37.5197 Guar gum

1. Aseptically weigh a 25-g analytical unit into a sterile beaker (250 mL) or other appropriate container.
2. Prepare a filter-sterilized 1.0% cellulase solution (add 1 g of cellulase to 99 mL of distilled water). Sterilize by filtration through a 0.45-µm membrane.
3. Add 225 mL sterile lactose broth and 2.25 sterile 1% cellulase solution to sterile wide-mouth, screw-cap jar (500 mL) or other appropriate container.
4. While vigorously stirring the cellulase/lactose broth with a magnetic stirrer, pour the 25-g analytical unit quickly through a sterile glass funnel into the cellulase/lactose broth.
5. Cap jar securely, and let stand for 60 min at room temperature.
6. Incubate the loosely capped container, without pH adjustment, for 24 ± 2 hr at 35°C.
7. Continue as directed under Section 37.52.

37.5198 Meats, meat substitutes, meat by-products, animal substances, glandular products, and meals (fish, meat, and bone)

1. Aseptically weigh 25-g analytical unit into a sterile blending container.
2. Add 225 mL of sterile lactose broth and blend for 2 min, and transfer blended homogenate to sterile Erlenmeyer flask (500 mL) or other appropriate container. For foods that do not require blending (powdered, ground, or comminuted foods), add 225 mL of sterile lactose broth to a 25-g analytical unit in a sterile Erlenmeyer flask (500 mL), and swirl thoroughly.
3. Continue as directed under Section 25.511, 2 to 4.
4. Add up to 225 mL of steamed (15 min) Tergitol Anionic 7 (undiluted) or Triton X-100 (undiluted), and mix well.
5. Loosen container caps 1/4 turn, and incubate food mixtures for 24 ± 2 hr at 35°C.
6. Continue as directed under Section 37.52.

37.52 Isolation of Salmonella

Tighten the lid and gently shake the incubated sample mixture. For foods with a high microbial load, transfer a 0.1-mL portion of the preenrichment cultures to 10 mL of RV medium and another 1.0 mL to 10 mL of TBG broth. For foods with a low microbial load, transfer replicate 1.0-mL portions to 10 mL of SC broth and to 10 mL of TBG broth.

For foods with a high microbial load, incubate RV medium for 24 ± 2 hr at 42 ± 0.2°C (waterbath for better temperature control) and TBG broth for 24 ± 2 hr at 43 ± 0.2°C (waterbath). For foods with a low microbial load, incubate SC and TBG broths for 24 ± 2 hr at 35°C (air incubator).

Vortex enrichment culture tubes and streak 3-mm loops of SC culture on BS, HE, and XLD agars.

Repeat plate inoculations with TBG and RV (as appropriate) enrichment cultures.

Incubate plates 24 ± 2 hr at 35°C.

Examine plates for suspect *Salmonella* colonies.

1. *BS agar.* Typical *Salmonella* colonies occur as black colonies with or without a metallic sheen. Medium surrounding *Salmonella* colonies gradually changes from brown to black with increased incubation to produce the so-called halo effect.
2. *HE agar.* Salmonellae generally produce blue-green to blue colonies with or without black centers. Strong H_2S-producing strains may produce colonies with large, glossy black centers or appear as completely black colonies. Atypical lactose-positive and/or sucrose-positive *Salmonella* strains and coliforms produce salmon-colored colonies.
3. *XLD agar.* Typical salmonellae occur as pink colonies with or without black centers. Strong H_2S-producing strains may yield colonies with large, glossy black centers or appear as completely black colonies. Atypical lactose-positive and/or sucrose-positive *Salmonella* strains and coliforms produce yellow colonies with or without black centers.

Inoculate two or more suspect colonies from each selective plating medium into TSI and LIA slants. BS agar plates with no suspect colonies or with no growth should be incubated for an additional 24 hr.

Touch the center of a suspect colony with a sterile inoculating needle and inoculate a TSI slant by streaking the slant and stabbing the butt. Without flaming the needle, inoculate an LIA slant by stabbing the butt twice and then streaking the slant. Loosen cap of TSI slant and close firmly LIA slant.

Incubate TSI and LIA slants at 35°C for 24 ± 2 hr. *Salmonella* in TSI cultures typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar). However, some lactose and/or sucrose-positive salmonellae will produce atypical reactions; i.e., acid slant and acid butt, with or without blackening. In LIA, *Salmonella* typically produces an alkaline (purple) butt from decarboxylation of lysine. Consider only a distinct yellow butt as a negative reaction. Do not eliminate cultures that produce yellow (lysine negative) discoloration of the butt solely on the strength of this reaction because lysine negative biotypes have been reported. Most *Salmonella* cultures produce H_2S in LIA.

Re-examine 48-hr BS agar plates for suspect *Salmonella* colonies. Pick two or more of these colonies and continue procedure.

Retain all presumptive-positive TSI cultures (alkaline slant and acid butt) for further biochemical and serological testing, whether corresponding LIA reaction is positive (alkaline butt) or negative (acid butt). Do not exclude a TSI culture that appears to be non-*Salmonella* if the corresponding LIA is typical (alkaline butt) for *Salmonella*. Treat these cultures as presumptive-positive and submit them to further biochemical/serological examination. LIA is useful for the detection of *S. arizonae* and atypical strains of *Salmonella* that utilize lactose and/or sucrose. Discard only cultures that appear not to be *Salmonella* on TSI (acid slant and acid butt) if corresponding LIA reactions are negative (acid butt) for *Salmonella*. Test retained presumed-positive TSI cultures as directed in Section 37.53 to determine if they are *Salmonella*. If original TSI cultures failed to indicate the presence of *Salmonella*, inoculate

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additional suspect colonies from appropriate selective agar plates into fresh TSI and LIA slants.

Apply biochemical and serological identification tests to three presumptive TSI cultures recovered from each set of plates streaked from SC, TBG, and RV broth cultures. Examine a minimum of six TSI cultures for each 25-g analytical unit.

37.53 Identification of Salmonella

37.531 Mixed TSI Cultures

Streak TSI cultures that appear to be mixed on MacConkey, HE, or XLD agar. Incubate plates 24 ± 2 hr at 35°C and examine plates for the presence of suspect *Salmonella* colonies.

1. *MacConkey agar*. Typical colonies appear transparent and colorless, sometimes with opaque center. *Salmonella* will clear areas of precipitated bile caused by lactose-fermenting microorganisms.
2. *HE agar*. See Section 37.52.
3. *XLD agar*. See Section 37.52.

Transfer at least two presumptive *Salmonella* colonies from the plates used to purify mixed TSI cultures to fresh TSI and LIA slants and proceed as described in Section 37.52.

37.532 Pure TSI Cultures

1. *Urease test (conventional)*. With a sterile needle, inoculate tubes of urea broth with growth from each presumptive-positive TSI culture and incubate 24 ± 2 hr at 35°C. An uninoculated negative control should be included because urea broth can produce a false-positive reaction (purple red) on standing.
2. *Optional urease test (rapid)*. Transfer two 3-mm loops of growth from each presumptive-positive TSI culture into a tube of rapid urea broth. Incubate for 2 hr in a waterbath at 37 ± 0.5°C.

Most salmonellae are urease-negative; however, urease-positive cultures have been reported. Retain all urease-negative cultures for further testing.

37.533 Serological Screening with Polyvalent Flagellar (H) Antisera or Spicer-Edwards Antisera

1. Perform the polyvalent flagellar (H) test at this point to eliminate false-positive TSI cultures, or later as described in Section 37.535. Inoculate growth from each urease-negative TSI slant into 1) brain heart infusion broth and incubate 4 to 6 hr at 35°C until visible growth occurs (to test on same day); or 2) trypticase (tryptic) soy-tryptose broth and incubate 24 ± 2 hr at 35°C (to test on following day). Add 2.5 mL formalinized physiological saline solution to 5 mL of either broth culture. Select two formalinized broth cultures from each 25-g analytical unit and test with *Salmonella* polyvalent flagellar (H) antisera. Mix 0.5 mL each of appropriately diluted *Salmonella* polyvalent flagellar (H) antiserum and formalinized antigen in a 10 × 75-mm or 13 × 10-mm serological test tube. Prepare a saline control by mixing 0.5 mL formalinized physiological saline solution with 0.5 mL formalinized test culture (autoagglutination). Incubate mixtures in a waterbath at 48 to 50°C. Observe at 15-min intervals for flocculation and record final results after 1 hr. A positive tube reaction consists of agglutination/flocculation in the test mixture and absence of agglutination in the control tube. A negative reaction corresponds to absence of agglutination in both the test and control tubes. A nonspecific autoagglutination reaction produces agglutination in both the test and control tubes. Cultures giving such nonspecific results should be tested with Spicer-Edwards antisera.
2. The Spicer-Edwards serological test can be used as an alternative to the polyvalent flagellar (H) screening test. Perform Spicer-Edwards flagellar (H) antisera test using Spicer-Edwards antisera 1, 2, 3, 4, in addition to the en, L, and 1 complexes.[8]
3. If both formalinized BHI or trypticase (tryptic) soy-tryptose broth cultures are serologically-negative, repeat serological tests on additional broth cultures to obtain a minimum of two serologically-positive cultures for additional serological and biochemical testing (Sections 37.533 and 37.536). If all TSI cultures from a given sample give negative serological flagellar (H) test results, check them for motility (Section 37.539).

37.534 Serological Somatic (O) Tests for Salmonella

(Pre-test all antisera with known *Salmonella* cultures.)

1. *Polyvalent somatic (O) test*. Using a wax pencil, mark off two sections about 1 × 2-cm each on the inside of a glass or plastic petri dish (15 × 100 mm). Commercially available sectioned slides may be used. Transfer a loopful of growth from a 24-hr TSI agar culture in the upper portion of both marked sections. Add 1 loopful of physiological saline solution to the lower portion of one section (negative control) and 1 drop of *Salmonella* polyvalent somatic (O) antiserum to the lower portion of the remaining (test) section. With a sterile loop or needle, emulsify the culture in the saline solution; repeat for the test section using a sterile loop or needle. Tilt mixtures back and forth for 1 min and observe against a dark background in good illumination. Consider any degree of agglutination (clumping) as a positive reaction. A positive sample shows agglutination in the test but not in the control section. A negative sample shows no agglutination in either the control or test section. A nonspecific reaction corresponds to agglutination in both the test and control sections.
2. *Somatic (O) group tests*. Test polyvalent (O)-positive cultures using individual group somatic (O) including Vi antisera. Cultures giving a positive Vi agglutination reaction should be examined as described in Section 967.28(B) of the *Official Methods of Analysis*.[8] Record cultures that agglutinate with individual somatic (O) antiserum as positive for that group.

37.535 Interpretation of Serological Tests

Cultures producing a positive polyvalent flagellar (H) test (Section 37.533) or polyvalent somatic (O) test (Section 37.534) should be further tested with Spicer-Edwards antisera to determine flagellar antigens (Section 37.533) and somatic (O) antigens using single grouping antisera (Section 37.534). Biochemically type using a rapid diagnostic kit (Section 37.132) or by traditional biochemical tests (Section 37.536).

If a specific somatic group (O) (Section 37.534) and flagellar antigen(s) (Section 37.533) reactions are obtained with one or more TSI cultures, the sample is positive; additional biochemical testing may be performed but is not required.

TSI cultures that produce negative, variable, or nonspecific serological reactions should be tested biochemically by a rapid diagnostic kit (Section 37.132) or traditional methods (Sections 37.536 and 37.537). Subculture isolate in broth and plate on nonselective plating medium, and then retest with antisera.

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37.536 Biochemical Testing of Cultures

Following is a list of tests for the biochemical screening of *Salmonella;* kits for the biochemical characterization of *Enterobacteriaceae* may be substituted (Section 37.132).

1. *Lysine decarboxylase broth.* If the LIA test was satisfactory, it need not be repeated. Use lysine decarboxylase broth for definitive determination of lysine decarboxylase activity if culture gives doubtful LIA reaction. Inoculate broth with a small amount of growth from presumptive-positive TSI slant. Replace cap tightly and incubate 48 ± 2 hr at 35°C, but examine after 24 hr of incubation. *Salmonella* species produce an alkaline reaction (purple) throughout the medium. A negative test is indicated by yellow color throughout the medium. If the medium is discolored (neither purple or yellow), add a few drops of 0.2% bromcresol purple dye and repeat the reading.
2. *Phenol red dulcitol broth or bromcresol purple broth base with 0.5% dulcitol.* Inoculate the broth with a small amount of growth from a presumptive-positive TSI culture. Replace the cap loosely and incubate 48 ± 2 hr at 35°C, but examine after 24 hr. Most *Salmonella* species give a positive test indicated by gas formation in an inverted fermentation vial and acidity (yellow) of the medium. Production of acid should be interpreted as a positive reaction. A negative test is indicated by the absence of gas formation and a red (phenol red) or purple (bromcresol purple) color throughout the medium.
3. *Tryptone (or tryptophan) broth.* Inoculate broth with small amount of growth from TSI culture. Incubate 24 ± 2 hr at 35°C and proceed as follows:
   a. *Potassium cyanide (KCN) broth.* Transfer 3-mm loopful of 24-hr tryptophan broth culture to KCN broth. Heat rim of tube so that a good seal is formed when tube is stoppered with wax-coated cork or other material not susceptible to corrosion. Incubate 48 ±2 hr at 35°C, but examine after 24 hr. Interpret growth (indicated by turbidity) as positive. Most *Salmonella* species do not grow in this medium, as indicated by lack of turbidity.
   b. *Malonate broth.* Transfer a 3-mm loopful of 24-hr tryptone broth culture to malonate broth. Since occasional uninoculated tubes of malonate broth turn blue (positive test) on standing, include an uninoculated tube of this broth as control. Incubate 48 ±2 hr at 35°C, but examine after 24 hr. Most *Salmonella* species give a negative test (green or unchanged color) in this broth, but many members of subspecies III (*S. arizonae*) are malonate-positive.
   c. *Indole test.* Transfer 5 mL of 24-hr tryptone broth culture to a sterile test tube and add 0.2 to 0.3 mL Kovacs' reagent. Most *Salmonella* cultures give a negative reaction (no deep color at the surface of the broth). Record varying shades of orange and pink as indeterminate.
4. Classify, as "confirmed" *Salmonella,* those cultures that exhibit typical *Salmonella* reactions for test nos. 1 to 11, shown in Table 1, or produce a positive flagellar (H) agglutination test and typical biochemical profile in an acceptable biochemical identification kit (Section 37.132). If one TSI culture from a single 25-g analytical unit or from a (15 × 25 g) composite is identified as *Salmonella,* further testing of other TSI cultures from the same 25-g analytical unit is unnecessary. Cultures that contain demonstrable *Salmonella,* flagellar (H) antigens but do not have biochemical characteristics of *Salmonella* should be purified (Section 37.531) and retested, beginning as in Section 37.532.

37.537 Additional Biochemical Tests

1. *Phenol red or bromcresol purple lactose broth.* Inoculate the broth with a small amount of growth from the TSI agar slant. Examine after 24 and 48 hr of incubation at 35°C. A positive reaction consists of acid production (yellow with either phenol red or bromcresol purple) and gas production in the inverted fermentation vial. A negative reaction consists of a red (with phenol red) or purple (with bromcresol purple) color throughout the medium. Most *Salmonella* are lactose-negative.
2. *Phenol red or bromcresol purple sucrose broth.* Follow procedure described in Section 37.537, item 1. Most *Salmonella* are sucrose-negative.
3. *MR-VP broth.* Inoculate the medium with a small amount of growth from each suspect TSI slant and incubate for 48 ± 2 hr at 35°C.
   a. *Voges Proskauer test.* Transfer 1 mL broth culture to a sterile test tube and incubate the remainder of the MR-VP culture for an additional 48 hr at 35°C. Add 0.6 mL alpha-naphthol and shake well. Add 0.2 mL 40% KOH solution and shake. To intensify and accelerate the reaction, add a few crystals of creatine; read reactions after 4 hr of incubation at room temperature. Development of a pink to ruby red color throughout the medium corresponds to a positive test. Most *Salmonella* cultures are Voges Proskauer-negative.
   b. *Methyl red test.* To 5 mL or MR-VP broth (96 hr) culture, add 5 to 6 drops of methyl red indicator. Read results immediately. Most *Salmonella* cultures give a positive test, as indicated by a diffuse red color in the medium. A distinct yellow color constitutes a negative test.
4. *Simmons citrate agar.* Inoculate the agar with growth from the TSI slant, using a sterile needle. Inoculate by streaking the slant and stabbing the butt and incubate for 96 ± 2 hr at 35°C. A positive reaction consists of visible growth, usually accompanied by a color change from green to blue. Most *Salmonella* cultures are citrate-positive. A negative reaction shows little or no growth with no color change in the medium.

37.538 Identification of Salmonella cultures

Typical *Salmonella* cultures produce reactions described in Table 1 or a suitable biochemical profile in diagnostic kits and positive flagellar (H) agglutination tests. If neither of two TSI cultures carried through these tests confirms the isolates as *Salmonella,* perform tests on remaining TSI cultures from the same 25-g analytical unit.

1. Confirm, as *Salmonella,* cultures producing specific Spicer-Edwards flagellar (H) test (Section 37.533) and somatic group (O) (Section 37.534) reactions.
2. Confirm, as *Salmonella,* a culture identified as presumptive *Salmonella* according to the criteria in Table 1 or with a commercial biochemical kit and positive *Salmonella* somatic (O) and flagellar (H) tests (polyvalent or Spicer-Edwards).
3. Discard cultures classified as non-*Salmonella* with commercial biochemical kits using data base-dependent numerical profiles and that fail to give positive flagellar (H) agglutination reactions (polyvalent or Spicer-Edwards). Also discard cultures that are negative for both somatic (O) and flagellar

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(H) tests (polyvalent or Spicer-Edwards) and produce atypical reactions in biochemical tests (Table 1).

4. Send presumptive positive cultures that do not conform to 1 or 2 to a reference laboratory for definitive serotyping and identification.

37.539 Treatment of Cultures Giving Negative Flagellar (H) Test

If biochemical reactions of a flagellar (H)-negative culture strongly suggest that it could be *Salmonella*, the negative flagellar agglutination may be the result of nonmotile organisms or insufficient development of flagellar antigen. Proceed as follows: Inoculate motility test medium held in a petri dish, using a small amount of growth from the TSI slant. Inoculate by lightly stabbing the medium once about 10 mm from the edge of the plate. Do not stab to the bottom of the plate or inoculate any other portion of the plate. Incubate 24 hr at 35°C. If organisms have migrated 40 mm or more, retest as follows: Inoculate a 3-mm loopful of the growth that migrated farthest into trypticase soy-tryptose broth. Repeat polyvalent flagellar (H) or Spicer-Edwards (Section 37.533) serological tests. If cultures are not motile after the first 24 hr, incubate an additional 24 hr at 35°C; if still not motile, incubate up to 5 days at 25°C. Classify a culture as nonmotile if the motility tests remain negative. If a flagellar (H)-negative culture is strongly suspected as *Salmonella* on the basis of its biochemical reactions or appearance on plating medium (i.e., BS agar), the culture should be sent to a reference laboratory for serotyping and final identification.

37.6 INTERPRETATION OF DATA—REFERENCE METHOD

If no suspect colonies are observed on isolation media (Section 37.52), or if none of the suspect colonies picked from isolation media produce reactions indicative of *Salmonella* in TSI or LIA, then the sample is negative for *Salmonella*.

If presumptive-positive cultures are obtained in either TSI or LIA, a minimum of 6 cultures must be subjected to serological and biochemical confirmation tests. However, if the first TSI culture confirms as *Salmonella*, then the remaining 5 TSI cultures do not need to be characterized biochemically and serologically. If fewer than 6 presumptive cultures are obtained, apply confirmation tests to all presumptive-positive cultures.

Confirm, as *Salmonella*, presumptive cultures that meet criteria 1 or 2 in Section 37.538. If one or more presumptive cultures meet either criterion, the sample is positive for *Salmonella*.

Discard, as non-*Salmonella*, cultures meeting criterion 3 in Section 37.538. If all presumptive cultures examined meet criterion 3, the sample is negative.

If presumptive-positive cultures cannot be classified as "confirmed" or "non-*Salmonella*," additional tests should be performed[4] until the cultures can be definitively identified.

37.7 RAPID SCREENING TESTS

Acceptance of rapid screening methods as reliable alternatives to conventional culture methods for detection of *Salmonella* in foods generally arises from comparative studies that establish the equivalence of standard culture and rapid test methods.[2] Because of the public health, regulatory, and potential legal significance of *Salmonella* in foods, validation of method performance by a widely accepted protocol is desirable. Numerous rapid methods have been proposed for detection of *Salmonella* in foods, employing a variety of technologies ranging from relatively simple rapid biochemical screening to sophisticated nucleic acid amplification and detection systems. These technologies are discussed in Chapter 10 of this book. The objective of this chapter is to introduce those rapid methods that have been validated against a standard method using a widely recognized validation protocol.[2a,2b]

Worldwide there are numerous method validation schemes in use. The most widely recognized and accepted of these schemes are the ISO and the AOAC collaborative study program. In 1992 the AOAC Research Institute (AOAC-RI) performance tested validation program was initiated. The AOAC-RI performance tested program was developed to specifically address the validation of test kits containing proprietary components. Through the mid-1980s up to 1997, several rapid methods for *Salmonella* have been evaluated via the AOAC-collaborative study and AOAC-RI programs. A number of rapid test kits has been evaluated against the ISO *Salmonella* method, but these evaluations have been performed using various validation schemes which are accepted in specific countries, but not on an international basis. Therefore, the methods described below are limited to those validated by AOAC collaborative study and AOAC-RI programs. These two validation schemes differ principally in that the AOAC-collaborative study program involves several laboratories, whereas the AOAC-RI requires independent laboratory verification of selected claims made by the manufacturer of the test kit. Both programs require that the manufacturer of the kit supply an extensive amount of data to verify performance parameters, e.g., sensitivity, specificity, range of application to products; inclusivity with regard to *Salmonella;* and exclusivity to competing organisms. The AOAC-RI program also includes verification of the manufacturer's quality assurance systems for production, shelf life of the kit, and ruggedness testing of the method across a range of conditions that might be encountered in routine use.

Most of the validated rapid methods are screening tests that require confirmation. In other cases, the rapid method may be as sensitive in detection of salmonellae as the cultural method but does not produce identical results; i.e., neither method provides 100% detection, but false-negative rates are similar. In comparing data, consider that the basis for detection of *Salmonella* by rapid methods may be different from that for isolation by culture methods. Positive results by culture methods require that three cultural conditions be met. First, *Salmonella* must grow to the level necessary to assure that cells are transferred into selective enrichments. Second, a sufficient number of salmonellae must be present in at least one of the selective enrichment cultures to ensure that sufficient *Salmonella* cells are streaked onto plates. Third, the relative proportion of salmonellae to other organisms capable of growth on the selective/differential isolation media must be such that at least one isolated colony of *Salmonella* can be obtained. Thus, the basis for obtaining positive results by culture is primarily selectivity, but the importance of pre-enrichment for resuscitation of injured cells cannot be minimized. Rapid screening methods require that a sufficient number of salmonellae/mL be present to allow detection by DNA hybridization, serological, or biochemical tests. High numbers of competitors might inhibit growth of salmonellae to the level necessary for detection by the rapid methods as well as prevent isolation by standard culture conditions. However, under certain conditions, sufficient numbers of salmonellae could develop to produce positive results by the rapid assays but not by the culture procedure because of large populations of competitive flora (e.g., swarming *Proteus*). Although it may be unrealistic to expect 100% agreement between conventional culture and rapid assay results, the high sensitivity

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of standard cultural methods for *Salmonella*[2] will generally provide for a high agreement between rapid and conventional culture methods. Rapid methods that have been evaluated by AOAC and/or AOAC-RI are discussed briefly, but not in the detail needed to perform the test. The published method in AOAC's *Official Methods of Analysis*[8] and the package insert for the kit (AOAC-RI) should be consulted for performance of the methods.

37.71 Fluorescent Antibody Method

Many of the rapid methods proposed for detection of salmonellae are based on immunological reactions. The FA technique was the first immunological method applied to the detection of salmonellae. Thomason et al.[120] reported that the O, Vi and H antigens of *S. typhi* could be stained with fluorescent-labeled antibodies and observed by fluorescence microscopy. With the advent of antisera of greater sensitivity and specificity, a protocol was developed for detection of salmonellae in foods.

The method was extensively evaluated and approved by AOAC in 1975. A historical summary of the development of the FA procedure has been published.[119] The AOAC-approved method employs a 24-hr pre-enrichment (for all foods but raw meat samples, which are placed directly in selective enrichment), and a 24-hr selective enrichment followed by a 4-hr post-enrichment.[47] Portions of the post-enrichment are then stained and examined for fluorescent cells. The presence of fluorescent cells morphologically typical of salmonellae constitutes a presumptive-positive result that must be confirmed by culture methods. Negative results are generally available one day earlier than with conventional culture methods. Thomason[119] compiled data from three studies and indicated a 97.6% agreement between standard culture methods and the FA method, and a false-negative and false-positive rate of 0.1% and 2.3%, respectively. However, some laboratories observed much higher false-positive rates, especially with certain products.[119] It was also established that staining and reading of stained slides is both laborious and subjective, requiring a high degree of technical training and experience. A detailed description of the FA method is not presented in this edition of the *Compendium of Methods for the Microbiological Examination of Foods* but appears in the second edition[73] and in the 16th edition of *Official Methods of Analysis*.[8]

37.72 Hydrophobic Grid Membrane Filtration[5]

The method uses a membrane filter impregnated with hydrophobic material in a grid pattern of 1,600 compartments. The hydrophobic material acts as a barrier, preventing the spread of "growth units" between adjacent compartments. An appropriate dilution of an enriched sample is passed through a HGMF filter and placed on a plate of appropriate agar medium. Following incubation, growth units are isolated within the grid.

HGMF has found application in the qualitative analysis of foods for *Salmonella*. Entis et al.[44] reported the use of an HGMF method for the detection of *Salmonella* in foods, and this method was subsequently subjected to an AOAC collaborative study. In the AOAC method,[8] originally approved for the analysis of six foods (nonfat dry milk, powdered eggs, cheese powders, pepper, chocolate, and raw poultry), the test sample was incubated in the appropriate pre-enrichment medium for 18 to 24 hr at 35° C. A 0.1-mL volume of the pre-enriched test sample was subcultured in 10 mL of TBG broth. After 6 to 8 hr of incubation, a 1-mL portion of diluted (raw poultry) or undiluted (the other five foods) selective TBG enrichment was filtered through the HGMF filter. The filters were then placed on selective agar plates. Following incubation of the selective lysine agar (SLA) (43°C) and the HE (35°C) agar plates for 24 hr, the filters were examined for suspect *Salmonella* growth units. If present, these growth units were confirmed by the conventional AOAC culture method.[8] Since the original collaborative study of the HGMF method, the method has been modified, and SLA and HE plates have been replaced by a single EF-18 agar plate. This method is now approved for all foods.

Use of the HGMF method has one limitation. The use of a single selective enrichment and a single selective plating agar may preclude the detection of strains that are more readily recovered by other selective enrichment broths and/or selective agars.

37.73 Immunodiffusion[33,54]

Detection of *Salmonella* in the self-contained and disposable *Salmonella* 1-2 TEST unit (BioControl Systems, Inc., Bellevue, Washington) is based on the formation of an opaque zone or band when *Salmonella* bacteria are immobilized by polyvalent H (flagellar) antibodies in a semi-solid motility medium. The clear, plastic device consists of two interconnected chambers: a motility chamber containing L-serine and a peptone-based, nonselective motility medium and an inoculation chamber containing L-serine and TBG broth.

Most food samples are pre-enriched for 24 ± 2 hr at 35°C as described in the culture reference method. The pre-enrichment culture is then seeded into the inoculation chamber of the 1-2 TEST system. Raw meats and other highly contaminated products are pre-enriched for 24 ± 2 hr at 35°C and then enriched in TBG broth and incubated 6–8 hours at 42 ± 0.5°C.[48,51,131] Enrichment cultures of raw meats and other highly contaminated products are then added to an empty inoculation chamber of the 1-2 TEST system.[8] Preparation of the 1–2 unit involves three steps. First, the TBG broth in the inoculation chamber is activated by the addition of one drop of iodine-iodide solution. Then one drop of a *Salmonella* antibody solution is added to the motility chamber.

Following removal of the chamber plug that seals the opening between the motility chamber and the inoculation chamber, a 0.1-mL portion of pre-enrichment (processed food) culture is added to the inoculation chamber. For raw meat and other highly contaminated products, 1.5 mL of the separately inoculated TBG broth is added to a previously emptied inoculation chamber. The 1-2 unit is incubated for a minimum 14 hr at 35°C. Motile *Salmonella* organisms, if present, migrate from the inoculation chamber into the motility chamber. A visible band of precipitation occurs where motile *Salmonella* and diffusing antibody interface. Such presumptive-positive reactions are then confirmed by streaking a loopful of TBG broth from the inoculation chamber onto selective plating media as described by the method.[8] Isolates are then confirmed using the reference method.

In addition to yielding presumptive-positive results for processed foods and raw meat samples one day earlier than with the conventional culture method, the *Salmonella* 1-2 TEST offers other advantages. It is easy to use, requires no specialized training or sophisticated equipment, and is readily adaptable for use in laboratories that handle small or large numbers of test samples.

Because the detection of *Salmonella* by the 1-2 TEST is based on immobilization, nonmotile serovars such as *S. gallinarum* and *S. pullorum* and other variants will not be detected.

In 1986, DeSmedt et al.[35] developed a motility enrichment technique based on the use of a semi-solid selective medium. The analytical procedure consisted of pre-enrichment for 20 hours,

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followed by motility enrichment in MSRV medium for 24 hr and serological tests on the migrated cultures. Since then, the method has undergone collaborative evaluation by comparing the method to the method of the International Office of Cocoa, Chocolate and Sugar Confectionery (IOCC) for detecting *Salmonella* in cocoa and chocolate products.[56] The rapid immunodiffusion method was at least as sensitive as the IOCC method, and, in some cases, provided superior detection of lactose-fermenting *Salmonella*. This method was subsequently compared to the AOAC reference method in a collaborative study. The method was approved by AOAC in 1992 for detection of *Salmonella* in cocoa and chocolate.[8]

37.74 Enzyme immunoassays[21,48,50,52,56,58,59]

Several EIAs have been proposed for detection of salmonellae in foods.[70,87] Several methods have been evaluated collaboratively and approved by AOAC.[49,52,56,58,59]

The first EIA method approved by AOAC was the BioEnzaBead™ method (Organon Teknika, Durham, NC, formerly Litton Bionetics). Comparative studies on the productivity of this method based on the reaction of two antibodies, MOPC 467 and 6H4, with *Salmonella* antigens were conducted by D'Aoust and Sewell,[32] Eckner et al.,[39] Flowers et al.,[61] and Todd et al.[121,122] The EIA method evaluated by Eckner et al.[38] employed a 24 ± 2-hr pre-enrichment, an 18-hr selective enrichment, and a 6-hr M-broth (35° C) post-enrichment.

Flowers et al.[52] reported on a collaborative study comparing the BioEnzaBead to the AOAC culture method. Twenty-five laboratories participated in the study, with each laboratory analyzing 12 samples of each of six food types. No significant difference appeared in the productivity of EIA and the culture procedure at the 5% probability level for any of the foods. Based on the results of this collaborative study[52] and an earlier comparative study,[59] the BioEnzaBead EIA method was approved by AOAC.[4] The approved method employed pre-enrichment in nonselective medium for 24 ± 2 hr at 35°C, selective enrichment in TBG/SC at 35°C for 18 to 24 hr, post-enrichment in M-broth for 6 hr (35°C), and a centrifugation/heating step prior to performing the EIA procedure. Subsequent work indicated that the incubation period for selective enrichment could be reduced and the centrifugation step eliminated with low-moisture foods.[59] A collaborative study was performed to compare the efficiency of the modified *Salmonella* method with the AOAC culture method.[59] The modified method required 18 to 24-hr pre-enrichment, 6 to 8-hr selective enrichment, at 35°C, and 14 to 18-hr post-enrichment at 35°C, and heating prior to EIA. The minimum time to complete the modified method was 40 hr, compared with 48 hr for the longer EIA method and 96 hr for the AOAC culture method. Based on the results of the collaborative study, which indicated no significant difference ($P > 0.05$) in detection of *Salmonella* by the modified EIA and the AOAC culture method, the modified BioEnzaBead™ method was approved for low-moisture foods by AOAC.[58]

The BioEnzaBead immunoassay originally used plastic-coated ferrous metal beads as the solid support phase. Monoclonal antibodies specific for *Salmonella* antigens were adsorbed onto the surface of the coated beads. The assay was initiated by dropping an antibody-coated metal bead into a microtiter dish well containing a heated extract of the food sample. Washing and subsequent steps in the assay were performed by moving the metal beads between plates containing wash solutions, conjugate, or substrate, using a magnetic transfer device. If *Salmonella* antigens were present in the sample, the conjugate would bind to the *Salmonella* antigens already attached to the antibody-coated bead. The beads were washed to remove any unbound conjugate and then placed in a substrate solution. Appearance of color indicated the presence of *Salmonella* antigen in the sample.

In 1988, this immunoassay was modified in favor of an antibody-coated microelisa 12-well strip-plate developed by Organon Teknika. This format (*Salmonella*-Tek™) offers several advantages over the bead assay, including the need for only one, rather than the eight, microtiter plates required by the bead assay and the use of a more sensitive substrate. Small numbers of samples can be tested by removing only the required number of strip wells. In addition, more antibody molecules are bound to the well surfaces of the strip-plate, providing for greater method sensitivity.

The *Salmonella*-Tek™ strip-plate assay was compared with the BioEnzaBead method using both inoculated and naturally contaminated samples.[73] This study demonstrated equivalent results by the BioEnzaBead assay and the *Salmonella*-Tek plate assay when compared to those obtained by the culture method. The *Salmonella*-Tek assay was more sensitive than the BioEnzaBead assay; i.e., it was capable of detecting lower levels of *Salmonella* in the post-enrichment cultures. Thus, the *Salmonella*-Tek assay can reliably replace the BioEnzaBead assay for detecting *Salmonella* in foods when the same enrichment protocol is employed. Consequently, it was recommended to AOAC that the *Salmonella*-Tek assay method replace the BioEnzaBead method and that the BioEnzaBead method be discontinued. Both assays employ the same two *Salmonella*-specific antibodies and thus should offer similar degrees of specificity. After boiling, portions of the cooled M-broth cultures are dispensed into the wells of the microelisa strips and incubated with agitation to allow capture of *Salmonella* antigens. The wells are then washed, and peroxidase-labeled conjugate binds to the captured *Salmonella* antigen (if present) to form an antibody-antigen antibody-peroxidase complex. Unbound conjugate is removed from the wells by washing, and enzyme substrate is added. If a blue color develops, the sample is presumptive-positive for *Salmonella*. The reaction is stopped with an acid solution that turns the blue color to an intense yellow. The plates are read on an EIA reader; all presumptive-positives must be confirmed culturally.

Four other EIA techniques which include enrichment protocols similar to *Salmonella*-Tek have undergone comparative and collaborative studies.

The TECRA™ EIA method (Bioenterprises Pty. Ltd., Roseville, NSW, Australia), in contrast to *Salmonella*-Tek, utilizes polyclonal, rather than monoclonal, antibodies. The assay is performed in microtiter plates or strip wells and can be read visually or spectrophotometrically.

The TECRA method is simple to perform, with no requirement for sophisticated instrumentation. Wash steps can be performed manually, and assay results can be readily obtained visually, thereby eliminating the need for an EIA spectrophotometer. Based on the results of the collaborative[55] and comparative studies,[53] the TECRA method was approved by AOAC.[6]

The Q-TROL *Salmonella* assay (Dynatech Laboratories, Inc., Chantilly, VA) utilizes monoclonal antibodies and a fluorogenic reagent. An AOAC collaborative study of the Q-TROL™ method based on 1,700 food samples, was performed in 1988.[56] Based on the results of this study, the method was approved by AOAC.[7] Subsequently, a colorimetric enzyme assay, using the substrate phenolphthalein monophosphate and the enzyme alkaline phosphatase, was developed and compared with the fluorogenic assay. The results indicated that the colorimetric assay was at least as sensitive as the previously approved fluorogenic assay.[21]

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The VIDAS SLM method for detection of *Salmonella* is an automated qualitative enzyme-linked fluorescent immunoassay. The VIDAS SLM method, including culture enrichment steps and assay, requires 48 hr for completion. The enrichment sequence is similar to other EIA methods discussed above, but has the advantage that the fluorogenic assay is fully automated. The VIDAS SLM has undergone comparative and collaborative studies and was approved by AOAC in 1997.[20]

The LOCATE EIA method for *Salmonella* (Rhone-Poulenc Diagnostics, Ltd.) received AOAC approval in 1998.[9] This is a microplate assay similar to the *Salmonella*-Tek and TECRA assays, incorporating similar enrichment steps.

The extensive evaluations of the EIA methods clearly indicate that this technology is an attractive alternative to conventional culture methods for detection of salmonellae in foods. This analytical approach provides detection of salmonellae one day earlier than by conventional culture methods, with low numbers of both false-negative and false-positive results for most processed products, although relatively high false-positive rates have been reported for some raw products.[31,32,39,52] Advantages of the method include automation and a specific end-point that contrasts with the subjective interpretation associated with culture methods.

37.75 DNA Hybridization

Genetic relatedness among related bacteria can be measured by the DNA hybridization (DNAH) technique that probes for unique nucleotide sequences in target organisms. The performance of a ^{32}P DNA hybridization assay for *Salmonella* in foods (GENE TRAK Systems, Hopkington, Mass.) was compared to the AOAC culture method.[60] The results showed that the sensitivity of the DNAH method compared favorably with the culture method under all test conditions and, in some cases, DNAH appeared to provide superior detection.

A collaborative study compared the sensitivity of the ^{32}P DNA procedure to the standard culture method for detection of *Salmonella* in six artificially contaminated foods: ground pepper, soy flour, dry whole egg, milk chocolate, nonfat dry milk, and raw deboned turkey.[57] The results of this collaborative study[60] and earlier comparative studies[52] led to an AOAC approval of the DNAH method for the rapid detection of salmonellae in foods.[5] The isotopic DNAH method includes pre-enrichment (18 to 24 hr), 6-hr selective enrichment in TBG/SC, and a 12- to 16-hr post-enrichment in GN broth for processed foods and 18-hr enrichment in TBG/SC + 6-hr GN broth for raw meats. After inoculation of the post-enrichment GN broth with 1.0 mL of enrichment (TBG/SC) culture, both TBG/SC enrichments are reincubated for confirmation for up to a total of 24 ± 2 hr. A portion of the GN enrichment culture is then filtered, and the DNAH assay performed on the filters.

Positive reactions are reported when counts for the test filter are >500 cpm above the average of three negative control filters. The assay itself requires about 4 hr to perform, and the culture steps require a minimum of 42 hr. Thus, the total time required for the DNAH method is 46 hr. The DNAH assay is a screening method, and positive results are confirmed by culture methods. Both post-enrichment and reincubated selective enrichment cultures are streaked to selective agar media for confirmation of positive DNAH assays.

Subsequent to approval of the isotopic DNAH method, a non-isotopic, colorimetric DNAH method was developed.[22] The colorimetric assay employed DNA probes different from those used in the isotopic method. The target for the colorimetric assay probes is ribosomal RNA; i.e., the assay is based on hybridization of the DNA probes to target rRNA. The assay, which is carried out in an aqueous environment, involves hybridization between rRNA and *Salmonella*-specific oligonucleotide probes, capture of rRNA target:DNA probe hybrids onto a solid support, and a colorimetric end-point detection. Following pre-enrichment, selective enrichment, and post-enrichment of test samples as described above for the DNAH assay, bacteria are lysed and *Salmonella*-specific fluorescent-labeled detection and polydeoxyadenylic (poly dA) capture probes are added. The mixture is then incubated to allow hybridization of both probes to target rRNA, if present. A polydeoxythymidylic (poly dT)-coated plastic dipstick solid phase is then introduced into the hybridization solution. Base pairing between the poly dA tail sequence of the capture probe and the poly dT on the dipstick facilitates capture of the DNA probe:rRNA target hybrids onto the dipstick. Unbound labeled probe is removed by washing the dipstick, followed by incubation in a solution containing anti-fluorescent antibody conjugated to horseradish peroxidase (HRP). The peroxidase conjugate binds to the fluorescent label on the hybridized detector probe. Unbound conjugate is washed away, and the dipstick is incubated in a substrate-chromogen solution. Reaction of HRP with substrate-chromogen results in development of a blue color. The enzyme:substrate reaction is stopped by addition of acid with a concomitant change in color from blue to yellow. Absorbance at 450 nm in excess of threshold value indicates the presumptive presence of *Salmonella* in the test samples.

The colorimetric DNAH assay can be completed in 2.5 hr following approximately 44 hr of culture enrichments. Chan et al.[14] compared the productivity of the colorimetric DNAH (cDNAH) method with the AOAC culture method,[8] and an AOAC collaborative study was performed in 11 laboratories to validate the cDNAH method.[22] No significant difference appeared in the ability of the methods to detect salmonellae in any of the six artificially-contaminated foods examined in the latter study.

37.76 Conductivity

The principle of conductance microbiology is that microorganisms growing in a broth culture increase the conductance of the medium as they metabolize neutral components to charged metabolites. This change in conductance can be measured by introducing platinum electrodes and applying a low-frequency voltage. Scanning the conductance at regular intervals generates a curve that is analogous to a growth curve. Change in conductance may arise from the metabolism of general components (e.g., peptones) or specific components (TMAO or lysine). TMAO is a neutral, uncharged molecule which, when reduced to trimethylmine (TMA), produces a large change in conductance[38]; decarboxylation of lysine produces the same result.

In a collaborative study, Gibson[65] showed no significant difference between the AOAC/BAM[8,129] and the conductance method (Malthus Instruments Ltd., The Manor, Manor Royal, Crawley, West Sussex, United Kingdom) for the detection of *Salmonella* in 6 food types by 17 participating laboratories.

37.8 AOAC-RI PERFORMANCE TESTED METHODS

Ten test kit methods for rapid detection of *Salmonella* spp. in foods have certified as Performance Tested (Table 4). Prior to 1998, AOAC-RI did not require that the data upon which the certification was based be published. Therefore, the validation data for the

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Table 4. AOAC-RI "Performance Tested Methods" for Detection of *Salmonella* in Foods and Related Products

Test kit	Manufacturer	Matrixes
Path-Stik	Lumac B.V.	food
Reveal	Neogen	food
Bioline ELISA	Bioline	food, feed
Salmonella Screen/ *Salmonella* Verify	Vicam L.P.	food, feed, stainless steel, concrete
Oxoid Rapid Test for *Salmonella*	Oxoid Ltd.	food, feed, environmental samples
Salmonella DLP Assay	GENE-TRAK Systems Corp.	food, feed, environmental samples
Dynabeads anti-*Salmonella*) Kit	Dynal S.A.	food, feed
BIND *Salmonella* Rapid Assay Kit	IDEXX Laboratories, Inc.	food, feed
Bax for Screening *Salmonella*	Qualicon	milk, chicken, turkey, beef, and pork
TECRA Unique *Salmonella*	TECRA Diagnostics	food and environmental samples

approval of methods listed (Table 4) are not readily available in the scientific literature. Information on the methods and their performance should be requested directly from the manufacturers.

A brief discussion of each method is provided below, along with the manufacturer's address.

37.81 Immunological Tests

Several of the "Performance Tested" methods are immunologically based. The *Salmonella* Screen/*Salmonella* Verify method (Vicam L.P., Watertown, MA, 0217) and the Dynabeads anti-*Salmonella* Kit (Dynal, Inc., Lake Success, NY, 11042) employ magnetic anti-*Salmonella* labeled beads to capture salmonellae from enrichment broths. The captured *Salmonella* are then plated directly onto selective/differential agar media for isolation and identification. The Vicam *Salmonella* Verify Method includes a step where colonies developing from the beads can be quickly screened by a latex agglutination technique to determine if they are presumptive *Salmonella* spp.

The TECRA Unique *Salmonella* screening test (TECRA Diagnostics, Roseville, NSW 2069, Australia) is a 22-hr dipstick test, which includes a 16-hr pre-enrichment step followed by: capture, wash, replication (4–5 hr at 35–37°C), conjugate binding, wash, and color development steps within a disposable device composed of a series of tubes containing the appropriate reagents. The Bioline ELISA (Bioline, DK-7100 Vejte, Denmark) is a 36-hr microtiter strip assay. The Reveal dipstick method (Neogen Corp., Lansing, MI, 48912) and Path-Stik methods (Celsis-Lumac Ltd., Cambridge Science Park, Cambridge CB4 4FX, UK) are other immunological dipstick methods that have received AOAC-RI approval for food testing. These methods include preenrichment and selective enrichment steps followed by the dipstick assay.

37.82 DNA Probe Assays

GENE-TRAK Systems (Hopkinton, MA) has received "Performance Tested" certification for the dipstick assay modification of their AOAC-OMA method.

Qualicon, a DuPont subsidiary (Wilmington, DE, 19880-0357) has developed a one-day *Salmonella* detection method, "BAX for Screening *Salmonella*," based on polymerase chain reaction (PCR) amplification of *Salmonella*-specific nucleic acid. After pre-enrichment (20 hr) and a short (3 hr) secondary enrichment step, cells are lysed and added to PCR tubes which contain the reagents required to perform the amplification. *Salmonella*-specific PCR gene product is detected using a gel electrophoresis procedure. The BAX *Salmonella* method requires approximately 28 hr including enrichment, amplification, and assay steps.

37.83 Other Methods

The Oxoid *Salmonella* Rapid Test (Unipath, Nepean, Ontario, K2E 7K3 Canada) is a biochemical screening method. Samples are pre-enriched (18–24 hr) in appropriate media and then aliquots transferred to a disposable culture vessel containing an elective medium and two tubes. Each tube contains a selective medium in the lower portion of the tube, and an indicator medium in the upper portion separated by a porous partition. *Salmonella* grow in the elective enrichment and migrate into the tubes through the lower selective media to the upper indicator media where their presence is indicated by a color change. After a 24-hr incubation of the enrichment/screening system, tubes are examined for the possible presence of *Salmonella*. Tubes producing positive results are screened using the Oxoid *Salmonella* Latex Test, and positive reactors reported as presumptively containing *Salmonella*.

The BIND (bacterial ice nucleation detection) *Salmonella* assay (Idexx Laboratories, Westbrook, ME, 04092) is a two-day *Salmonella* screening method which includes preenrichment followed by a simple assay system based on bacteriophage infection of *Salmonella* resulting in expression of ice nucleation proteins that lead to ice crystal formation which can be detected with a colorimetric assay.

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UNITED STATES DEPARTMENT OF AGRICULTURE
FOOD SAFETY AND INSPECTION SERVICE
WASHINGTON, DC

FSIS DIRECTIVE	10,010.1, Revision 1	3/31/04

MICROBIOLOGICAL TESTING PROGRAM AND OTHER VERIFICATION ACTIVITIES FOR *Escherichia coli* O157:H7 IN RAW GROUND BEEF PRODUCTS AND RAW GROUND BEEF COMPONENTS AND BEEF PATTY COMPONENTS

NOTE: ***FSIS PESONNEL ARE NOT TO IMPLEMENT THIS DIRECTIVE UNTIL MAY 17, 2004***

Part I – General

I. PURPOSE

This directive provides Food Safety and Inspection Service (FSIS) inspection program personnel, program investigators, and import inspection personnel instructions for sampling raw beef products as part of verification testing for *Escherichia coli* O157:H7 (*E. coli* O157:H7) to ensure the protection of public health. It also outlines actions FSIS will take when a raw ground beef product sample, raw ground beef component sample, or raw beef patty component sample is found to be positive for *E. coli* O157:H7. Attachment 1 provides questions and answers for further clarification.

II. CANCELLATION

FSIS Directive 10,010.1, dated 2/1/98
FSIS Notice 11-03, dated 4/18/03
FSIS Notice 47-02, dated 11/20/02

III. REASONS FOR REISSUANCE

This directive has been rewritten in its entirety to be consistent with the Agency's current policies regarding *E. coli* O157:H7. No establishment that produces raw ground beef products, raw ground beef components, or raw beef patty components will be exempt from FSIS sampling and testing for *E. coli* O157:H7. This directive provides new instructions: 1) for the policy that non-intact raw beef products contaminated with *E. coli* O157:H7 are adulterated; 2) for follow-up actions taken after an initial FSIS sample tests positive; and 3) for verifying the control of beef products that are presumptive positive or positive for *E. coli* O157:H7.

DISTRIBUTION: Inspection Offices; T/A Inspectors; Plant Mgt; T/A Plant Mgt; TRA; ABB; TSC;Import Offices

OPI: OPPD

IV. REFERENCES

Federal Meat Inspection Act
9 CFR 318.2, 325.10, 416, 417, and 500
FSIS Directives 5000.1, Revision 1, 5000.2, and 8080.1, Revision 3
Federal Register Notices: Policy on Beef Products Contaminated with *E. coli* O157:H7 (64 FR 2803, 1/19/99); Recent Developments Regarding Beef Products Contaminated with *Escherichia coli* O157:H7; Public Meeting (65 FR 6881, 2/11/00); Availability of and Request for Comment on FSIS Draft Risk Assessment for *Escherichia coli* O157:H7 in Ground Beef (66 FR 55912, 11/05/01); and *E. coli* O157:H7 Contamination of Beef Products (67 FR 62325, 10/7/02).

V. BACKGROUND

Non-intact raw beef products contaminated with *E.* coli O157:H7 are adulterated. Non-intact beef products include ground beef; beef that has been injected with solutions; beef that has been mechanically tenderized by needling, cubing, Frenching, or pounding devices; and beef that has been reconstructed into formed entrees. Intact raw beef products contaminated with *E. coli* O157:H7 that are intended to be processed into non-intact products are also adulterated. Establishment records and HACCP documents (e.g., the flow chart and hazard analysis) should identify the intended use of intact raw beef products. Manufacturing trimmings (e.g., pieces of meat remaining after steaks, roasts, and other intact cuts are removed) are an example of intact raw beef product that may be intended to be used for non-intact product. Raw beef products contaminated with *E. coli* O157:H7 may, however, be further processed at official establishments to destroy the pathogen.

On October 7, 2002, FSIS published a notice requiring establishments that had not already reassessed their Hazard Analysis and Critical Control Point (HACCP) plans for raw beef products in light of relevant *E. coli* O157:H7 data to do so to determine whether *E. coli* O157:H7 contamination was reasonably likely to occur in their production process for raw beef products (67 FR 62329). In that notice, FSIS advised that it intended to scrutinize very closely the hazard analyses and HACCP plans of those slaughter or deboning establishments that had conducted a reassessment and decided that an intervention was not necessary. Also in that notice, FSIS stated that establishments receiving product for grinding should address *E. coli* O157:H7. FSIS explained that these establishments could employ validated Critical Control Points (CCPs) in their HACCP plans to address *E. coli* O157:H7, or the establishments could incorporate purchase specifications in their HACCP plans, Sanitation Standard Operating Procedures (Sanitation SOPs), or other prerequisite programs to prevent *E. coli* O157:H7-contaminated product from entering their establishments.

This directive focuses on raw ground beef products and the beef products that are used to produce raw ground beef products. These products will be the focus of FSIS' verification sampling program for *E. coli* O157:H7. Products that FSIS may sample are listed in Parts II and VI.

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This directive discusses the significance of a finding that a sample is "presumptive positive." A sample is presumptive positive when analytical steps of microbiological analysis indicate the strong possibility that *E. coli* O157:H7 is present, but additional steps are needed to confirm the presence or absence of the organism.

A sample is confirmed to contain the bacterial isolate of *E. coli* O157:H7 through testing by either FSIS or non-FSIS laboratories when biochemical, serological, or genetic testing results in a finding of *E. coli* Serotype O157:H7, O157:H7:NM (nonmotile), or O157:H7-indeterminate.

FSIS recognizes that many establishments test their raw ground beef products, raw ground beef components, and raw beef patty components for *E. coli* O157:H7. The Agency applauds and encourages this practice. FSIS points out, however, that if an establishment finds a sample of one of these products to be presumptive positive for *E. coli* O157:H7, that product would only be allowed to move off site under appropriate controls for proper disposition at official establishments, landfill operations, or renderers. If the establishment's confirmation testing finds the sample negative for the pathogen, that product may be shipped in commerce under normal procedures. Product that is confirmed positive for *E. coli* O157:H7, through FSIS or establishment testing, may also be moved off site under appropriate controls for proper disposition. If product is confirmed positive, or is presumptive positive and no additional testing confirmed the product negative, such product destined for an official establishment for further processing that will destroy the pathogen would have to move under company control (e.g., through company seals) or under FSIS control (e.g., under USDA seal or accompanied by FSIS Form 7350-1). Such product destined for a landfill operation or renderer would have to move under company control.

According to 9 CFR 325.10, if product is found to be adulterated or misbranded after it has been transported from an official establishment, transportation back to the establishment that originally produced the product or to another official establishment must be authorized. According to 9 CFR 318.2(d), inspection program personnel must place a U.S. retained tag at the time of reinspection on all products suspected of being adulterated. FSIS will allow product that is positive or presumptive positive for *E. coli* O157:H7 to move under company control (e.g., through company seals) or under FSIS control (e.g., under USDA seal or accompanied by FSIS Form 7350-1), rather than as required in 9 CFR 325.10 and 318.2(d) to facilitate proper disposition of product that may be adulterated with *E. coli* O157:H7. FSIS intends to modify these regulations to reflect this policy.

5. If a sample must be held overnight, it must be refrigerated. If a sample must be held longer than overnight, it must be frozen.

6. After the establishment completes the pre-shipment review, inspection program personnel should prepare the sample to be sent to the laboratory on the first available Federal Express pick-up.

Part III – Supplier Information

A. What actions does FSIS take when there is an FSIS presumptive *E. coli* O157:H7 positive for a raw ground beef product sample?

1. Every FSIS verification sample that is eventually confirmed positive by FSIS for *E.* coli O157:H7 goes through three stages of analysis. The results of each stage are reported to IIC's on LEARN. These samples are initially screened and, as appropriate, are reported as "Potential Positives." At the next stage, based on laboratory results, some samples are reported as "Presumptive Positives." Because most "Presumptive Positives" are eventually confirmed, the contact person in the District where the establishment is located needs to immediately inform the establishment that the sample is a "Presumptive Positive." At the same time, the District contact person also informs the establishment management that if the results are confirmed positive, FSIS will collect the following information regarding the suppliers of the source materials used in the production of the product (9 CFR 320.1):

a. name of the supplying establishment, point of contact (name, title, e-mail address, and fax number), and phone number of supplying establishment;

b. supplier lot number; and

c. production date, name of supplied material, and any additional information to clearly identify the material used to the management of the supplying establishment.

2. If the source materials are from a foreign establishment, the District contact person should inform the establishment that FSIS will also collect the following information, should the product be confirmed positive for *E. coli* O157:H7:

a. country of origin;

b. foreign establishment number;

c. shipping mark;

d. I-house; and

e. barcoding or any other information that identifies the origin of the product.

3. The District contact person advises the establishment that it should begin to gather the information above, along with distribution information.

B. What information does FSIS collect when a raw ground beef product sample collected by FSIS for verification testing at an official establishment is confirmed positive for *E. coli* O157:H7, and whom does FSIS notify concerning the positive?

1. When a sample is confirmed positive, inspection program personnel collect from the establishment the information in Part III. A. Inspection program personnel make note of any information that the establishment is unable to provide.

2. Inspection program personnel forward the information by e-mail to the designated DO contact, with a "cc" to the front-line supervisor.

3. The DO will access the System Tracking *E. coli* O157:H7 – Positive Suppliers (STEPS), open a case file for the incident, and follow STEPS procedures.

4. STEPS automatically e-mails the DO that has jurisdiction over the supplying establishment. The DO notifies the IIC at the supplying establishment to perform a HACCP 02 and other activities described in Part VI.

5. The DO notifies all of the supplying establishments in the District, by telephone, of the positive finding and provides the suppliers the production date for the product that the supplier provided to the grinder, the lot number of the supplied product, and other information that would be useful to the supplier to help identify the *E. coli* O157:H7 positive lot. The DO documents the date and time of this oral notification in the STEPS system.

6. After all necessary information on the supplying establishment has been entered into the STEPS system, the DO reviews the information in the STEPS system and sends an e-mail notification to the supplier about the *E. coli* O157:H7 positive product.

7. The supplier information is maintained within the STEPS system and is maintained on FSIS' network. Users must be given access to this site.

NOTE: If the confirmed positive sample came from product which was made, in whole or in part from imported product, the DO provides information about the supplier to the Office of International Affairs (OIA), Import-Export Programs Staff, by telephone, and documents the date and time of this oral notification in the STEPS system. The DO then provides information about the supplier to OIA, Import-Export Programs Staff, through an e-mail message to importexport@fsis.usda.gov. OIA, in turn, forwards this information to the head of the inspection service in the country where the supplying establishment is located.

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Part IV – Enforcement Actions in Official Establishments

A. What actions do inspection program personnel take if an FSIS sample taken from an official establishment is confirmed positive for *E. coli* O157:H7?

1. The DO is notified of a positive through the Biological Information Transfer and E-mail System (BITES).

2. Inspection program personnel, the DO, and Recall Management Staff (RMS) work together to determine the necessity of product retention, detention, or recall. The Technical Services Center (TSC) and OPHS may also serve as technical resources to assist in the decision making process. The DO will contact inspection program personnel and program investigators as necessary (see FSIS Directive 8080.1, Revision 3).

3. As set out in FSIS Directive 5000.1, Revision 1, inspection program personnel are to:

a. issue an NR under the appropriate 03 ISP code using the "verification" trend indicator; and

b. as soon as possible after the establishment has implemented its corrective action, perform a HACCP 02 procedure for the specific production that tested positive for *E. coli* O157:H7 and verify that the establishment implements corrective action that meets the requirements of:

i. 9 CFR 417.3(a) if *E. coli* O157:H7 is addressed in the HACCP plan;

ii. 9 CFR 417.3(b) if *E. coli* O157:H7 is not addressed in the HACCP plan or if it is addressed in prerequisite programs; or

iii. 9 CFR 417.3(b) and 416.15 if *E. coli* O157:H7 is addressed in the Sanitation SOPs.

4. If disposition of the positive product will be delayed, inspection program personnel should work with their front-line supervisors to determine how to work with the establishment to ensure proper and timely disposal of the product.

5. If product disposition is to occur off site, inspection program personnel are to verify that the establishment that produced the positive product maintains appropriate control of the product by conducting the following activities when performing the 02 procedure:

a. obtaining the identity of the official establishment or obtaining the name and address of any renderer or landfill that will receive the product;

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b. notifying, through e-mail, the contact person in the District that covers the establishment that produced the positive product that adulterated product is being transferred and providing the DO contact person the establishment number of the establishment where disposition will occur or the name and address of the landfill operation or renderer. The District contact person will notify the District where the establishment that will further process the product, landfill operation, or renderer is located, if the establishment, landfill operation, or renderer that is to receive the product is located in another District;

c. for product being transferred to a landfill operation or renderer, verifying that the establishment will maintain control of the positive product while it is in transit (e.g., through company seals);

d. for product being transferred to an official establishment, verifying that either 1) the establishment that produced positive product will maintain control of the product while it is in transit (e.g., through company seals) or 2) the product will move under FSIS control (e.g., under USDA seal or accompanied by FSIS Form 7350-1); and

e. verifying that records are available that show that the positive product received the proper disposition, including documentation evidencing proper disposal of the product from the official establishment, landfill operation, or renderer where disposition occurred. The HACCP 02 procedure at the establishment that produced the positive product cannot be completed for this specific production until the establishment has conducted pre-shipment review of the corrective action record and has received documentation evidencing proper disposal from the official establishment where disposition occurred or landfill operation or renderer where disposition occurred.

6. If inspection program personnel find noncompliance with paragraph 5, they are to contact the DO. The DO will investigate to determine whether the establishment committed the prohibited act of selling or transporting adulterated articles that have not been inspected and passed.

Part V – Follow-up Sampling

A. Are FSIS follow-up samples taken at official establishments after an FSIS-sample of raw ground beef is confirmed positive for *E. coli* O157:H7?

1. If inspection program personnel identify no significant problems through the HACCP 02 procedure (see Part IV. A. 3. b.), inspection program personnel are to contact OPHS through an Outlook e-mail message to **Sampling Forms – Headquarters** mailbox, so a form can be sent for the collection of a follow-up verification sample. Inspection program personnel should copy (CC) their front-line supervisor and the DO designated representative on their e-mail message. The request must include the establishment number, the number of forms (in this case 1), the type of sample to be collected (i.e., a product sample), the purpose of the request (i.e., follow-

up sampling in response to a confirmed positive in raw ground beef), the sample form number of the original positive sample triggering this request, and the DO official approving the request. Instructions for follow-up sampling will be provided on FSIS Form 10,210-3, Requested Sample Programs, or in revisions to FSIS Directive 10,210.1, under the appropriate project. Inspection program personnel should collect the follow-up sample as soon after the establishment has taken its corrective action as possible. See Part V. A. 3., for actions to take if disposition of the positive product is delayed.

2. If inspection program personnel identify regulatory noncompliance, they should document the noncompliance in accordance with FSIS Directive 5000.1, Revision1, Chapter IV. If inspection program personnel find that the establishment may have moved positive product without appropriate controls or if they find the establishment may not have records showing that positive product received proper disposition, they should contact the DO. Inspection program personnel should also collect one follow-up sample as soon after the establishment has taken its corrective action as possible. Inspection program personnel are to contact OPHS so a form can be sent for the collection of a follow-up verification sample. See Part V. A. 1., for information on e-mailing OPHS to request a follow-up sampling form. See Part V. A. 3., for actions to take if disposition of the positive product is delayed.

3. If disposition of the positive product will be delayed, inspection program personnel should work with their front-line supervisors to determine when it would be appropriate to collect the follow-up sample and how to work with the establishment to ensure proper and timely disposal of the product.

4. If the inspection program personnel have concerns regarding whether the design of the HACCP system is adequate to ensure food safety, they should not collect a follow-up sample. They should notify their front-line supervisor, who will determine whether it is necessary to bring in an Enforcement Investigations and Analysis Officer (EIAO) to the establishment to conduct a comprehensive assessment of the food safety systems. If the EIAO determines that the establishment's corrective actions appear to be appropriate and effective, the EIAO will contact OPHS so a form can be sent to inspection program personnel for the collection of a follow-up verification sample. See Part V. A. 1., for information on e-mailing OPHS to request a follow-up sampling form. Inspection program personnel are to take the sample as soon as possible after they receive the form. See Part V. A. 3., for actions to take if disposition of the positive product is delayed.

5. If a follow-up sample is found positive, the DO is notified through BITES, and the DO will determine the appropriate follow-up action.

6. If the EIAO determines that the corrective actions are inappropriate or ineffective, the EIAO will recommend an enforcement action as described in 9 CFR 500.3 or 500.4 (e.g., Notice of Intended Enforcement (NOIE), withholding, or suspension).

EXHIBIT

7. If the District Office decides to either defer a decision on suspending the establishment, or a suspension action is taken and then put into abeyance (see FSIS Directive 5000.1, Revision 1, Chapter IV), FSIS will conduct follow-up sampling to verify that the corrective action taken by the establishment is appropriate and effective. The DO will determine the number of follow-up samples. Guidance on how to determine the number of follow-up samples will be provided to the DO. The DO should contact OPHS so the appropriate number of forms can be sent to inspection program personnel for the collection of follow-up verification samples. See Part V. A. 1., for information on e-mailing OPHS to request follow-up sampling forms. The guidance is designed to provide enhanced statistical confidence for finding low levels of *E. coli* O157:H7 but is not designed to provide validation of the establishment's food safety system.

PART VI - FSIS' Verification Activities at Establishments Producing Raw Ground Beef Components or Raw Beef Patty Components

A. If FSIS confirms raw ground beef product at an official establishment or retail facility positive for *E. coli* O157:H7, and a second official establishment supplied the product used to produce the ground product, what verification activities does FSIS conduct at the supplying establishment?

The IIC at the supplying establishment ensures that the inspection program personnel perform a HACCP 02 procedure to verify that the establishment met the applicable regulatory requirements at all CCPs in the HACCP plan (monitoring, verification, recordkeeping, corrective actions, and reassessment) for the production lots sent to the establishment or retail facility where FSIS found the positive. If inspection program personnel find noncompliance, they take appropriate action as described in FSIS Directive 5000.1, Revision1, Chapter IV.

B. If a grinding establishment or retail facility receives incoming product for grinding, and FSIS finds the raw ground product positive for *E. coli* O157:H7, will FSIS test product from suppliers? If so, how do inspection program personnel collect samples?

1. When FSIS conducts sampling at official establishments or at retail, and a sample tests positive for *E. coli* O157:H7, FSIS may test raw ground beef components and raw beef patty components at the supplying establishment.

2. If inspection program personnel are requested to collect raw ground beef components or raw beef patty component samples, they are to follow the instruction in Part II of this directive and collect samples as described in Attachment 2. The types of product inspection program personnel may collect are:

Raw Ground Beef Components: These components include raw esophagus (weasand) meat, head meat, and cheek meat; beef manufacturing trimmings (e.g., 90/10, 85/15, 75/25, 65/35, 50/50); boneless beef; beef from AMR systems; and lean finely textured beef (LFTB).

Raw Beef Patty Components: These components include all the components listed above in Raw Ground Beef Components, as well as partially defatted chopped beef (PDCB), finely textured PDCB; heart; and partially defatted beef fatty tissue (PDBFT).

3. Also, inspection program personnel are to only collect samples of raw ground beef components or raw beef patty components that are intended for use in raw non-intact product. To determine the intended use of the products, inspection program personnel are to review establishment records and HACCP documents. In cases where the establishment records and HACCP documents (e.g., flow chart and hazard analysis) are unclear about the intended user, FSIS will handle the product as if it were intended for use in raw non-intact product. If the establishment has not identified the intended use or consumers of the finished product, the establishment is out of compliance with 9 CFR 417.2(a)(2).

C. If FSIS finds raw ground beef product at an official establishment positive for *E. coli* O157:H7, and the ground product was derived from raw ground beef components produced at the same establishment, would FSIS sample raw ground beef components at that establishment?

FSIS may sample and test raw ground beef components at an establishment that produces raw ground beef products from such components if FSIS finds the ground beef product positive. If instructed to sample such products, inspection program personnel should follow the sampling procedures in Part VI. B.

D. What enforcement actions do inspection program personnel take if FSIS finds a raw ground beef component or raw beef patty component positive for *E. coli* O157:H7?

Inspection program personnel are to follow the instructions in Part IV. A. The enforcement actions inspection program personnel are to take when FSIS finds a raw ground beef component or raw beef patty component positive for *E. coli* O157:H7 are the same as the enforcement actions inspection program personnel are to take when FSIS finds a raw ground beef product positive for *E. coli* O157:H7. Similarly, the controls necessary for movement of presumptive positive or positive raw ground beef products are also necessary for movement of presumptive positive or positive raw ground beef components or raw beef patty components.

PART VII – Inspection program personnel responsibilities for an establishment's testing of product for *E. coli* O157:H7

EXHIBIT 5

A. Can establishments conduct pre-shipment review for product that is not at the producing establishment?

FSIS has taken a consistent position that establishments can conduct pre-shipment review when the product is at locations other than at the producing establishment provided that the product does not leave the control of the producing establishment. Some establishments analyze samples for *E. coli* O157:H7 while the product is being moved but is still under the establishment's control. FSIS is providing the establishments the flexibility to move this product prior to pre-shipment review being conducted when the establishment is conducting testing for *E. coli* O157:H7 and maintains control of the product. FSIS has instructed inspection program personnel that they have access to the results of any testing and of any monitoring activities that are performed that may have an impact on the establishment's hazard analysis (FSIS Directive 5000.2). Inspection program personnel must review these results on at least a weekly basis.

B. What do inspection program personnel verify if an establishment conducts verification testing for *E. coli* O157:H7?

1. Inspection program personnel are to review the records associated with any *E. coli* O157:H7 testing conducted by an establishment. If inspection program personnel find a presumptive positive or confirmed positive *E. coli* O157:H7 result in the testing records, they should verify that the establishment is implementing corrective actions that meet the regulatory requirements as part of a HACCP 02 procedure as described in Part IV.

2. If establishment records show that the establishment transports product that it has found presumptive positive or positive for *E. coli* O157:H7 to another establishment for appropriate disposition, or if establishment records show that the establishment moves product before *E. coli* O157:H7 test results become available, inspection program personnel should verify that the establishment—

a. maintains records identifying the official establishment, renderer, or landfill operation that received presumptive positive or positive product;

b. maintains records identifying the official establishment that is to receive product for which results are pending;

c. maintains control of product that is destined for a landfill operation or renderer while the product is in transit (e.g., through company seals);

d. maintains control of product that is destined for an official establishment while the product is in transit (e.g., through company seals) or ensures such product moved under FSIS control (e.g., under USDA seal or accompanied by FSIS Form 7350-1);

e. maintains records that show that presumptive positive or positive product, including product that moved pending test results, received the proper disposition, including documentation evidencing proper disposal of the product from the official establishment, renderer, or landfill where disposition occurred; and

f. completes pre-shipment review for product from a lot that has tested positive or presumptive positive and that was moved pending test results only after it has the records described in paragraph e. for that particular product.

3. If inspection program personnel are aware that an establishment has found product presumptive positive or positive for *E. coli* O157:H7, and that the establishment is currently moving the product for further processing to destroy the pathogen or for destruction, they should verify that the establishment moves the product using the appropriate controls identified in Part VII. B. 2. Inspection program personnel should also notify the DO where the establishment that produced positive or presumptive positive product is located, through e-mail, of the establishment number or name and address of the renderer or landfill operation that is to receive the product. The DO contact person will notify the contact person in the District where the establishment, landfill operation, or renderer that is to receive the product is located, if that establishment, landfill operation, or renderer is located in another District.

4. If inspection program personnel find noncompliance with Part VII. B., 1., 2., or 3., they should contact the DO. The DO will investigate to determine whether the establishment committed the prohibited act of selling or transporting adulterated articles that have not been inspected and passed.

5. The HACCP 02 procedure for a specific production at the establishment that produced the positive or presumptive positive product cannot be completed until that establishment completes pre-shipment review, including review of the corrective action record, and has received documentation evidencing that product has been properly disposed of from the official establishment where disposition occurred or renderer or landfill operation where disposition occurred.

NOTE: When an establishment tests product, a presumptive positive or positive result alone does not warrant an NR. Inspection program personnel are only to issue an NR in response to an establishment's presumptive positive or positive finding if the establishment fails to take the appropriate actions to meet the requirements in 9 CFR 417.3.

PART VIII – Receiving raw ground beef products, raw components, and raw beef patty components that are positive for *E. coli* O157:H7

EXHIBIT

What should inspection program personnel do at an establishment that receives raw ground beef products, raw ground beef components, and raw beef patty components that FSIS or an establishment has found positive for *E. coli* O157:H7?

When inspection program personnel perform a HACCP 01 or 02 procedure at an establishment that has received product from a lot that was found positive for *E. coli* O157:H7 product, they are to verify that:

1. the establishment documents the receipt of presumptive positive or positive product, as required under 9 CFR 417.5;

2. the establishment maintains control of the product; and

3. *E. coli* O157:H7 is addressed in the establishment's hazard analysis and HACCP plan, so that the positive product will receive an adequate lethality treatment to destroy the pathogen.

If inspection program personnel find noncompliance, they take appropriate action as described in FSIS Directive 5000.1, Revision 1, Chapter IV.

Part IX -- Verification Procedures Involving Instructional or Disclaimer Statements Concerning *E. coli* O157:H7

A. What is an instructional or disclaimer statement concerning *E. coli* O157:H7?

1. An instructional statement concerning *E. coli* O157:H7 is a statement that addresses how the product should be prepared or handled to ensure that the pathogen is eliminated or reduced to an undetectable level. Examples of instructional statements concerning *E. coli* O157:H7 in raw ground beef components, raw beef patty components, and raw ground beef products may include, "for full lethality treatment" or "for cooking only." "Cooking" is applying heat to a product at a sufficient temperature and for a sufficient period of time to eliminate *E. coli* O157:H7 or reduce the pathogen to an undetectable level, and "full lethality treatment" may be cooking or another process that eliminates *E. coli* O157:H7 or reduces the pathogen to an undetectable level, such as fermentation or salt curing.

2. A disclaimer statement concerning *E. coli* O157:H7 is a statement regarding the type of verification activities addressing the pathogen that were NOT used in the production of the product. An example of a disclaimer statement concerning *E. coli* O157:H7 is, "product has not been tested for *E. coli* O157:H7."

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B. What type of products can bear these labeling statements?

Establishments can only place these statements on product for use at other official establishments. When the Labeling and Consumer Protection Staff (LCPS) approves the use of instructional labeling statements, LCPS specifies that such statements can only be used on products destined for official establishments that ensure these products receive adequate lethality treatment. When LCPS approves the use of disclaimer labeling statements, LCPS specifies that such statements can only be used on products destined for official establishments that address *E. coli* O157:H7 in their HACCP plan. Establishments' use of these statements is entirely optional.

C. What verification activities should inspection program personnel conduct at establishments that place instructional or disclaimer statements concerning *E. coli* O157:H7 on the labeling of raw ground beef products, raw ground beef components, or raw beef patty components?

1. When conducting an 04B04 procedure, inspection program personnel are to verify that the establishment has received sketch approval from LCPS and that it is maintained in the company's required labeling records (see 9 CFR 317.4(a)).

2. If inspection program personnel find that the establishment did not receive sketch approval or does not maintain that sketch approval in its official labeling records, they are to document the noncompliance on an NR under the Inspection System Procedure (ISP) code 04B04, and they are to document noncompliance with 9 CFR 317.4(a).

3. When performing a HACCP 01 or 02 procedure to verify the HACCP regulatory requirements are met for the production of such products, inspection program personnel are to verify that:

a. the instructional or disclaimer statement does not serve as a control or CCP to address *E. coli* O157:H7;

b. the establishment has not used the statement to justify its determination that *E. coli* O157:H7 is NOT a hazard reasonably likely to occur in the production of these products;

c. the use of any instructional statements is reflected in the establishment's decisionmaking documents (9 CFR 417.5) or hazard analysis (9 CFR 417.2(a)(1)); and

d. the establishment's HACCP plan for products on which it places a disclaimer statement includes a validated intervention for *E. coli* O157:H7.

4. If inspection program personnel find that the establishment's use of instructional statements does not meet the criteria in paragraph 3. a., b., or c. or that the establishment's use of disclaimer statements does not meet the criteria in paragraph 3. a. or b., they are to document the noncompliance on an NR as described in FSIS Directive 5000.1, Revision 1, Chapter IV using the 03-01 or 03-02 ISP code and the appropriate regulatory citation.

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5. If inspection program personnel find that the establishment's HACCP plan for product on which it places a disclaimer statement does not include an intervention for *E. coli* O157:H7, they are to document the noncompliance on an NR as described in FSIS Directive 5000.1, Revision 1, Chapter IV, using the 03-01 or 03-02 ISP code and the appropriate regulatory citation. If inspection program personnel are concerned about product moving outside the establishment, they should initiate a regulatory control action (9 CFR 500.2).

D. What verification activities should inspection program personnel conduct at establishments receiving raw ground beef components, raw beef patty components, or raw ground beef products with instructional or disclaimer statements concerning *E. coli* O157:H7?

1. When performing an 01 or 02 procedure to verify the HACCP requirements are met for products produced using such incoming products, inspection program personnel are to verify that establishments that receive such incoming products:

a. have addressed the use of incoming product with disclaimer statements in their HACCP plan as if the product may be contaminated with *E. coli* O157:H7; and

b. are following any instructional statements on the incoming products.

2. If inspection program personnel find that the establishment has not met the criteria in paragraph 1., they are to document the noncompliance on an NR as described in FSIS Directive 5000.1, Revision 1, Chapter IV using the 03-01 or 03-02 ISP code and the appropriate regulatory citation.

3. Inspection program personnel should retain product produced using such incoming products under the following conditions:

a. the establishment is not following the instructional statement, or the establishment is receiving product bearing a disclaimer statement and its hazard analysis or decisionmaking documents do not address the use of the incoming product as if it were contaminated with *E. coli* O157:H7;

b. the establishment's process may not be adequate to eliminate or reduce *E. coli* O157:H7 to undetectable levels; and

c. the product is not intended for further processing that would destroy the pathogen.

4. If inspection program personnel retain product, they are to document the noncompliance on an NR as described in FSIS Directive 5000.1, Revision 1, Chapter IV using the 03-01 or 03-02 ISP code and the appropriate regulatory citation. Inspection program personnel should also notify the DO through supervisory channels of the conditions observed in association with the use of instructional or disclaimer statements. The DO may send an EIAO into the establishment to conduct a comprehensive food safety assessment or invoke an enforcement action as described in 9 CFR 500.3 or 500.4.

PART X -- Retail Sampling

A. How is raw ground beef product sampling conducted at retail?

1. Retail sampling continues to be an important part of FSIS' *E. coli* O157:H7 sampling program. The likelihood that a specific retail facility will be sampled will depend on what the Agency learns about how raw ground beef product is handled at that facility.

2. When OPHS schedules samples to be taken at retail facilities, OPHS will send OPEER offices FSIS Form 10,210-3, "Requested Sample Programs." Specific information will be provided for the samples to be collected.

3. Program investigators are to make an effort to notify the retail facility the day before they plan to collect the raw ground beef product samples, so that the retail facility can prepare to hold the expected sampled lot. However, in cases when this is not possible, program investigators should try to get to the retail facility as close to the beginning of the grinding operation as possible.

4. Program investigators do not collect raw ground beef product that is received and sold as case-ready product or raw ground beef product that is only re-packaged at the retail store. Program investigators also do not collect raw ground beef product that is ground at retail if the retail facility only regrinds product previously ground at official establishments and does not conduct any practices that would introduce *E. coli* O157:H7 in the product (examples of situations in which samples should be taken include when the store mixes irradiated and un-irradiated beef; adds store trim; or grinds case-ready coarse ground product in a grinder also used to grind store trim if the sanitation program is not well documented, monitored, and verified for effectiveness).

5. When they collect the sample, program investigators obtain from the retail facility the names and establishment numbers of the establishments supplying the source materials for the lot of raw ground beef product sampled.

NOTE: When the source material for the sampled product is store-g[illegible] the program investigator obtains and records the names and establishment numbers of the establishments that produced the product from which the store-generated trim was derived.

EXHIBIT

6. The supplier information is recorded on the retail worksheet that is used specifically for collection of raw ground beef products at retail.

7. In addition, the program investigator records the supplier lot number, production date, and other identifying information that would be useful to the supplier if it is later notified of a positive sample.

B. If a sample of raw ground beef product from a retail facility is confirmed positive for *E. coli* O157:H7, what actions does FSIS take to ensure that adulterated product is kept out of commerce?

The retail facility is notified of the positive *E. coli* O157:H7 result by the program investigator. FSIS will request a recall if any product in the sampled lot has been made available for retail sale. Program investigators and RMS are to work together to determine the necessity of product retention, detention, or recall. (See FSIS Directive 8080.1, Revision 3).

C. Whom does FSIS notify when a raw ground beef product sample at a retail facility is confirmed positive for *E. coli* O157:H7, and how is the notification given?

1. OPEER is notified of a retail positive through the Biological Information Transfer and E-mail System (BITES) and enters supplier information into the STEPS system.

2. The OPEER contact person accesses the STEPS system site with the list of suppliers for the sampled product that tested positive and follows the procedures for notifying suppliers in Part III. B.

D. If FSIS finds raw ground beef product produced at retail positive for *E. coli* O157:H7, does FSIS conduct follow-up sampling at the retail facility?

After an FSIS sample tests positive, program investigators should contact OPHS through an Outlook e-mail message to **Sampling Forms – Headquarters** mailbox, so a form can be sent for the collection of a follow-up sample. The request must include the retail facility name and address, the number of forms (in this case, 1), the type of sample to be collected (i.e., product sample), the purpose of the request (i.e., follow-up sampling in response to a confirmed *E. coli* O157:H7 positive in raw ground beef), the sample form number of the original positive sample triggering this request, the date by which the form is needed, and the program investigator's name and work address. Instructions for follow-up sampling will be provided on FSIS Form 10,210-3, "Requested Sample Programs" or in revisions to FSIS Directive 10,210.1, under the appropriate project. In addition, when feasible, FSIS will schedule

verification activities, including testing, at the supplying establishment following an FSIS positive sample from a retail facility.

PART XI -- Import Sampling

A. How is raw ground beef product sampling conducted at import establishments?

1. OPHS works with the Office of International Affairs (OIA) to send import inspection personnel FSIS Form 10,210-3, "Requested Sample Programs." Certain information will be provided specific to the sample to be collected. Import inspection personnel are to follow the corresponding instructions found in the Import Manual of Procedures (Part 3, Section 5). When OPHS begins sending the form electronically, the Automated Import Information System (AIIS) will schedule samples and send the form electronically to import inspection personnel.

2. Import inspection personnel notify the import establishment management of the reason a sample is being collected for *E. coli* O157:H7 testing (routine FSIS verification testing, increased sampling, or intensified sampling). Imported products may be under increased sampling if OIA has determined that product may be at risk of being contaminated with *E. coli* O157:H7. When a shipment is to be sampled for FSIS testing, the importer, broker or applicant has an opportunity to voluntarily hold the product until the results are reported. Positive samples from imported products result in an intensified level of sampling of subsequent shipments from the foreign establishment. When a foreign establishment is under intensified sampling for *E. coli* O157:H7, FSIS holds the product to be sampled until negative results are reported by the laboratory.

B. If a sample of imported raw ground beef product collected from an import establishment is confirmed positive for *E. coli* O157:H7, what actions does FSIS take to ensure that adulterated product is kept out of commerce?

1. If the product is on hold at the import establishment, whether on FSIS hold or voluntary hold, import inspection personnel will initiate refused entry procedures on the entire lot.

2. FSIS will request a recall if any product in the sampled lot has been released into commerce. Program personnel, including OIA, the DO, and RMS, work together to determine the necessity of product retention, detention, or recall. OIA will coordinate with the DO to provide information to inspection program personnel and program investigators as necessary.

C. Whom does FSIS notify when an imported raw ground beef product collected at an import establishment is confirmed positive for *E. coli* O157:H7, and how is notification given?

EXHIBIT

1. If the lot has not moved into commerce, import inspection personnel notify establishment management, which is responsible for notifying the importer of record. Import inspection personnel should refer to Part 4, Section 11 of the Import Manual of Procedures for guidance on refused entry procedures.

2. If the lot has moved into commerce from the import establishment:

a. the import inspection personnel should send a copy of FSIS Form 9540-1 and the foreign health certificate via facsimile to OIA/Import Inspection Division.

b. OIA notifies the head of the inspection service in the country of origin of the sample that has been confirmed positive for *E. coli* O157:H7 and requests that appropriate action be taken.

D. If FSIS finds raw ground beef product collected at an import inspection establishment positive for *E. coli* O157:H7, does FSIS conduct follow-up sampling of product from the foreign establishment?

Positive samples from imported products result in an intensified level of sampling of subsequent shipments from the foreign establishment. An intensified level of sampling is automatically generated by the AIIS for the next 15 consecutive shipments of product from the foreign establishment presented at port-of-entry anywhere in the United States. Under an intensified level of sampling, the shipment is placed on FSIS hold when the sample is collected, until results are reported. Import inspection personnel should follow the procedures outlined in the Import Manual of Procedures (Part 3, Section 5) for guidance.

All questions related to this directive should be directed through normal supervisory channels.

Philip S. Derfler /s/

Assistant Administrator
Office of Policy and Program Development

Attachment 1

Questions and Answers

1. Will FSIS sample trimmings and other ground beef and beef patty components?

FSIS may sample and test beef manufacturing trimmings and other raw ground beef and beef patty components at a supplying establishment when that establishment has supplied product to grinders that tested positive for *E. coli* O157:H7 after it was ground. In the future, FSIS intends to develop a random sampling and testing program for raw ground beef components and beef patty components and non-intact beef products other than ground beef, such as mechanically tenderized and injected steaks and roasts.

2. Will an establishment that has incorporated testing of trimmings and ground beef products for *E. coli* O157:H7 into its HACCP plan as a verification procedure be exempt from FSIS sampling and testing?

No establishment that produces raw ground beef products, raw ground beef components, or raw beef patty components will be exempt from FSIS sampling and testing for *E. coli* O157:H7. However, FSIS' verification testing will become more risk-based. Establishments that have designed and implemented sampling and verification testing, with a high degree of confidence of finding the pathogen in both trim and finished ground product, presumably present a lower risk for producing adulterated product than one that conducts this activity only on trim or only on finished ground product and, therefore, will be sampled less frequently than other establishments.

3. What factors will be considered by FSIS in establishing risk-based verification testing for *E. coli* O157:H7 in federally-inspected establishments?

FSIS will weight its sample scheduling process so that an establishment producing a large volume of raw ground beef products will be sampled more frequently than an establishment with a lower volume of production of raw ground beef products. In addition, FSIS will also consider seasonality of *E. coli* O157:H7 prevalence and other factors, such as the number of suppliers, in developing a sampling plan based on risk. FSIS will also sample ground beef product at inspected establishments that form ground beef patties but do not grind the product. However, FSIS will also sample ground beef product at these establishments less frequently than at a plant that grinds product.

4. What factors are considered by FSIS in ensuring that retail sampling focuses on the highest risk product?

Retail sampling focuses on higher risk products by focusing on products that includes store-generated trim or was ground using equipment that had been used to grind store-generated trim without being adequately cleaned.

EXHIBIT

5. Can an establishment have a CCP for product disposition based on finished product testing?

If a grinder has internal controls for *E. coli* O157:H7 and receives product from suppliers (both slaughter and fabrication establishments) that have controls for *E. coli* O157:H7, and the grinder and its suppliers conduct rigorous verification testing at multiple points during the production process, a CCP for disposition based on finished product testing for *E. coli* O157:H7 may be appropriate. A CCP for disposition based on finished product *E. coli* O157:H7 testing should employ testing at a level sufficient to find the organism if present at very low frequency. Corrective and preventive action in response to a positive in finished product testing should accompany an examination of the whole system, not merely disposition of the product.

6. Can the FSIS guidance materials suffice for supporting documentation for validation of CCPs, or does FSIS expect the scientific supporting documents to be more specific than a copy of the FSIS guidance materials?

The guidance materials that FSIS has developed for slaughter establishments, grinders, and suppliers on minimizing the risk of *E. coli* O157:H7 contamination included the parameters of certain studies. If establishments can demonstrate that their process meets the parameters of those studies, the FSIS guidance materials would be sufficient documentation of their validation. However, if the process parameters in the establishment differ from those in the FSIS guidance materials, in-house validation would be necessary.

7. If the establishment or FSIS tests raw ground beef products, raw ground beef components, or raw beef patty components for *E. coli* O157:H7 and finds more than one positive, do these findings signify a HACCP failure?

The establishments' or FSIS' finding more than one positive would not alone be a HACCP failure. However, FSIS would expect the establishment to identify *E. coli* O157:H7 as a hazard reasonably likely to occur (if it has not already done so). In addition, the establishment should attempt to determine the cause of the positive findings and would likely need to examine its intervention methods to determine why they are not working. Some establishments have adopted intensive raw material and finished product testing and supplier controls within their Sanitation SOPs and HACCP systems. In these situations, inspection program personnel should verify that the establishments control procedures to determine whether a HACCP failure is occurring. In other situations, the establishment may decide to conduct carcass mapping to identify areas of carcass contamination (if the establishment conducts slaughter or fabrication). In addition, if FSIS testing finds *E. coli* O157:H7, the establishment may decide to intensify its verification program or may decide to ensure that the sensitivity of its testing method is equivalent to FSIS' testing method.

8. Can an inspector collect and submit a ground beef sample prior to pre-shipment review being performed by the establishment?

Inspection program personnel should become familiar with the production process and provide notification to the establishment that a sample will be collected in time for the establishment to hold the sampled product. Some establishments have an extensive verification testing program, sample every "lot" of ground beef product produced, and have a CCP for product disposition. In this scenario, the establishment cannot conduct pre-shipment review until the result from the sample has been received. If the establishment has no interventions in place after the product is sampled that address the presence of the pathogen of concern, the establishment could conduct a pre-shipment review on this product up to this point with a note indicating that the product is being held pending laboratory analysis. Inspection program personnel could verify that the establishment meets the corrective action requirements of 9 CFR 417.3, if a positive result is received by the establishment. If disposition of product is delayed, inspection program personnel should work with their front-line supervisors to determine how to work with the establishment to ensure proper and timely disposal of the product. When the results for both samples (FSIS sample and the establishment sample) have been received, the establishment could then conduct a "final" pre-shipment review. In a scenario similar to this, inspection program personnel could submit the sample prior to the final pre-shipment review being conducted.

9. If an establishment makes case-ready product and requests that the inspector give it notice the day before the inspector is to take a sample, so that the establishment can adjust its production levels to fill its orders but still hold the sampled lot, should the inspector accommodate the request?

Yes. The purpose of FSIS sampling is to provide verification that the establishment's process is producing product that is not adulterated by *E. coli* O157:H7. It is not to compromise the establishment's ability to fill its orders.

10. What is the purpose of follow-up sampling by FSIS after FSIS finds that a sample of product is positive for *E. coli* O157:H7?

FSIS generally always will collect at least one supplemental verification sample of product immediately following corrective actions by the establishment when FSIS finds a sample of product from an official establishment positive for *E. coli* O157:H7. This follow-up verification sample is expected to be larger (e.g., double the size of the regular verification sample), and FSIS expects to double the number of sub-samples that it analyzes. The results will be reported as either positive or negative, like other routine verification sample results. FSIS' follow-up sampling is one of several activities FSIS conducts to verify the adequacy of the establishments' corrective actions following an FSIS positive *E. coli* O157:H7 finding.

11. Is FSIS notified of *E. coli* O157:H7 positive Agriculture Marketing Service (AMS) results? If so, what actions do inspection program personnel take in response to such notification?

Yes, FSIS is notified of *E. coli* O157:H7 positive AMS results. AMS reports potential positives and confirmed positives to FSIS. When the DO is notified of an AMS potential positive, FSIS reacts as if the product were found presumptive positive by FSIS (see Part III, A.). If the product is confirmed positive by AMS, the establishment needs to ensure its proper disposition and to conduct appropriate corrective actions. An AMS result is an official government result.

12. Why must establishments obtain sketch approval from FSIS to use labeling that includes instructional or disclaimer statements on raw ground beef products, raw ground beef components, or raw beef patty components?

The labeling of ground beef products, single-ingredient raw ground beef components, or single-ingredient raw beef patty components that includes special instructions or disclaimer statements concerning *E. coli* O157:H7 cannot be generically approved because FSIS considers these special instructions or disclaimers to be special claims (see 9 CFR 317.5(b)(2)).

13. If FSIS finds that establishments have been using labeling that includes instructional or disclaimer statements on raw ground beef products, raw ground beef components, or raw beef patty components without sketch approval from FSIS, will FSIS request that the establishments recall the product?

No. FSIS will not request that establishments recall product that has already been shipped with unapproved labels because use of such product will not result in adverse health consequences. However, FSIS will rescind such labels, and the establishment would need to submit them to FSIS for sketch approval.

14. Can instructional or disclaimer statements serve as controls or CCPs to address *E. coli* O157:H7?

Labeling is not a means to prevent, eliminate, or reduce pathogens. Therefore, instructional or disclaimer statements cannot be used as CCPs or interventions for *E. coli* O157:H7. If the establishment has determined that *E. coli* O157:H7 is a hazard reasonably likely to occur in its production of raw ground beef products, raw ground beef components, or raw beef patty components, the establishment must have an intervention to address the hazard.

15. Can establishments use instructional or disclaimer labeling statements to justify a determination that *E. coli* O157:H7 is not a hazard reasonably likely to occur in their production of beef products?

No. Because labeling is not a means to control pathogens, establishments may not use these labels to justify their determination that *E. coli* O157:H7 is NOT a hazard reasonably likely to occur in their production of these products.

16. Can product labeled "for cooking only" go to an establishment that cooks product intended for additional further processing?

Yes. Even if the product will undergo further processing after it leaves the cooking establishment, as long as the cooking establishment cooks the product at a sufficient temperature and for a sufficient period of time to eliminate or reduce *E. coli* O157:H7 to an undetectable level, the cooking establishment would be complying with the labeling instructions.

17. How should the placement of instructional statements be reflected in HACCP plan documents?

The placement of any instructional statement addressing *E. coli* O157:H7 on labels of raw ground beef products, raw ground beef components, or raw beef patty components must be reflected in an establishment's decisionmaking documents and hazard analysis.

For example, if an establishment is placing the statement "for cooking only" or "for full lethality treatment" on raw ground beef products, raw ground beef components, or raw beef patty components, the establishment's hazard analysis should show how the establishment is ensuring that the product will go for cooking only or for other full lethality treatment only. If the establishment places a "for cooking only" statement on the product and cooks the product in the establishment, the establishment's flow chart should show the cooking steps the product will undergo. If the establishment places a "for cooking only" statement on the product and ships it to outside establishments, the shipping establishment should have controls in place to ensure that the product goes only to establishments that cook it. If the shipping establishment also produces product that is not intended for cooking, it should have controls in place to segregate product intended for cooking from product not intended for cooking. If an establishment places the statement "for cooking only" on its finished product, but the establishment has not addressed the intended use of its finished product in its decisionmaking documents or hazard analysis, the establishment's hazard analysis and decisionmaking documents would not be consistent with the information contained in the instructional statement, and the establishment would not be in compliance with 9 CFR 417.5.

18. Why are establishments that place labels on raw beef products that include a disclaimer statement concerning *E. coli* O157:H7 required to have an intervention for the pathogen in their HACCP plan?

An establishment may use a disclaimer statement, such as, "not tested for *E. coli* O157:H7," on labels of raw ground beef products, raw ground beef components, or raw beef patty components only if it has an intervention for the pathogen in its HACCP plan for these products. A disclaimer that the product has not been tested for *E. coli* O157:H7 implies that *E. coli* O157:H7 may be a food safety hazard reasonably likely to occur in the product in the absence of controls. Therefore, the information contained in the disclaimer statement would be inconsistent with a determination in the hazard analysis that it is unnecessary to address this hazard in the HACCP plan, and the HACCP plan may be determined inadequate (9 CFR 417.6).

19. How are inspection personnel to document noncompliances involving labeling and disclaimer statements?

Inspection program personnel are usually to cite 9 CFR 417.5 and to use the recordkeeping trend indicator when documenting on an NR most of the possible noncompliances involving labeling and disclaimer statements. Under 9 CFR 417.5, required records documenting the establishment's HACCP plan include: a written hazard analysis, supporting documentation of the hazard analysis, a written HACCP plan, and decisionmaking documents associated with selection and development of CCPs and critical limits.

a. If the establishment's use of instructional statements concerning *E. coli* O157:H7 is not reflected in its decisionmaking documents or hazard analysis, the establishment is not in compliance with 9 CFR 417.5, because its records do not show that the establishment has considered its use of these instructional statements in its hazard analysis or HACCP plan.

b. If the instructional or disclaimer statements serve as controls or CCPs to address *E. coli* O157:H7, the establishment is not in compliance with 9 CFR 417.5 because the establishment's decisionmaking documents or hazard analysis cannot support its use of instructional or disclaimer statements as controls or CCPs.

c. If the establishment has used instructional or disclaimer statements to justify its determination that *E. coli* O157:H7 is NOT a hazard reasonably likely to occur, the establishment is not in compliance with 9 CFR 417.5 because the establishment's decisionmaking documents or hazard analysis incorrectly concluded that labeling statements would prevent *E. coli* O157:H7 from becoming a hazard reasonably likely to occur in the establishment.

d. If an establishment receiving product with instructional or disclaimer statements has not addressed the use of such products in its decisionmaking documents or hazard analysis, or does not have data to validate that these products will receive adequate lethality treatment, the establishment is not in compliance with 9 CFR 417.5 because its records do not show that the establishment has adequately addressed the use of these incoming products in the hazard analysis for those products in which such incoming products will be used.

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PROCEDURES FOR SAMPLING RAW GROUND BEEF COMPONENTS AND RAW BEEF PATTY COMPONENTS:

Refer to the page in FSIS Directive 10,210.1 that corresponds to the project code in Block 14 of FSIS Form 10,210-3, Requested Sample Programs, for further collection instructions.

Sample Size:
The FSIS laboratory requires approximately 1.5 pounds (24 ounces or 680 grams) but no less than 1.25 pounds (20 ounces or 570 grams) of product.

Sample Chilling:
If the sample is warmer than 40°F/4.4°C when the sample is taken, place it in a cooler to chill it before shipping.
Prior to shipping the sample, pre-chill the shipping container in a refrigerated cooler that is between 28°F and 45°F for at least 8 hours.

Randomized or Representative Sampling:
As best as practical, select a representative sample by one of the two following procedures:

1. **Time**: Throughout the production lot, as defined by the establishment, as boxes or combo bins are filled, collect samples at random times. Use standard procedures for identifying "random times".
   If random times are not practical use the "Space" option below.

2. **Space**: At or toward the end of the production lot, as defined by the establishment, note the number of boxes or combo bins containing the requested product types. Take the square root of that number and round up to the next whole number (i.e., if the number of boxes is 29, the square root is 5.38; the next whole number is 6). That number, or no more than 10, is the number of containers to be sampled.
   Use a standard random number procedure to select which containers to sample. Select representative samples from the top of the filled boxes or combo bins. These pieces should have collected representative bacteria from the product contact surfaces during the course of production.

Sampling Procedure:
There will be three basic sampling procedures based on size of sample pieces:

1. Very small pieces less than the size of an ordinary thumb, such as AMR, or LBT/LFTB.
   For very small pieces, use the laboratory-supplied scoop or spoon to collect the sample.

2. Small pieces less than the size of an ordinary palm, such as head meat or trimming.
   For small pieces, use the laboratory-supplied scoop, tongs, or hook to collect the sample.

3. Chunks and pieces larger than an ordinary palm, such as chucks and plates:
Call for help from an establishment employee with an establishment knife and laboratory-supplied hook. Have the employee sanitize knife and hook in the same manner as is done on the boning/trim line. From each of the designated containers (or during the day) "Grab sample" pieces with the hook. From each piece, slice a thumb to palm-sized piece of surface, no thicker than ½ inch (or 1 cm). Place the samples into the sterile sample bag, using the hook or laboratory-supplied tongs.

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Exhibit P

ICMSF methods studies. I. Comparison of analytical schemes for detection of *Salmonella* in dried foods[1,2,3]

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Silliker, J. H., and D. A. Gabis. 1973. ICMSF methods studies. I. Comparison of analytical schemes for detection of *Salmonella* in dried foods. Can. J. Microbiol. 19: 475–479.

Twenty-six low-moisture food samples naturally contaminated with salmonellae were analyzed to compare the efficiency of sixty 25-g, fifteen 100-g, and three 500-g subsamples in recovery of salmonellae. In addition, the efficacy of wet compositing in recovery of salmonellae was studied by pooling in groups of five, the pre-enrichment broths from the 25-g and 100-g subsamples. Eighteen of the samples were positive by all sampling procedures. Four of the samples were negative when sixty 25-g subsamples were analyzed, four were negative by the fifteen 100-g sub-samples, and two were negative by the three 500-g subsamples. Wet-compositing results indicate the method is as sensitive in detecting salmonellae as the analysis of individual subsamples. The overall results indicate samonellae may be detected with equal facility by any of the sampling and analytical methods tested.

Silliker, J. H., et D. A. Gabis. 1973. ICMSF methods studies. I. Comparison of analytical schemes for detection of *Salmonella* in dried foods. Can. J. Microbiol. 19: 475–479.

On a analysé vingt-six échantillons d'aliments, ayant un taux peu élevé d'humidité et contaminés naturellement par des salmonelles. Le but de cette étude était de comparer l'efficacité de 60 sous-échantillons de 25 g, 15 de 100 g et 3 de 500 g pour la récupération des salmonelles. De plus, on a étudié l'efficacité d'un mélange mouillé dans la récupération des salmonelles en répartissant, par groupes de cinq, les bouillons de pré-enrichissement de sous-échantillons de 25 et 100 g. Quelle que soit la méthode d'échantillonnage; dix-huit des échantillons ont été positifs, quatre furent négatifs dans l'analyse de 60 sous-échantillons de 25 g; quatre furent également négatifs pour 15 sous-échantillons de 100 g et, finalement deux furent négatifs dans l'analyse de 3 sous-échantillons de 500 g. Cette méthode des mélanges mouillés s'est avérée aussi efficace dans la détection des salmonelles que l'analyse des sous-échantillons individuels. Dans leur ensemble les résultats démontrent que les salmonelles peuvent être facilement décelées quelles que soient les méthodes d'analyses ou d'échantillonnages utilisées.
[Traduit par le journal]

The sensitivity of the analytical methods is most important in the design of *Salmonella* control systems for foods. Increasing the amount of sample examined from a particular lot decreases the probability of accepting a positive lot. There has been a trend to analyzing increasingly larger samples, but the sensitivity of detecting *Salmonella* in large samples has not been adequately determined. The testing of 60 individual 25-g subsamples with negative results was recommended by the National Research Council (NRC) Committee on *Salmonella* (2) before high-risk foods could be released for consumption by aged and infirm persons and infants. It is obvious that following this recommendation would not be economically feasible on a routine basis. Examination of fifteen 100-g subsamples would require one-fourth the laboratory work but would be warranted only if the sampling and analytical scheme were as efficient in detecting positive lots as one involving the analysis of sixty 25-g subsamples. Similarly, if an analytical method using three 500-g subsamples from the lot were equally capable of detecting contamination, then the laboratory cost of statistical quality control over *Salmonella* would be greatly reduced, and more widespread application of statistical control programs would be encouraged.

Compositing of multiple subsamples (lumping) and wet compositing of pre-enrichment cultures have been tested as economical approaches to *Salmonella* control. Both approaches are being used widely by the food industry in the United States, but little has been published on the efficacy of either technique.

Silliker (appendix C of reference 2) examined 389 lots of dried egg products by analyzing

[1]Received August 31, 1972.
[2]International Commission on Microbiological Specifications for Foods (ICMSF).
[3]This study was sponsored by the Agricultural Research Service (ARS), Eastern Utilization Research and Development Division, United States Department of Agriculture, under the terms of a cooperative agreement between ARS and ICMSF.

400-g composite samples as well as multiple 25-g samples. His data suggested some loss in sensitivity caused by the use of 400-g samples. Huhtanen *et al.* (1) analyzed 25 meat-and-bone meal samples for salmonellae, comparing a single 300-g sample with ten 30-g samples. Seventeen were positive on the testing of a single 300-g sample; 18 were positive on the testing of ten 30-g samples.

Silliker (appendix D of reference 2) reported that of 106 wet composites derived from 1060 individual samples, 39 were positive for *Salmonella* and 66 were negative. In seven instances negative results were obtained despite the fact that one or more of the 10 individual samples comprising the wet composite did contain salmonellae. Thus, these seven were false negative results. Price *et al.* (3) reported that as many as 25 pre-enrichment broth cultures could be pooled without apparent loss in sensitivity.

The present work compares five analytical systems for the detection of salmonellae in dried foods. In each system 1500 g of sample was tested. The studies were designed to compare the following analytical schemes: (1) Sixty 25-g subsamples; (2) fifteen 100-g subsamples; (3) three 500-g subsamples; (4) wet compositing of 25-g pre-enrichment cultures in groups of five; and (5) wet compositing of 100-g pre-enrichment cultures in groups of five. The work is part of a broad study program of analytical microbiological methods for foods being conducted by the International Commission on Microbiological Specifications for Foods.

Methods

Source of Samples

All test samples were commercial products, believed to be naturally contaminated with salmonellae. Each sample comprised 70–80 kg; the major portion of this was consumed in primary testing.

Sample Preparation

Certain highly contaminated samples were diluted with *Salmonella*-free material of the same composition, as shown in Table 1.

Chocolate-almond bars and milk chocolate were ground in a meat grinder through a plate with holes 8 mm in diameter, then hand-mixed and reground through a plate with holes 3 mm in diameter. The other foods (with exceptions noted below) were mixed for 1 h in a stainless steel liquid–solids blendor (Patterson-Kelley Company, Inc., East Stroudsburg, Pa.).

The fish meal used in test 22 and the coconut used in test 25 were not mixed. In test 26 contaminated coconut was diluted 1:3 with uncontaminated coconut, mixed by hand, and then ground twice in the meat grinder through a plate containing holes 3 mm in diameter.

Sampling

In tests 1–21 the mixed products were sampled directly, i.e., sixty 25-g, fifteen 100-g, and three 500-g subsamples were weighed directly into the pre-enrichment broth. In tests 22–26, 180 25-g subsamples were weighed into 180 sterile sample bottles. Sixty of the 180 subsamples were randomly selected for the 25-g subsamples. The 100-g subsamples were prepared by randomly pooling four 25-g subsamples to form 15 groups. The 500-g subsamples were prepared by randomly pooling groups of twenty 25-g subsamples.

Pre-enrichment

Chocolate-almond and milk-chocolate samples were enriched in reconstituted 10% (w/v) non-fat dry milk containing 0.002% brilliant green dye. Black pepper, whole egg solids, egg yolk solids, egg albumen, and isolated soy protein samples were enriched in lactose broth (Difco, Detroit, Michigan). Meat meal, cottonseed meal, fish meal, and desiccated coconut samples were enriched in lactose broth containing 1% (v/v) Tergitol Anionic 7 (sodium heptadecyl sulfate, Union Carbide Company, Chicago, Illinois).

The 25-g subsamples were enriched in 225 ml of broth contained in 500-ml jars with screw-cap lids. The 100-g subsamples were enriched in 900 ml of broth contained in 1500-ml bottles with screw-cap lids. The 500-g subsamples were enriched in 4500 ml of broth in 8-liter polyethylene containers (Rubber-Maid Company, Commercial Division, Winchester, Va.). The containers were covered with aluminum pie pans with 20-cm diameters. Since the polyethylene containers were not resistant to 121C, all media were pasteurized in flowing steam for 20 min. The temperature of the media after this treatment was greater than 85C. The media were used immediately after cooling to room temperature.

Subculturing of Pre-enrichment Cultures

All pre-enrichment cultures were incubated for 24 h at 35C. After incubation, 1 ml from each of the cultures was introduced into 9 ml selenite–cystine broth (Difco) and 9 ml tetrathionate broth containing brilliant green dye (Difco) (4).

Sixty 25-g pre-enrichment cultures were randomly assigned to 12 groups of five. Each of these groups was used to prepare wet composites as follows: 1 ml from each of the five pre-enrichment cultures was introduced both into 45 ml selenite broth and 45 ml tetrathionate broth. Similarly, the fifteen 100-g pre-enrichment cultures were randomized and assigned to three groups of five. Wet composites of the 100-g pre-enrichment cultures were prepared in the same manner as described above. The selenite and tetrathionate subcultures were incubated 24 h at 35C.

Selective and Differential Media

After incubation, each of the selenite and tetrathionate broth cultures was streaked onto brilliant green (BG), *Salmonella–Shigella* (SS), and bismuth sulfite (BS) agars (Difco) and incubated at 35C. The SS and BG plates were examined after 24 h, the BS plates after 48 h.

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Identification

Suspect colonies were identified by conventional biochemical and serological procedures (4). The serological identification included slide agglutinations using group specific O antisera (Difco) and tube agglutinations using Spicer-Edwards H antisera (Difco).

Results

Eighteen of the 26 samples were positive for salmonellae by all analytical procedures (Table 1). Four of the samples were negative when sixty 25-g subsamples were used, but the same samples were positive by one or more of the other analytical procedures. The twelve 25-g wet composites derived from each of these four samples were also negative. Four of the test samples were negative in the fifteen 100-g subsamples, and the wet composites derived from these aliquots were likewise negative. In test 18, three of the fifteen 100-g subsamples were positive, but all of the wet composites were negative. A minimum of one and a maximum of three positives would be expected from the wet composites; thus one or more of the negative results on the 100-g wet composites in test 18 must be considered as "false negative." Noteworthy was the detection of *Salmonella* in 24 of 26 test samples when three 500-g subsamples from each test sample were studied.

Table 1 indicates that the failure of one or more of the analytical procedures to detect salmonellae was generally associated with samples containing a low level of contamination.

TABLE 1

Comparison of analytical methods for detection of salmonellae in dried foods

		Salmonella-positive results				
		Dry subsamples			Wet composites	
		Wt. of aliquots, g				
		25	100	500	25	100
		No. of aliquots				
Test	Product	60	15	3	12	3
1	Chocolate almond bar	59	15	3	12	3
2	As test 1 diluted 1:10	39	13	3	12	3
3	As test 1 diluted 1:40	9	5	3	6	3
4	As test 1 diluted 1:100	3	2	0	2	1
23	As test 1 diluted 1:40	5	2	1	2	1
5	Milk chocolate	0	0	1	0	0
6	Black pepper	0	1	1	0	1
7	Whole egg solids	0	1	1	0	1
8	Egg yolk solids	2	1	1	2	1
9	Egg albumen solids lot 1	59	14	3	12	3
10	Egg albumen solids lot 2	60	15	3	12	3
12	As test 10 diluted 1:2.6	45	13	3	11	3
13	As test 10 diluted 1:26	2	0	0	2	0
11	Mixture albumen solids lots 1 and 2 diluted 1:4	56	15	3	12	3
14	Isolated soy protein	0	0	1	0	0
15	Meat meal lot 1	28	15	2	11	3
17	As test 15 diluted 1:4	4	0	1	4	0
16	Meat meal lot 2	6	2	1	4	1
21	Meat meal lot 3	58	15	3	12	3
18	Cottonseed meal	7	3	2	4	0
19	Fish meal lot 1	22	10	3	8	2
20	Fish meal lot 2	20	11	3	11	3
22	Fish meal lot 3	60	15	3	No test	No test
24	As test 22 diluted 1:100	10	11	3	4	3
25	Desiccated coconut	43	15	3	11	1
26	As test 25 diluted 1:3	2	3	1	2	2

By assuming that the contamination in samples negative by one or more of the methods was sufficiently low so that a single positive resulted from a single *Salmonella*, then the number of salmonellae per 500 grams may be estimated.

Table 2 represents in condensed form the patterns of salmonellae recovery from the 26 samples and the estimated level of contamination in samples negative by one or more methods. The estimation of the *Salmonella* contamination was derived by calculation. For example, in test 4 a total of five positives were obtained from the sixty 25-g, fifteen 100-g, and three 500-g subsamples. Assuming that any positive resulted from a single *Salmonella*, then, in the total of 4500 g of sample analyzed (wet composites not included), there were five salmonellae. The best estimate of contamination in 500 g would be five positives in 4500 g divided by nine, or 0.56 salmonellae per 500 grams. Estimates of the number of salmonellae per 500 grams have been calculated for each lot found negative by one or more of the five analytical procedures.

The estimates of the level of contamination are in the right-hand column of Table 2. In seven of the eight test samples found negative by any one of the analytical schemes, the level of salmonellae was calculated to be less than one *Salmonella* in 500 g. If the concentration of salmonellae in the lot is one in 500 g, the probability of finding all sixty 25-g subsamples negative is about 5% (2). It is assumed that the same probability is provided by the other analytical procedures. Thus in seven of eight negative samples, the level of salmonellae was below that which could be detected with 95% confidence. The exception was cottonseed meal used in test 18 in which the estimated *Salmonella* level was 1.32 per 500 grams. This sample was positive in three 100-g subsamples, but all three wet composites were negative. In such a case one or more of the negative wet composites must be considered as "false negative."

Twenty-five *Salmonella* serotypes were obtained from the 17 product-samples tested.

Discussion

Analytical results from 26 samples of naturally contaminated dried foods indicate that *Salmonella*-positive samples may be detected with equal facility by analyzing sixty 25-g subsamples, fifteen 100-g subsamples, or three 500-g subsamples. Pooling sixty 25-g or fifteen 100-g pre-enrichment cultures into groups of five (wet compositing) followed by selective enrichment of the composites apparently results in the same assurance of detection of positive lots as the analysis of individual samples.

Limited data suggest that the analysis of a single 1500-g subsample is feasible. Eleven of the samples were analyzed by pre-enriching a single 1500-g subsample in 13 500 ml of broth. Ten of 11 samples were *Salmonella*-positive with the negative result coming from the material used

TABLE 2

Patterns in the recovery of salmonellae by five different sampling and analysis methods

Sampling and analytical method						
Dry			Wet composites			
Wt. of subsamples, g						
25	100	500	25	100		
No. of subsamples					Samples positive for each pattern	Estimated *Salmonella* level/500 g
60	15	3	12	3		
+*	+	+	+	+	18	
+	+	−†	+	+	1	0.56
+	+	+	+	−	1	1.32
+	−	+	+	−	1	0.56
−	+	+	−	+	2	0.22; 0.22
+	−	−	+	−	1	0.22
−	−	+	−	−	2	0.11; 0.11

*Plus (+) indicates one or more of the subsamples were positive.
†Minus (−) indicates none of the subsamples were positive.

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for test 24, diluted fish meal. Failure to isolate *Salmonella* from this 1500-g subsample is probably a "false negative," because the sample had a relatively high level of contamination.

The efficacy of using large samples for *Salmonella* analysis permits statistical quality control which is economically feasible. This is extremely important, because the best way to discover a *Salmonella* problem is through constant surveillance of finished product and the plant environment. In addition, sampling plans being recommended for certain foods require examination of a large number of samples to attain a high probability of safety.

Routine surveillance requires collection and examination of large numbers of samples, especially in a large plant producing many products. If the samples from individual lots can be lumped as described here, the monetary and time savings are obvious. Similarly, substantial savings would result by compositing in the application of strict sampling plans such as those recommended by the NRC, Committee on *Salmonella* (2) and the International Commission on Microbiological Specifications for Foods (report in preparation). For example, the NRC Committee recommended sixty 25-g samples for category I foods (powdered infant foods, therapeutic diet supplements, geriatric food and drugs), which are to be used by populations having high susceptibility to a *Salmonella* infection. The present study indicates that these products could be as effectively controlled by analyzing three 500-g samples per lot. Category II includes high-risk foods which contain a sensitive ingredient (s), have no final pasteurization, and are capable of supporting *Salmonella* growth. The recommended sampling plan requires analysis of twenty-nine 25-g samples for a total of 725 g. The results of this study show that analysis of two 500-g subsamples would permit a higher measure of control over such products than analysis of twenty-nine 25-g samples, since this entails the analysis of an additional 375 g.

Similarly, the less critical products in categories III, IV, and V could be statistically controlled by analyzing a single pool of twenty 25-g (500 g) samples, since the recommended analytical scheme entails the analysis of thirteen 25-g (325 g) samples.

The NRC report directed its attention to the problems of *Salmonella* control in the United States. The difficulties encountered in the control of foods in international commerce are much greater, since it is more likely that the history of a given lot of product is unknown. Here the need for statistical quality control over the *Salmonella* defect is correspondingly greater. At present, cursory or the complete absence of sampling affords little protection to the consumer.

Attribute sampling plans assume random distribution of a defect in a lot. It should be emphasized that in the present studies the sample received for testing was thoroughly mixed in the laboratory to promote random distribution of salmonellae. The question of the proper procedures to be used in obtaining representative material from lots of food for laboratory testing remains moot and was not a part of this investigation.

The foods tested in these experiments were naturally contaminated with salmonellae, and the diversity of *Salmonella* serotypes was limited. Nevertheless, 25 serotypes were isolated from the samples examined, and it appears that the results of the study were not serotype-dependent.

The present study involved dried foods, but similar sampling procedures for high-moisture products, such as meats, poultry, and bulk eggs are being investigated.

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Exhibit Q

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Salmonella Testing of Pooled Pre-Enrichment Broth Cultures for Screening Multiple Food Samples

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A method has been described for testing multiple food samples for *Salmonella* without loss in sensitivity. The method pools multiple pre-enrichment broth cultures into single enrichment broths. The subsequent stages of the *Salmonella* analysis are not altered. The method was found applicable to several dry food materials including nonfat dry milk, dried egg albumin, cocoa, cottonseed flour, wheat flour, and shredded coconut. As many as 25 pre-enrichment broth cultures were pooled without apparent loss in the sensitivity of *Salmonella* detection as compared to individual sample analysis. The procedure offers a simple, yet effective, way to increase sample capacity in the *Salmonella* testing of foods, particularly where a large proportion of samples ordinarily is negative. It also permits small portions of pre-enrichment broth cultures to be retained for subsequent individual analysis if positive tests are found. *Salmonella* testing of pooled pre-enrichment broths provides increased consumer protection for a given amount of analytical effort as compared to individual sample analysis.

Positive test results are relatively rare among the many samples of food ingredients and products which are analyzed for *Salmonella*. Therefore, a test procedure that gives a single negative answer for many negative samples offers an important saving in the analytical effort required for quality control. These studies support the conclusion that *Salmonella* pre-enrichment broths can be pooled for analysis without loss in sensitivity to *Salmonella* in the individual samples.

MATERIALS AND METHODS

Salmonella analyses were carried out as described in the *Bacteriological Analytical Manual* (BAM) (2) except for the pooling of pre-enrichment broths described in the following paragraph.

The procedure for *Salmonella* testing of pooled pre-enrichment broths is illustrated in Fig. 1. Samples are individually pre-enriched followed by transfer from multiple pre-enrichments to single selenite and tetrathionate broths at the enrichment stage. During pooling, a portion of each pre-enrichment is transferred to a sterile culture tube and retained at 4 C for later reference to the individual samples. If the *Salmonella* test of the pooled pre-enrichment broths is positive, individual tests on the retained samples can be made to determine which sample or samples contributed *Salmonella* to the pool.

Pre-enrichment broths consisted of 0.5% lactose broth for all foods except nonfat dry milk, for which sterile distilled water containing 0.002% Brilliant Green was employed.

Salmonella-positive test samples were prepared by blending the dry test food with one of three dry inocula. Inoculum 1 was a dry enzyme drain cleaner which had been found to contain multiple *Salmonella* serotypes including E_1, z_{15}; G, z_{29} (*S. cubana*); and C_1, g complex. Inoculum 2 was a freeze-dried, skim milk suspension of serotypes *S. anatum*, *S. binza*, *S. tennessee*, *S. worthington*, *S. cubana*, *S. bruenderup*, and a B_1, g complex. Inoculum 3 consisted of four laboratory isolates of serotypes G, z_{29} (*S. cubana*); E_4, b; E_1, l complex; and C_1, l complex grown in a sterile aqueous suspension of cottonseed flour and freeze dried.

RESULTS AND DISCUSSION

Dried egg albumen, cocoa, and nonfat dry milk were inoculated with inoculum 1 at levels ranging from 6 salmonellae per 100 g to 3,000 salmonellae per g. Pre-enrichment broths of the *Salmonella*-inoculated samples were analyzed both individually and after pooling with 5, 9, and 14 pre-enrichments of uninoculated

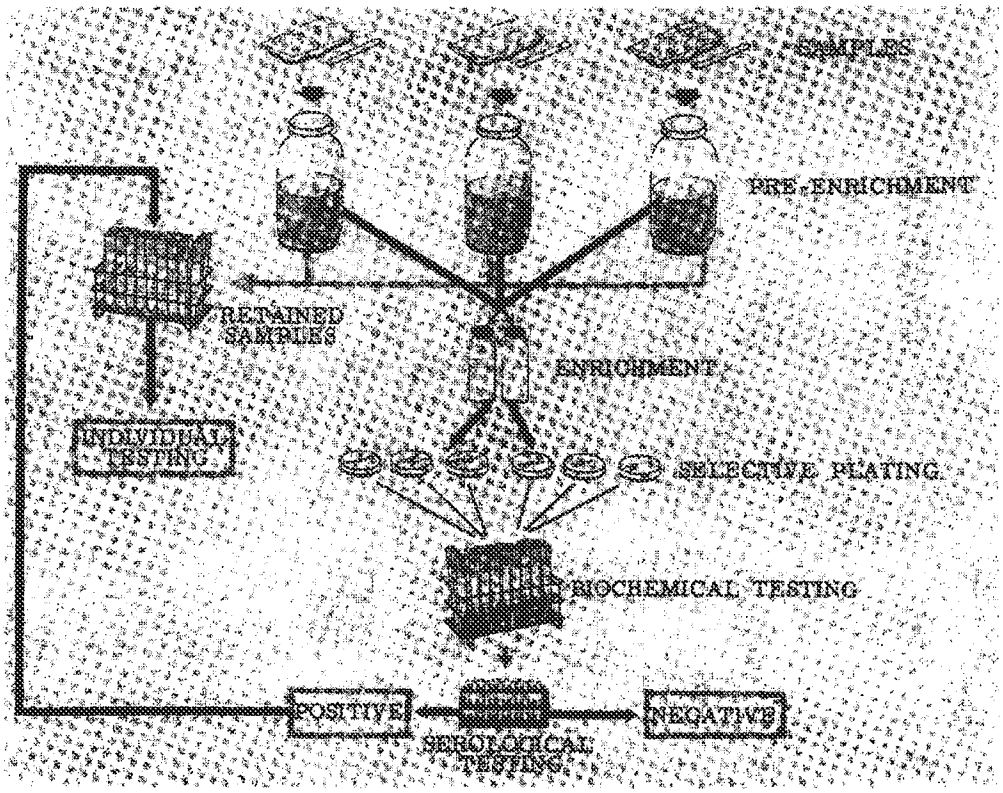


FIG. 1. *Salmonella testing of pooled pre-enrichment broths.*

TABLE 1. *Effect of pooling pre-enrichment broth cultures on Salmonella recovery from food samples*

Inoculation level[a] (per g)	Nonfat dry milk				Cocoa				Dried egg albumin			
	P[b]	P + 5N	P + 9N	P + 14N	P	P + 5N	P + 9N	P + 14N	P	P + 5N	P + 9N	P + 14N
Series I												
600	+	+		+	+	+		+	+	+		+
60	+	+		+	−	−		−	+	+		+
6	+	+		+	−	−		−	+	+		+
0.6	+	+		+	−	−		−	+	+		+
0.06	−	−		−	−	−		−	−	−		−
Series II												
3,000	+		+		+		+		+		+	
20	+		+		+		+		+		+	
0.2	+		+		+		+		+		+	

[a] The most-probable-number estimate (1) of the original *Salmonella* inoculum (inoculum 1 containing serotypes E,z_{15}; G,z_{25}; and C,g complex) was 93,000/g with 95% confidence limits ranging from 15,000 to 380,000/g. The inoculation level represents a suitable dilution of inoculum 1, into an otherwise *Salmonella*-negative sample, to provide the levels as shown.

[b] Abbreviations: P, positive sample inoculated with naturally contaminated dry enzyme drain cleaner; N, negative (uninoculated) sample; +, *Salmonella* recovery positive; −, *Salmonella* recovery negative.

samples. The positive inoculated samples were detected in pooled pre-enrichment broth cultures as effectively as when they were analyzed individually (Table 1). The level of inoculation affected the recovery of *Salmonella* equally for both individual and pooled analyses. Detection of *Salmonella* in cocoa was less sensitive than in the other two test materials. A possibly sim-

ilar antimicrobial effect of cocoa on strains of *S. gallinarum* and *S. typhimurium* was reported by Busta and Speck (3).

Similarly, *Salmonella* was detected in wheat flour, coconut, and cottonseed flour samples without loss in sensitivity, with both inoculum 1 and inoculum 2, when single pre-enrichments were pooled with from 9 to 24 pre-enrichments of uninoculated samples.

Pooled samples versus pooled pre-enrichment broths. Silliker (4) reported a loss in sensitivity when several samples were pooled in a large container of pre-enrichment broth as compared to individual sample analysis. This is not surprising since, to be detected, the desiccated and debilitated *Salmonella* cells in a dried food sample must grow out in pre-enrichment broth in competition with other bacteria in the sample. If many food samples are pooled at the pre-enrichment stage, the salmonellae face competition from the flora of all the samples in the pool. If, on the other hand, the samples are individually pre-enriched, the actively growing salmonellae might be expected to compete more favorably with organisms introduced from other samples when pooled at the enrichment stage. In addition, pooling pre-enrichment broths avoids the hazards and inconvenience of handling very large flasks of culture.

A study was made of *Salmonella* detection in pooled samples and pooled pre-enrichment cultures in the presence of added interfering organisms, i.e., non-*Salmonella* bacteria having colonies that resemble *Salmonella* on the selective agar media. The study was carried out with nonfat dry milk and egg albumin, using inoculum 3. About 500 cells each of four interfering organisms, *Pseudomonas* sp., *Proteus* sp., a lactose-positive *Citrobacter freundii*, and a lactose-negative Bethesda-Ballerup were added at the pre-enrichment stage.

Pooled samples were prepared by placing nine 25-g *Salmonella*-negative samples and one 25-g inoculated sample together in a single jar containing 2,250 ml of pre-enrichment broth. A pool of 10 samples was considered to be a practical maximum for convenient handling. Pre-enrichment pooling was carried out as previously shown in Fig. 1 except that 1 positive and 14 negatives were included in the pool.

Table 2 compares pooling of samples and of pre-enrichment broth cultures for nonfat dry milk and dried egg albumin at six levels of *Salmonella* inoculation. The results show that a higher number of positives was recovered by pooling pre-enrichments rather than by pooling samples. In addition, pre-enrichment pooling yielded positives at lower levels of inoculation. Similar results were found in tests with *Salmonella*-inoculated cocoa, shredded coconut, and wheat flour.

TABLE 2. *Effect of pooling stage on Salmonella detection*[a]

Inoculation[b] level (per g)	Nonfat dry milk		Dried egg albumin	
	Pooled samples[c] (No. positive 3 replicates)	Pooled pre-enrichments[d] (No. positive 3 replicates)	Pooled samples[c] (No. positive 3 replicates)	Pooled pre-enrichments[d] (No. positive 3 replicates)
840	3	3	3	3
420	3	3	3	3
17	2	3	3	3
8	2	3	0	1
0.33	3	2	0	1
0.07	0	3	0	2
Total	13/18	17/18	9/18	13/18

[a] Approximately 500 cells each of *Pseudomonas* sp., *Proteus* sp., *Citrobacter freundii*, and Bethesda-Ballerup were added to the pre-enrichment broth of each procedure.

[b] The most-probable-number estimate of *Salmonella* in inoculum 3 (containing serotypes G,z_{28}; E_1, b; E_1,L complex; and C_1,L complex) was 2,800/g with 95% confidence limits ranging from 1,000 to 15,000/g. The inoculation level represents a suitable dilution of inoculum 3 into an otherwise negative sample to provide the levels as shown.

[c] Pooled samples = one positive and nine negative samples.

[d] Pooled pre-enrichments = pre-enrichment broths from 1 positive sample and 14 negative samples.

The pooling of broth cultures permits small portions of each individual culture to be retained for subsequent analysis if a positive test is detected following enrichment, thus maintaining the individuality of each test sample. On the other hand, the direct pooling of food samples into pre-enrichment results in the loss of individual sample identity.

Number of isolates examined. Two or more colonies are picked from each positive selective plate for biochemical and serological testing by the BAM *Salmonella* method (2) to increase the chances of detecting *Salmonella*.

By following this procedure, 180 isolates could be tested if 15 samples were tested individually, whereas only 12 isolates could be tested at the same rate of picking from a pool of 15 samples.

To test the importance of the reduced number of isolates, 238 isolates were picked at

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the rate of one colony per positive selective plate from the pooled pre-enrichment culture tests of the three food materials: dry milk, egg albumin, and cocoa. Forty-four per cent of the isolates were *Salmonella*. In a similar evaluation of the tests on flour and coconut, 60% of 72 isolates picked at the rate of two colonies per plate were *Salmonella*. The high proportion of *Salmonella* indicated that pooled pre-enrichment provided an adequate number of isolates to detect the presence of *Salmonella*.

Whether pooled pre-enrichments are less sensitive than individual tests in the presence of interfering organisms probably is not known. For the present, we would recommend that, if numerous non-*Salmonella* colonies that resemble *Salmonella* on selective agar media are encountered in the pooled test, the retained pre-enrichment cultures should be tested individually. Alternatively, problems with interfering organisms may warrant the use of direct selective enrichments, two selective enrichments, or possibly even a reduction in the incubation time of the pre-enrichment cultures.

Effect of volume of pre-enrichment broth transferred. The BAM method (2) for *Salmonella* detection in egg products calls for the transfer of 1 ml of pre-enrichment broth culture to 10 ml of enrichment broth. In the multiple pooling of pre-enrichment broth cultures that has been described, 1-ml quantities of cultures were transferred, generally, to a volume of enrichment broth determined by the number of samples to be pooled to maintain a fixed pre-enrichment-to-enrichment ratio. Thus, 150 ml of enrichment broth was used to receive 15 1-ml poolings, 100 ml for 10 1-ml poolings, etc. However, for routine laboratory operations, it is more desirable to dispense enrichment broth at a constant volume. Since the number of samples to be pooled may vary considerably from time to time, it is important to know whether variations in enrichment ratio would significantly affect the detection of *Salmonella*.

Accordingly, a study was undertaken to test the effect of 1/10, 1/50, and 1/100 enrichment ratios on the recovery of *Salmonella* in inoculated food systems. The study included evaluation for both pooled samples and pooled pre-enrichment broth cultures. Each pooled pre-enrichment test was comprised of 15 25-g samples, whereas each pooled sample test consisted of 10 25-g samples. Six different tests were included for each ratio. Table 3 summarizes the results of this study.

TABLE 3. *Effect of enrichment ratio on Salmonella detection in inoculated food materials*[a]

Method of detection	No. positive/18 tests		
	Enrichment ratio		
	1/10	1/50	1/100
Pooled pre-enrichments	11	13	15
Pooled samples	9	9	9

[a] Chi-square tests and tests for linear trend between the number of positive tests and the enrichment ratio indicated no significant differences at the $P = 0.05$ level.

No significant differences in detection were noted among the three ratios tested as measured by the chi-square test and the test for linear trend. The tentative conclusion drawn is that the enrichment ratio over the range investigated and for the food samples tested (nonfat dry milk, dry egg albumin, and cocoa) was not critical to *Salmonella* detection.

We have also examined the effect of 1-ml and 2-ml transfer volumes of the pre-enrichment cultures on *Salmonella* detection and found no significant differences in recoveries. This finding has prompted the standard use of a 1-ml transfer for routine *Salmonella* analyses.

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ICMSF methods studies. II. Comparison of analytical schemes for detection of *Salmonella* in high-moisture foods[1,2,3]

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Twenty-one naturally contaminated high-moisture food samples were analyzed to compare the relative efficiency of using thirteen 25-g, three 108.3-g, one 325-g, and one 500-g subsamples in recovery of salmonellae. In addition, recovery by preenrichment in lactose broth was compared with direct selective enrichment in selenite cystine and tetrathionate brilliant green broths. Analysis of preenriched large lumped subsamples, prepared by combining multiple 25-g subsamples, detected positive samples as effectively as the analysis of individual 25-g subsamples. In the analysis of 25-g subsamples, direct enrichment detected as many positive "lots" as preenrichment, but in the analysis of lumped subsamples, direct enrichment gave dramatically reduced recovery. Nevertheless, directly enriched 25-g subsamples resulted in a significantly lower rate of recovery than preenriched subsamples. The results indicate that analysis of a single lumped 325-g preenriched subsample is as efficient in the recovery of salmonellae as the analysis of thirteen 25-g preenriched subsamples. Further, it is apparent that preenrichment of high-moisture foods, such as meat, poultry, and eggs is to be recommended over direct enrichment for the detection of salmonellae.

GABIS, D. A., et J. H. SILLIKER. 1974. ICMSF methods studies. II. Comparison of analytical schemes for detection of *Salmonella* in high-moisture foods. Can. J. Microbiol. 20: 663–669.

On a analysé vingt et un échantillons d'aliments à forte teneur d'humidité naturellement contaminés dans le but de comparer, lors de la recherche de salmonelles, l'efficacité relative entre treize prises d'essai de 25-g, trois de 108.3-g, une de 325-g et une de 500-g. On a aussi comparé l'isolement par pré-enrichissement en bouillon lactosé par rapport à l'enrichissement direct sélectif en bouillon sélénite-cystine ou tétrathionate-vert brillant. En analysant un échantillon pré-enrichi en bloc en combinant plusieurs prises d'essai de 25-g, on peut détecter des échantillons positifs avec autant de satisfaction qu'en analysant individuellement des prises d'essai de 25-g. A l'analyse des prises de 25-g, l'enrichissement direct permet de détecter autant de "lots" positifs que par pré-enrichissement; lorsque les prises sont analysées en bloc l'enrichissement direct ne permet qu'une très faible récupération. Néanmoins, il y a un taux de récupération significativement plus bas dans les prises d'essai de 25-g directement enrichies par rapport aux prises préenrichies. Les résultats indiquent qu'une prise d'essai unique préenrichie en bloc de 325-g est aussi efficace pour mettre les salmonelles en évidence que l'analyse de treize prises de 25-g préenrichies. Il ressort de plus que dans le cas d'aliments à forte teneur d'humidité soit la viande, la volaille, les oeufs, le préenrichissement est à recommander par rapport à l'enrichissement direct dans la détection des salmonelles. [Traduit par le journal]

Silliker and Gabis (7) reported that *Salmonella*-positive lots of low-moisture foods could be detected with equal certainty by analysis of sixty 25-g individual, fifteen 100-g, or three 500-g lumped samples. The purpose of the present investigation was to determine whether multiple 25-g subsamples could be similarly lumped to provide large sample units for the analysis of high-moisture foods, such as meats, poultry, and eggs. The economic advantage of lumping samples is obvious; indeed, this approach makes statistical quality control over the *Salmonella* defect economically feasible, particularly for high-risk foods where analysis of as many as sixty 25-g subsamples has been recommended (5). The United States Food and Drug Administration (12) has officially recognized the lumping of multiple 25-g samples to produce 375-g samples for analysis.

It is generally recognized that the detection of salmonellae in high-moisture foods presents problems different from those encountered in the analysis of dried products. In particular, meats, poultry, and eggs are apt to contain large num-

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[2]International Commission on Microbiological Specifications for Foods (ICMSF) of the International Association of Microbiological Societies.
[3]This study was sponsored by the Agricultural Research Service (ARS), Eastern Regional Reasearch Center (ERRC), United States Department of Agriculture, under the terms of a cooperative agreement between ARS and ICMSF.

bers of competing microorganisms. Consequently, a number of the recommended procedures for the analysis of such foods specify direct selective enrichment in contrast to preenrichment which is used in the analysis of dried foods (1, 3, 10, 12). Recently, however, Edel and Kampelmacher (4) and Moran (personal communication) reported that preenrichment of meats in a non-selective medium results in greater sensitivity of *Salmonella* detection than does direct selective enrichment. In the present work, the efficacy of lumping subsamples was investigated in conjunction with the influence of preenrichment as compared to direct selective enrichment.

Materials and Methods

Source of Samples

All samples were frozen raw products. Skeletal meat samples were obtained from human food processors. Organ meat and organ–skeletal meat mixtures were obtained from a pet food processor. Salmonellae in these samples were natural contaminants.

Sample Preparation

Meat, poultry, and casing samples were passed once through a meat grinder with a plate containing holes 8 mm in diameter. The samples were then hand mixed and reground twice through the meat grinder with a plate containing holes 3 mm in diameter. Egg samples were mixed thoroughly by hand in a 20-liter can using a large ladle. Mixing of the samples was a preliminary step to analysis to promote even distribution of contaminating organisms within the samples as received.

Twenty-five-gram subsamples were then weighed from each mixed sample in sufficient number for analysis. The 25-g subsamples were randomly selected to form lumped samples as described below.

Sampling

The acceptance criteria for category III products in the NRC report (5) was followed. Products in this category are accepted if thirteen 25-g subsamples are analyzed for *Salmonella* and found negative. Accordingly, 13 of the 25-g units were used for analysis as individual subsamples. Three lumped subsamples of 108.3 g each were prepared by combining four 25-g units and weighing an additional 8.3-g subsample from one of the 25-g units. A 325-g lumped subsample was prepared by combining thirteen 25-g units. In addition, a 500-g subsample was included in view of our previous experience with the analysis of 500-g dry food samples (7). Thus for each analytical procedure, thirteen 25-g, three 108.3-g, one 325-g, and one 500-g subsamples were analyzed.

Enrichment Procedures

All samples for non-selective preenrichment were introduced into sufficient volumes of lactose broth containing 1% (v/v) Tergitol Anionic 7 (sodium heptadecyl sulfate, Union Carbide, Chicago) to obtain a 1:10 dilution of the sample (7). For direct selective enrichment, the samples were introduced into appropriate volumes of tetrathionate broth containing brilliant green dye and selenite cystine broth (7, 10). All media were obtained from Difco, Detroit, Michigan. Preenrichment and direct enrichment cultures were incubated 24 h at 35C.

Subculturing of Enrichment Cultures

One-milliliter portions of the lactose preenrichment cultures were transferred to 9 ml of tetrathionate broth and 9 ml of selenite cystine broth, and these cultures were incubated 24 h at 35C. After incubation, the selective enrichment cultures, originating both from lactose preenrichment cultures and direct enrichment, were streaked onto brilliant green (BG), *Salmonella-Shigella* (SS), and bismuth sulfite (BS) agar plates which were then incubated at 35C. The BS and SS plates were examined after 24 h and the BS plates after 48 h.

Identification of Salmonella

Suspect colonies from the selective-differential agar plates were identified by conventional biochemical and serological procedures (10). Serological identification was accomplished using slide agglutinations with group specific O antisera and tube agglutinations using Spicer-Edwards H antisera (Difco).

Results

The *Salmonella*-recovery patterns of 21 high-moisture food samples are summarized in Table 1. All samples contained *Salmonella*; however, none of the samples was positive for salmonellae by all methods. Lactose preenrichment of three 108.3-g subsamples of each sample resulted in the detection of salmonellae in 18 of the 21 samples. Eighteen positive samples were also detected using direct enrichment in selenite broth of thirteen 25-g subsamples. Preenrichment of thirteen 25-g subsamples, as well as a single 500-g lumped subsample effected the detection of 17 positive samples. Lactose preenrichment of a single 325-g subsample from each sample resulted in the detection of only 14 positive samples.

Direct enrichment in tetrathionate broth was far less sensitive than direct enrichment in selenite broth. The use of 25-g subsamples inoculated directly into tetrathionate broth detected 13 positive samples, but with increasing weights of lumped samples the sensitivity decreased dramatically. With both 325 and 500-g lumped subsamples direct enrichment in tetrathionate broth resulted in the detection of only 2 of 21 positive samples. A similar decrease in sensitivity was noted with lumped samples inoculated directly into selenite broth.

Table 2 summarizes detailed recovery data for each of the enrichment procedures, namely,

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TABLE 1

Patterns in the recovery of salmonellae by three different sampling and analytical methods

Products	Direct selective enrichment																	
	Lactose preenrichment*						Selenite											
	Tetrathionate						Wt. of aliquots, g											
	Wt. of aliquots, g						25		108.3		325		500					
	25		108.3		325		500		No. of aliquots									
	No. of aliquots																	
	13	3	1	1	13	3	1	1	13	3	1	1	13	3	1	1	13	3	1	1
Beef trimmings	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−
Beef liver	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−
Beef liver	+	+	+	+	+	+	−	−	+	+	−	−	+	−	−	−	+	−	−	−
Beef kidney	+	+	+	+	+	+	−	−	+	+	−	−	+	+	+	−	+	+	−	−
Pork trimmings	−	−	−	−	−	−	−	−	+	−	−	−	+	+	+	−	+	−	−	−
Pork lungs	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	−	+	−	−	−
Pork liver	+	+	+	+	+	+	−	−	+	+	−	−	+	+	+	−	+	+	−	−
Pork liver	+	+	+	+	+	+	−	−	+	+	−	−	+	+	+	−	+	−	−	−
Pork kidney	+	+	+	+	+	+	−	−	+	+	−	−	+	+	+	−	+	−	−	−
Pork kidney	+	+	+	+	+	+	−	−	+	+	−	−	+	−	+	−	+	+	−	−
Pork kidney	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+
Pork spleen	−	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	−	−	−
Pork spleen	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	−
Hog casings (unsalted)	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−	+	+	−	−
Chicken (whole)	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	−	−	−
Chicken (whole)	+		−	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
Chicken (whole)-liver (beef)	+	+	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	−	−	−
Chicken (whole)-liver (beef)	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Whole egg	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Whole egg	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Egg albumen	+		−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Total samples positive	17	18	14	17	17	13	6	2	2	2	2	2	18	13	6	6	6	3	1	1

*Followed by selective enrichment.

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TABLE 2

Influence of sample size and enrichment procedure in the recovery of salmonellae from high-moisture foods

Products	Lactose preenrichment*				Direct selective enrichment: Tetrathionate				Direct selective enrichment: Selenite				Minimum estimated level/125 g
	Wt. of aliquots, g				Wt. of aliquots, g				Wt. of aliquots, g				
	25	108.3	325	500	25	108.3	325	500	25	108.3	325	500	
	No. of aliquots				No. of aliquots				No. of aliquots				
	13	3	1	1	13	3	1	1	13	3	1	1	
Beef trimmings	0	0	0	0	0	0	0	0	1	0	0	0	<0.08
Beef liver	13	3	1	1	4	3	1	0	7	2	1	1	NA
Beef liver	13	1	1	1	3	3	0	1	2	0	0	0	NA
Beef kidney	5	2	1	1	3	1	0	0	4	1	0	0	NA
Pork trimmings	9	3	1	1	3	0	0	0	7	2	0	0	NA
Pork lungs	0	0	0	1	0	0	0	0	2	1	1	1	0.08
Pork liver	12	2	1	1	7	1	0	1	11	1	0	0	NA
Pork liver	3	3	1	1	4	0	0	0	11	1	0	0	NA
Pork kidney	13	3	1	1	12	2	1	0	1	3	0	0	NA
Pork kidney	13	3	1	1	12	3	0	0	10	3	1	1	NA
Pork kidney	12	3	1	1	1	0	0	0	5	2	0	0	NA
Pork spleen	0	1	0	0	0	0	0	0	1	0	0	0	0.08
Pork spleen	13	3	1	1	3	0	0	0	7	2	1	0	NA
Hog casings (unsalted)	1	2	1	1	0	0	0	0	2	0	0	1	NA
Chicken (whole)	8	3	1	1	4	0	0	0	7	1	1	1	NA
Chicken (whole)	2	1	0	1	0	0	0	0	1	0	0	0	0.34
Chicken (whole)-liver (beef)	12	3	1	1	7	0	0	0	8	3	1	1	NA
Chicken (whole)-liver (beef)	6	2	1	1	2	0	0	0	6	2	0	0	NA
Whole egg	1	0	0	0	0	0	0	0	0	0	0	0	0.08
Whole egg	0	1	0	0	0	0	0	0	0	0	0	0	0.08
Egg albumen	1	1	0	1	0	0	0	0	0	0	0	0	0.08
Total samples positive	137	40	14	17	65	13	2	2	93	24	6	6	
Total samples analyzed	273	63	21	21	273	63	21	21	273	63	21	21	
% positive	50.2	63.5	66.7	81.0	23.8	20.6	9.5	9.5	34.1	38.1	28.6	28.6	

*Followed by selective enrichment.
Note: NA = not applicable.

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lactose preenrichment, direct enrichment in tetrathionate broth, or direct enrichment in selenite broth. An estimation of the level of salmonellae has been made for samples with a low level of contamination. A total of 1475 g of each product was analyzed using lactose preenrichment. Assuming that each positive result from the lightly contaminated subsample was derived from a single *Salmonella*, then from the total number of positive subsamples from that product an estimated minimum *Salmonella* level per 125 grams may be calculated. For example, in the second chicken sample there were two positives in the 25-g subsamples, none in the 325-g, and one in the 500-g subsample. The estimated minimum number of salmonellae was calculated to be 0.34 per 125 grams based on four positives in 1475 g of product (4:1475:X:125). The estimation is made for the level of *Salmonella* in 125 g, since according to the acceptance criteria in the NRC report (5), negative results of thirteen 25-g subsamples indicates a probability of about 95% that the level of contamination is no greater than one organism in 125 g. Stated differently, only one time in 20 should there be failure to isolate *Salmonella* from a sample containing one organism in 125 g, if 325 g of the sample is analyzed.

When 25-g subsamples were used, no sample containing >1 organism in 125 g was found negative by any of the three enrichment procedures. Further, when lactose preenrichment was used no sample containing >1 *Salmonella* in 125 g was found negative by the other three sampling procedures, i.e., three lumped 108.3-g or single lumped 325-g and 500-g subsamples. The results for lumped samples directly enriched in tetrathionate and selenite broths, however, were quite different. Nine samples containing >1 *Salmonella* in 125 g were negative in one or more instances in which lumped samples were directly enriched in tetrathionate broth. Similarly, six of the samples containing >1 *Salmonella* in 125 g were found negative in one or more cases when lumped samples were enriched directly in selenite broth.

From the foregoing it may be concluded that if the level of salmonellae in a given sample is >1 cell in 125 g, one may expect to detect that sample as positive if thirteen 25-g subsamples are analyzed by either lactose preenrichment or direct enrichment in either tetrathionate or selenite broth. It would be expected that the sample would also be found positive if a like amount of product were analyzed using three lumped 108.3-g subsamples or one lumped 325-g subsample, but only if these lumped subsamples were preenriched. The data indicate that analysis of such lumped subsamples through direct enrichment in either tetrathionate or selenite broths would give a significant number of negative results.

This perplexing loss of sensitivity when lumped samples were directly enriched was studied further. Consideration was given to the fact that 13 sets of differential plates were streaked when 25-g subsamples were enriched; whereas only three sets were inoculated from the three 108.3-g lumped subsamples and only one set from the 325-g lumped subsample. Accordingly, three samples (pork liver, pork spleen, and chicken), each of which was naturally contaminated with salmonellae, were inoculated directly into tetrathionate broth. Instead of subculturing the usual three sets of plates from the three 108.3-g subsamples, four sets of plates were streaked from two of the tetrathionate enrichment cultures and five from the third, giving a total of 13 sets of differential plates from the three 108.3-g subsamples. Similarly, 13 sets of plates were streaked from the single 325-g lumped subsample. Thus, the same number of differential agar plates were examined for each series of subsamples. Recovery results gave no indication that the inferior recovery of salmonellae from lumped 108.3-g and 325-g subsamples could be overcome by streaking 13 instead of 3 sets of plates.

The possibility that the use of lumped subsamples resulted in overgrowth of non-salmonellae was investigated. Quantitative determinations of total Enterobacteriaceae were carried out (10) on each of the tetrathionate cultures. There was no significant difference between the numbers of Enterobacteriaceae in the incubated 25-g subsamples and those for either the 108.3 and 325-g subsamples. Furthermore, there was no indication of significantly different levels of Enterobacteriaceae in the *Salmonella*-positive tetrathionate cultures compared with those in the *Salmonella*-negative cultures. Similar experiments were not conducted on samples directly enriched in selenite broth. The loss in sensitivity resulting from the direct inoculation of selenite and tetrathionate broths remains unexplained.

Whereas *Salmonella*-positive samples containing >1 cell in 125 g can be detected as posi-

tive if thirteen 25-g subsamples are analyzed either by lactose preenrichment or by direct enrichment in either tetrathionate or selenite broths, a striking aspect of the results is the greater sensitivity of the lactose preenrichment procedure. In considering the 25-g subsamples only, 50.2% of the 273 meat samples were found positive using preenrichment. In contrast, direct enrichment in tetrathionate and selenite broths resulted in only 23.8% and 34.1% positive tests, respectively. The difference between preenrichment followed by selective enrichment and selective enrichment without preenrichment is highly significant ($P < 0.001$).

Discussion

The results clearly indicate that lumping of 25-g subsamples to produce larger samples for analysis is as sensitive for the detection of positive samples as is the analysis of individual 25-g subsamples. This finding parallels the results of our previous study (7) on dried foods. The importance of this is obvious and has been discussed.

The present findings indicate that high-moisture foods should be preenriched in a non-selective medium. This is generally not done in the analysis of such products as eggs, meat, and poultry (1, 2, 10). The first edition of *Recommended methods for the microbiological examination of foods* (1), published in 1958 by the American Public Health Association (APHA), prescribes direct enrichment of food in selenite broth. This, however, was before North's (6) recognition that preenrichment of dried egg albumen in lactose broth increased subsequent sensitivity of direct enrichment procedures. Since that time, the advantage of preenrichment of dried foods has been widely appreciated. The second edition of the APHA book on microbiological methods (2), published in 1966, suggests a possible advantage of preenriching dried eggs and other dehydrated foods but makes no mention of enrichment procedures for high-moisture foods. Thatcher and Clark (10) indicate that non-selective enrichment is not required for raw meat, poultry, and unpasteurized eggs. The *Microbiology laboratory guidebook* of the United States Department of Agriculture (11), published in 1969, states that ordinarily raw meat is inoculated directly into tetrathionate broth, but adds parenthetically "a lactose pre-enrichment step may be used at the discretion of the investigator." A private communication (A. Moran, USDA) indicates that the USDA laboratories have, in fact, practiced non-selective enrichment in lactose broth since 1967. Published reports from USDA laboratories indicate that non-selective enrichment is used (8, 9). The latest edition of the *Bacteriological analytical manual* of the United States Food and Drug Administration (3) states that raw foods and finished products that have been grossly contaminated after processing should be examined by direct enrichment in selective broths.

Edel and Kampelmacher (4) reported that in the analysis of 150 naturally contaminated meat samples, 91.6% of the total number of positive samples were detected as positive by preenrichment in buffered peptone water, followed by selective enrichment in tetrathionate broth containing brilliant green, the latter incubated at 43C. On the other hand, only 69.4% of the total positive samples were detected by direct enrichment in tetrathionate broth incubated at 43C. The present results are in agreement with those of Edel and Kampelmacher. Clearly the examination of raw meats, poultry, and eggs for salmonellae should involve preenrichments of samples in a non-selective medium.

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