

Approach to Validating an Opsonophagocytic Assay for *Streptococcus pneumoniae*

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Received 31 August 2004/Returned for modification 28 September 2004/Accepted 9 December 2004

***Streptococcus pneumoniae* (pneumococcus) polysaccharide serotype-specific antibodies that have opsonophagocytic activity are considered a primary mechanism of host defense against pneumococcal disease. In vitro opsonophagocytic assays (OPAs) with antibody and complement to mediate opsonophagocytic killing of bacteria have been designed and developed as an adjunct to the standardized serum immunoglobulin G antipneumococcal capsular polysaccharide enzyme immunoassay to assess the effectiveness of pneumococcal vaccines. OPA presents challenges for assay standardization and assay precision due to the multiple biologically active and labile components involved in the assay, including human polymorphonuclear leukocytes or cultured effector cells, bacteria, and complement. Control of these biologically labile components is critical for consistent assay performance. An approach to validating the performance of the assay in accordance with International Conference for Harmonization guidelines, including its specificity, intermediate precision, accuracy, linearity, and robustness, is presented. Furthermore, we established parameters for universal reagents and standardization of the use of these reagents to ensure the interlaboratory reproducibility and validation of new methodologies.**

Streptococcus pneumoniae, a gram-positive diplococcal bacterium, is responsible for an estimated 1 million deaths yearly in children under 5 years of age, and these occur mostly among children in the developing world (40, 41). The burden of disease is greatest among young children, elderly individuals, and patients with predisposing conditions, such as immunosuppressive illnesses (8, 39). In the United States alone, an estimated 7 million cases of otitis media, 0.5 million cases of pneumonia, 50,000 cases of bacteremia, and 3,000 cases of meningitis occur each year (4). The sequelae for survivors are often serious neurological deficits and hearing loss. This organism also accounts for about 25 to 50% of the cases of community-acquired pneumonia among elderly individuals, who require hospitalization and among whom community-acquired pneumonia leads to an estimated 40,000 deaths per year (8, 19, 27). It is a significant burden for the health care system.

The development of an effective vaccine for the prevention of pneumococcal infections plays a crucial role in reducing the disease burden worldwide (2, 18, 21, 23, 26, 32). At present two types of pneumococcal vaccines are available: a pneumococcal capsular polysaccharide (PnPs) vaccine and a polysaccharide (Ps) protein conjugate vaccine (4, 5, 22, 24). The primary considerations for regulatory agencies for approval of a new vaccine product are the safety and efficacy of the product when it is used by the intended population. The ability to show the noninferiority or bioequivalence of a new vaccine to existing vaccines in clinical trials is key to extending the application of existing vaccine formulations to individuals in different age groups or of different ethnicities or to approving the licensure of new vaccine formulations. In 2002, the World Health Organization sponsored a meeting of experts to review the available serological data in order to draft guidelines for the evaluation of new vaccine for-

mulations on the basis of serological correlates for protection against pneumococcal disease and to formulate a series of recommendations for the establishment of a licensing pathway for new pneumococcal vaccine formulations (13). The concentration of immunoglobulin G (IgG) antibody against PnPs in serum, as measured by a standardized enzyme immunoassay (EIA) (6, 25, 38) after a three-dose priming series, was considered the primary end point that was predictive of efficacy (13). The killing activity measured by the opsonophagocytic assay (OPA) after a three-dose priming series provides auxiliary information and an in vitro mechanism to assess the functionalities of antibodies (3, 12, 13, 20, 34, 36). The result of OPA was recommended as a secondary end point to support pneumococcal vaccine licensure (13).

Vaccine potency data are collected from many clinical trials and over many years, and the test methods that measure the immunogenicities of vaccines must be validated for the intended purpose of the vaccines. The International Conference on Harmonization (ICH) published guidelines for the validation of analytical procedures that were adopted by the Food and Drug Administration (FDA) for the validation of bioanalytical test methods (9, 10, 11). This guidance provides a roadmap for validation: test method performance should be assessed in regard to the guidance parameters and typical acceptance limits thereof. A serum IgG anti-PnPs EIA that measures both functional and nonfunctional antipneumococcal antibodies has been standardized and validated according to ICH guidelines (6, 25, 38). However, the in vitro OPA, which measures functional antibodies, may provide a better surrogate for protection but is not completely standardized or validated (1, 3, 14, 30). Herein, we report on the specifications for the pneumococcal OPA and provide guidance to laboratories that perform these functional assays that will facilitate interlaboratory method standardization.

MATERIALS AND METHODS

Sera and antibodies. Sera from healthy adults (nonvaccinated) were prepared from plasma (American Red Cross, Rochester, N.Y.). Sera from infants were

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obtained from a clinical trial evaluating an experimental polyvalent pneumococcal conjugate (Wyeth, Pearl River, N.Y.). Synthetic bovine fetal clone I (FCI) serum, demonstrated to be devoid of pneumococcal activity by OPA, was obtained from HyClone (catalog no. 30080.03). Monoclonal antibodies specific for the cell markers CD35 and CD71 were obtained from Becton Dickinson (catalog no. 555452) and Caltag (catalog no. MHCD7104-4), respectively.

Bacterial strains. The sources of the target *S. pneumoniae* strains used in the OPA were serotype 1, 18C, and 23F strains (Wyeth); serotype 4 and 14 strains (Dana Farber Cancer Institute, Boston, Mass.); serotype 5, 6B, and 9V strains (Centers for Disease Control and Prevention [CDC], Atlanta, Ga.); and a serotype 19F strain (catalog no. 6319; American Type Culture Collection [ATCC]). ACM Clinical Laboratory (Rochester, N.Y.) confirmed the identities and the purities of these strains by World Health Organization methods for isolate detection (39). For safety purposes, only pneumococcal strains with defined antibiotic susceptibility profiles were used. Throughout these studies, bacteria were freshly grown in Todd-Hewitt broth with 5% Bacto yeast extract (catalog no. 212740; Becton Dickinson) at 37°C with 5% CO₂. Bacterial growth was monitored, and the bacteria were harvested for use in the OPA at the late log phase (target optical density at 550 nm, 0.7 to 0.8) to ensure the adequate formation of a capsule for each pneumococcal bacterial strain.

Complement. Multiple lots of baby rabbit serum complement were screened for potency and nontoxicity prior to use as an exogenous complement source in the OPA (catalog no. 31038; Pel-Freez Clinical Systems). Potency was acceptable when the new lot yielded titers within twofold of the known titers (± 1 well dilution, obtained when a previously qualified lot of baby rabbit serum was used) in an OPA performed with a minimum of three different positive human serum samples. Additionally, the acceptable lots demonstrated a low level of nonspecific killing in the OPA performed in the absence of human serum with every pneumococcal serotype of interest. This is discussed further in the Results section.

Phagocytic cells. Either human polymorphonuclear leukocytes (PMNs) or differentiated HL60 promyelocytic leukemia (HL60) cells were used as effector cells. Human PMNs from several healthy adult donors were freshly isolated by dextran sedimentation and Ficoll-Histopaque density gradient centrifugation (30); they were pooled for use in the OPA to reduce variable phagocytic activity due to the polymorphisms of IgG receptors on phagocytic cells in the human population (7, 28). HL60 cells were obtained from ATCC (catalog no. CCL240, lot no. 1473975); they were maintained, passaged, and differentiated into granulocytes (with 100 mM dimethylformamide [DMF]) by the protocol described by Romero-Steiner and coworkers (30). The HL60 cells were confirmed to be mycoplasma-free (catalog no. M-100; Bionique Testing Laboratories, Inc.). The viabilities of the differentiated HL60 cells were assessed by trypan blue exclusion and annexin V-propidium iodide staining (Caltag). Acceptable viability was demonstrated if either (i) greater than 90% of the cells demonstrated exclusion of trypan blue or (ii) less than 35% of the differentiated cells were stained by annexin V-propidium iodide (indicative of apoptotic and necrotic cells). HL60 cells from day 3, 4, or 5 postdifferentiation were used as long as CD35 (complement receptor 1) expression was up-regulated by $\geq 55\%$ of the cell population and CD71 (transferrin receptor) expression was down-regulated by $\leq 15\%$ of the cell population, as assessed by flow cytometry (FACS Calibur 4) (15, 33).

OPA. The OPA used in this study is a modification of the method of Romero-Steiner and coworkers (30). In brief, heat-inactivated human serum specimens were serially diluted in eight twofold steps in a 96-well microtiter plate with Hanks balanced salt solution containing 0.1% gelatin (10 μ l/well) and were then incubated with cells of the different *S. pneumoniae* serotypes (~2,000 CFU per well) and complement (baby rabbit serum [final concentration, 12.5%]) for 30 min at 37°C (final volume, 40 μ l/well) on an orbital shaker (model 4518; Forma Scientific), as specified below (opsonization step). The optimal shaking speed was determined (see Results) for each bacterial strain to minimize nonspecific killing or overgrowth. Plates containing serotypes 1 and 4 were shaken at 250 rpm, those containing serotypes 18C and 19F were shaken at 225 rpm, that containing serotype 5 was shaken at 200 rpm, those containing serotypes 6B and 23F were shaken at 100 rpm, and those containing serotypes 9V and 14 were shaken at 50 rpm. Freshly isolated human PMNs or differentiated HL60 cells (effector cells) were added at a 400:1 ratio to the bacterium (target)-complement-serum mixture (final volume, 80 μ l/well), and the mixture was incubated at 37°C for 45 min with shaking on an orbital shaker at 250 rpm (phagocytic step). After incubation, the solution in each test well was diluted with an equal volume of 0.9% NaCl; then, a 10- μ l aliquot was removed and applied onto a tilted blood agar plate, and the plate was incubated overnight at 37°C with 5% CO₂. The colonies were counted with a semiautomated colony counter (Sorcerer image analyzer; Perceptive Instruments), and the OPA titer was calculated as the reciprocal of the serum dilution that caused a 50% reduction of the CFU (killing) compared to the CFU from the control wells containing all reagents except human serum (i.e.,

TABLE 1. Controls for system suitability testing in pneumococcal OPA

Control type	Bacteria	Active complement	Heat-inactivated complement	Effector cells ^a	Serum specimen
Baseline reference T_0 ^b control	+	—	—	—	—
Complement control ^c	+	+	—	—	—
Background T_{75} ^d /effector cell control	+	+	—	+	—
Antibiotic therapy control ^e	+	—	+	—	+

^a PMNs or HL60 cells.

^b T_0 , CFU at time zero, which is used as a baseline reference.

^c Used daily to monitor for changes in susceptibilities of pneumococci to complement toxicity.

^d T_{75} , CFUs after 75 min of incubation (i.e., 30-min opsonization step and 45-min phagocytic step), which is used to calculate the 50% reduction in CFUs to determine the opsonophagocytic activity of each serum specimen; it is also used as an effector cell control.

^e This control is performed for every test specimen in one pneumococcal serotype OPA.

controls at 75 min [T_{75}]). The lowest titer of opsonophagocytic antibody that could be measured by our method was 8, based on the dilution of undiluted serum in the incubation well. Serum specimens not demonstrating a 50% reduction of CFU in the OPA at the lowest serum dilution (1:8) were assigned a titer of 4, enabling statistical analyses of the data sets. There is no upper limit for this pneumococcal OPA method, since the serum specimens were diluted until the number of CFU fell into the linear range of the assay. Prior to the assay run on each day, the performance of the semiautomated colony counters was calibrated by using plates with various amounts of CFU to ensure the counting reliability of the equipment.

System suitability testing. Additional control wells were included in each run of the assay to ensure that test performance met the preestablished criteria for acceptance, as described in Table 1. These controls were assays in which specified components were omitted: human test serum, complement source, or effector cells or a combination of these components. Furthermore, each specimen was assessed with an antibiotic control well. The antibiotic control well lacked active complement and effector cells. If inhibition of bacterial growth occurred in the presence of the test specimen but in the absence of active complement and effector cells, it was suggested that antibiotics (or another inhibitory substance) may have been present in the test specimen and that the specimen could not be analyzed. The T_0 control wells, which contained only bacteria, were plated out without incubation (time zero [T_0]) and were used as the CFU baseline reference. The T_{75} control wells lacked the test serum but contained bacteria, complement, and effector cells. Upon plating after the opsonization and phagocytic step incubations (75 min total), the T_{75} wells had to yield counts between 80 and 200 CFU per well for assay acceptance. This observed number of CFU from the T_{75} control wells then provided the basis for calculation of a 50% reduction in CFU by the test serum for the OPA titer determination. Four human adult serum samples with anti-PnPs IgG levels ranging from 0.41 to 34.81 μ g/ml were included as positive OPA controls and were randomly assigned to different assay plates (one control serum sample per assay plate) to monitor the performance of the assay. The OPA titers of the control sera should not exceed the median value, which was previously established by testing over a significant period of time, by twofold.

OPA specificity. The specificity of the pneumococcal OPA was determined by addition of a competitive inhibitor to the assay wells. A total of 125 μ g of homologous or heterologous PnPs, an unrelated meningococcal serogroup C polysaccharide (MnCPs), or buffer with no inhibitor per ml was mixed with a fixed concentration of human test serum and subsequently analyzed by the OPA. The dilution of a serum sample chosen for use in this competitive OPA was the greatest dilution that yielded complete killing of the targeted bacteria; if the OPA titer was ≤ 128 , then undiluted sera were used. The percent inhibition of killing was calculated as [(CFU in the presence of Ps inhibitor – CFU in the buffer with no inhibitor)/(CFU of the background T_{75} control counts – CFU in the buffer with no inhibitor)] $\times 100$.

RESULTS

We have validated the pneumococcal OPA using the guidelines for analytical and bioanalytical method validation described by ICH and FDA (9, 10, 11). Guidance parameters, including assay specificity, intermediate precision, linearity, accuracy, and

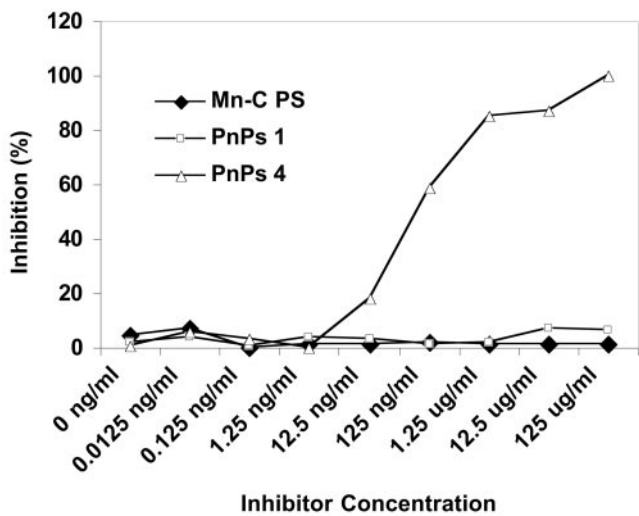


FIG. 1. The specificity of the pneumococcal serotype 4 OPA was evaluated by determination of the inhibition of opsonophagocytic activity when homologous PnPs (PnPs 4), heterologous PnPs (PnPs 1), unrelated polysaccharide (MnCPs), or no competitor (0 ng/ml) was added to the OPA.

robustness, were assessed by using pneumococci of nine serotypes (serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F). The outcomes from the assessment of these parameters allow the determination of whether the performance of the OPA is acceptable and suitable for use in clinical studies to analyze samples for the presence and the relative concentration of antibodies that mediate opsonophagocytosis of pneumococci in a serotype-specific fashion.

Specificity. The specificity of the pneumococcal OPA was assessed by competitive inhibition, in which the opsonophagocytic activity was evaluated after the addition of homologous or heterologous PnPs, which may or may not compete for anti-PnPs-specific antibody binding in the opsonization step. The specificity of the OPA was considered acceptable when the homologous PnPs inhibited $\geq 80\%$ of opsonophagocytic activity and when heterologous PnPs or unrelated polysaccharide inhibited $\leq 20\%$ of opsonophagocytic activity.

The specificity of the OPA was evaluated with sera from nonvaccinated adults. An example of the specificity of a typical OPA experiment is demonstrated in Fig. 1, which shows the results of competitive inhibition of a pneumococcal serotype 4 OPA in a dose-response fashion. Summary specificity data for nine pneumococcal serotypes, obtained with four serum specimens per serotype, are shown in Fig. 2. In the absence of

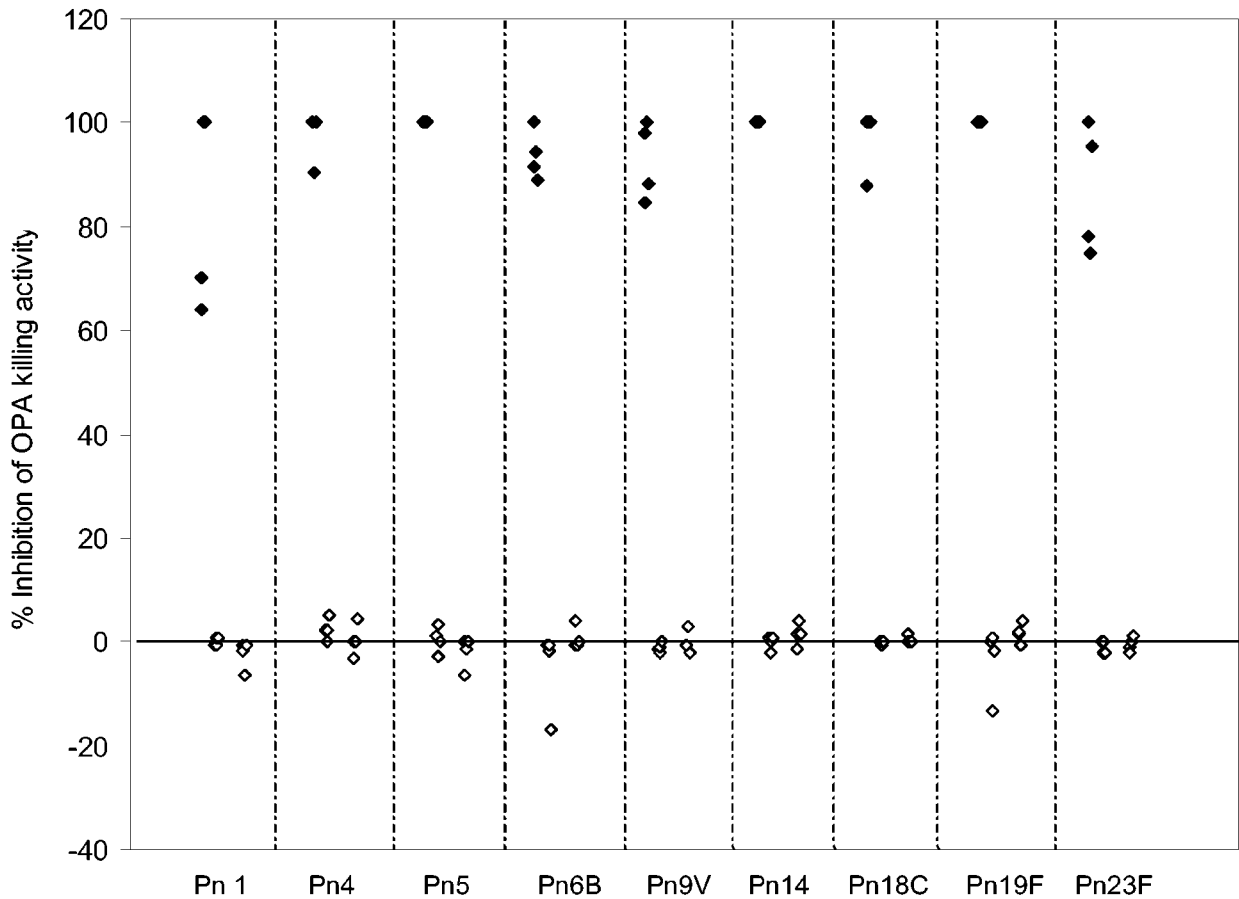


FIG. 2. The specificity of the pneumococcal OPA was evaluated by determination of the inhibition of opsonophagocytic activity with sera from nonvaccinated adults with 125 μ g of homologous, heterologous PnPs (PnPs 1 for the serotype 4, 5, 6B, and 9V OPAs and PnPs 5 for the serotype 1, 14, 18C, 19F, and 23F OPAs), or unrelated MnCPs per ml. Homologous inhibition is shown as solid symbols, while heterologous or unrelated MnCPs inhibition is shown as hollow symbols. Pn, pneumococcal serotype.

TABLE 2. Assessment of intermediate precision of pneumococcal OPA^a

Specimen no.	Mean OPA titer (no. of replicates, frequency ^b) by pneumococcal serotype								
	1	4	5	6B	9V	14	18C	19F	23F
1	2,048 (28, 93)	2,048 (23, 96)	2,048 (32, 100)	1,024 (29, 100)	4,096 (23, 96)	4,096 (22, 95)	1,024 (25, 88)	512 (25, 96)	8,192 (23, 100)
2	1,024 (28, 100)	512 (21, 86)	256 (25, 84)	2,048 (30, 97)	4,096 (23, 74)	2,048 (26, 85)	128 (24, 96)	256 (20, 100)	1,536 (22, 82)
3	128 (29, 100)	128 (21, 86)	64 (30, 97)	512 (27, 96)	256 (24, 83)	128 (20, 90)	48 (24, 92)	128 (19, 89)	256 (21, 100)
4	64 (25, 85)	64 (23, 100)	64 (26, 100)	256 (21, 90)	512 (23, 100)	512 (25, 96)	2,048 (27, 100)	32 (20, 80)	256 (20, 90)

^a Four serum specimens from nonvaccinated adults were analyzed over 6 months.

^b Percentage of serotype-specific OPA titers within twofold of the median titer for that specimen.

competitor, the OPA titers for these four human adult serum samples ranged from 64 to 2,048 for serotypes 1, 4, 5, 18C, and 19F and from 256 to 8,192 for serotypes 6B, 9V, 14, and 23F (data not shown). In the majority (32 of 36) of these competition assays, greater than 80% inhibition of killing was observed when 125 µg of homologous PnPs per ml was included. In the other four assays, the inhibition by homologous Ps was still high: 70 and 64% inhibition in the serotype 1 OPA and 78 and 75% inhibition in the serotype 23F OPA. The latter specimens likely had non-PnPs-specific opsonizing antibodies (30). In contrast, in all cases less than 10% inhibition of opsonophagocytic activity was observed in assays with 125 µg of heterologous PnPs per ml (PnPs 1 for the serotype 4, 5, 6B, and 9V OPAs and PnPs 5 for serotype 1, 14, 18C, 19F, and 23F OPAs) or MnCPs. Thus, the specificity of the pneumococcal OPA was established.

Intermediate precision. Intermediate precision is the degree of agreement of OPA results when serum specimens are tested multiple times (i.e., on different days or by different analysts). This parameter was assessed in two ways: (i) a small panel of four control adult serum samples was analyzed repeatedly over a period of 6 months; and (ii) larger panels of test sera comprising specimens with low, medium, and high OPA titers were evaluated in three assay runs. The acceptance criteria for this parameter are that the results for $\geq 70\%$ of the specimens in the panel be in agreement. Agreement is defined as a titer within twofold of the established median OPA titer (i.e., ± 1 well dilution of the median titer) (30). For example, if the median titer is 512, then titers of 256, 512, and 1,024 would be in agreement.

Four serum samples from nonvaccinated adults with high, medium, and low serotype-specific OPA titers were used to assess the intermediate precision of the OPA over a 6-month period by using various preparations of freshly grown bacteria and PMNs as effector cells. Each specimen was tested 19 to 32 times during this period. The median OPA titers of each control serum for serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F are listed in Table 2. There was $\geq 80\%$ agreement (range, 80 to 100%) for 35 of 36 control assays. For one assay, with control serum sample 2 in the serotype 9V OPA, the agreement was slightly lower (74%).

Assay precision was also assessed with large panels of sera from infants vaccinated with an experimental multivalent pneumococcal polysaccharide conjugate vaccine. Sera were analyzed on three different days, with different analysts performing the assay on each day (i.e., three assay runs for each serotype tested). A panel comprising 21 serum specimens was used in the OPAs for pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F; a 45-specimen panel was used in the serotype 1

and 5 OPAs. The frequency of agreement of the OPA titers for each specimen was determined (Table 3). In this set of assay runs, for the result for a specimen to be designated in agreement, all three titers for that specimen had to be within twofold of the median titer. For all serotypes, there was greater than 80% agreement of OPA titers on a per-specimen basis (range, 81 to 100%). Furthermore, when the data were stratified on the basis of low, medium, or high OPA titers, there was no indication of substantial differences in the frequency of agreement. For example, for the serotype 1 OPA, the percentages of agreement for the low-titer (≤ 32 ; $n = 19$), medium-titer (> 32 to ≤ 256 ; $n = 17$), and high-titer (> 256 ; $n = 9$) specimens were 89, 83, and 89%, respectively. Overall, the agreement for all OPAs with either infant or adult sera exceeded the minimum acceptance criterion (70%) for intermediate precision.

Linearity. Linearity is defined as the ability of the OPA to yield titers that are directly proportional to the concentration of serotype-specific pneumococcal antibody in the specimen. Each serotype-specific OPA was performed with four different initial concentrations of each serum sample, and the resulting titers were compared by linear correlation analysis. Acceptable linearity was obtained if the calculated Pearson correlation coefficient (r), which describes the relationship between the measured OPA titers and the initial concentrations of the test serum sample, was greater than 0.9 and the slope of the regression line was between -0.65 and -1.35 . Two serum specimens from nonvaccinated adults with high and medium serotype-specific OPA titers, respectively, were tested (at four initial concentrations) by the nine serotype-specific pneumococcal OPAs (i.e., serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F). Acceptable linearities were obtained in all cases (Table 4); an example of a typical assay result displaying such linearity, in this case, for the pneumococcal serotype 5 OPA, is shown in

TABLE 3. Intermediate precision of pneumococcal OPA^a

Pneumococcal OPA serotype	% Agreement ^b	No. of serum specimens tested
1	87	45
4	95	21
5	93	45
6B	100	21
9V	90	21
14	100	21
18C	81	21
19F	95	21
23F	95	21

^a A panel of serum specimens from vaccinated infants were analyzed on three different days by different analysts.

^b Percentage of specimens with titers within twofold of the median titer.

TABLE 4. Linearity of pneumococcal OPA^a

Pneumococcal serotype	Serum specimen 1		Serum specimen 2	
	<i>r</i>	Slope	<i>r</i>	Slope
1	1.000	-1.000	0.996	-1.350
4	1.000	-1.000	0.976	-0.900
5	0.996	-1.200	0.994	-1.150
6B	0.983	-1.300	0.984	-1.050
9V	0.993	-1.250	0.990	-0.850
14	0.996	-1.350	0.994	-1.150
18C	1.000	-1.000	0.994	-1.150
19F	0.994	-1.150	0.982	-1.300
23F	0.997	-1.300	0.997	-1.200

^a Sera with known serotype-specific OPA titers were tested at four serial dilutions. Effector cells were PMNs used at a PMN/bacterium ratio of 400:1.

Fig. 3. All slopes were within the acceptable range, and the Pearson correlation coefficient for both specimens in each of the serotype-specific OPAs ranged from 0.976 to 1.000. Thus, the data support good linearity.

Accuracy. Accuracy is the closeness of agreement of a value obtained from the assay to the reference or known value. In the absence of a standard reference serum specimen for the OPA, a surrogate approach involving the spiking of different amounts of OPA-positive serum into OPA-negative serum was used to estimate accuracy. The expected OPA titer of the spiked serum specimen was derived from the observed OPA titer and the dilution factor of the OPA-positive serum specimen. The criterion for acceptable assay accuracy is that the observed OPA titer of the spiked serum specimen be within twofold of the expected OPA titer. Bovine FCI is a synthetic serum specimen that is negative in each of the nine serotype-specific OPAs (data not shown). FCI was separately spiked with two different amounts of two human serum specimens known to be OPA positive and was tested two to four times in each serotype-specific OPA. Whenever possible, the human sera tested were selected such that they represented specimens with moderate and high expected OPA titers in that serotype-specific OPA. As such, different human sera were tested in different assays; the sera were diluted into FCI at 1:2, 1:4, or

TABLE 5. Accuracy of pneumococcal OPA^a

Pneumococcal serotype	Serum specimen 1		Serum specimen 2		Overall agreement ^c (%)
	OPA titer ^b	Agreement ^c	OPA titer ^b	Agreement ^c	
1	1,024	8/8	2,048	8/8	16/16 (100)
4	4,096	8/8	2,048	8/8	16/16 (100)
5	4,096	8/8	128	8/8	16/16 (100)
6B	1,024	8/8	256	8/8	16/16 (100)
9V	4,096	8/8	256	8/8	16/16 (100)
14	8,192	7/8	512	6/8	13/16 (81)
18C	2,048	8/8	1,024	8/8	16/16 (100)
19F	512	8/8	256	8/8	16/16 (100)
23F	1,024	8/8	512	4/8	12/16 (75)

^a Human OPA-positive sera were added to an OPA-negative serum specimen (FCI) and assessed for the agreement of the observed and the expected titers in each serotype-specific OPA. Effector cells were PMNs used at a PMN/bacterium ratio of 400:1.

^b The expected OPA titer of the selected human serum specimen.
^c Data represent the number of specimens with the expected titer/total number of specimens tested. Agreement is defined as an observed OPA titer of the spiked specimen within twofold of the expected OPA titer (on the basis of the dilution factor).

1:8 for the assessment of accuracy. The results of this analysis (Table 5) indicate that there was 100% agreement in seven of the nine serotype-specific OPAs. For assays with other serotypes (serotypes 14 and 23F), the rates of agreement were high: 81 and 75%, respectively.

Robustness. The pneumococcal OPA comprises interactions of multiple biological components. As such, assay results are easily influenced by changes in the components and the testing conditions. Robustness is the susceptibility of these results to deliberate changes in assay conditions. OPA performance, especially intra- and interlaboratory precisions, may be affected by changes in the bacterial strain, the exogenous complement source, the shaking rate during the incubation period, the criteria for qualified effector cells, and the effector cell-to-target bacterium ratio.

Selection of the pneumococcal strain(s) with an optimal capsule may be critically important for OPA performance. Pneumococcal strains with capsules of different sizes (as de-

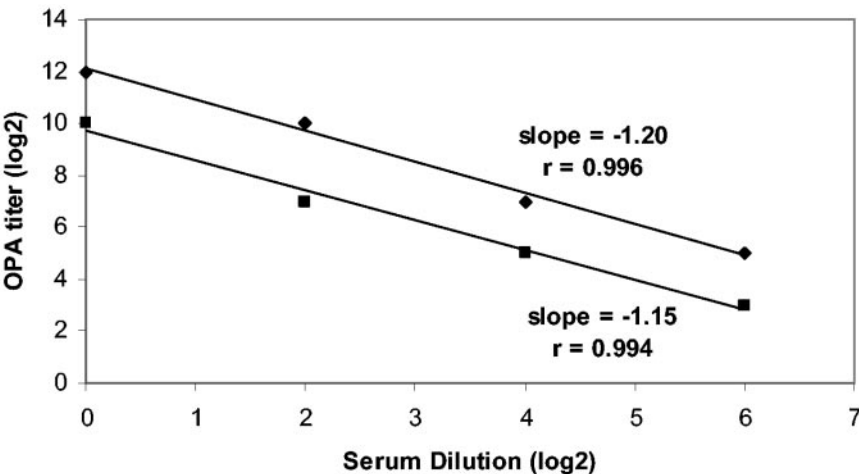


FIG. 3. Linearity of the results for two human serum specimens in the pneumococcal serotype 5 OPA. Each specimen was tested at four different initial concentrations. Linear correlation analysis was performed after log₂ transformation of the data. The resulting slopes and the Pearson correlation coefficients (*r*) for each line are indicated.

TABLE 6. Effect of target bacterial strain selection and complement source in pneumococcal serotype 4 OPA^a

Strain source	Capsule size	Relative no. of CFU (%) ^b	
		Complement source 1	Complement source 2
CDC	Small	35	ND ^c
ATCC	Small	115	20
Dana Farber	Moderate	ND	85

^a Effector cells were PMNs used at a PMN/bacterium ratio of 400:1. Viability (i.e., the relative number of CFU) was derived from the comparison of the numbers of CFU for the T_0 and T_{75} controls for each experiment.

^b Percentage of CFU for the T_{75} control relative to that for the T_0 control.

^c ND, not done.

terminated in a Quellung reaction) were tested for their susceptibilities to nonspecific complement-mediated killing in the OPA. An example of the results of an experiment involving serotype 4 strains and two different sources of complement is shown in Table 6. Nonspecific killing was estimated by comparing the CFU obtained from the T_0 and T_{75} controls (defined in Table 1). Strains with a small polysaccharide capsule yielded variable results. With one strain (from CDC), a high rate of nonspecific killing (65% reduction in CFU) by the complement from source 1 was observed, while an acceptable 15% increase in CFU was observed with the same complement when a second small-capsule strain (from ATCC) was used. In contrast, the latter strain was susceptible to nonspecific complement-mediated killing when the complement from source 2 was used. Acceptable results were obtained when a pneumococcal strain with a capsule of a medium size was used in conjunction with the complement from source 2: nonspecific killing was minimal (15% reduction in CFU). Thus, the susceptibility of a pneumococcal strain to nonspecific killing by complement must be carefully evaluated so that the numbers of CFU of bacteria from the T_{75} control are within the acceptable range (80 to 200 CFU).

Previous observations led to the notion that the shaking rate may have a critical impact on the viability of the pneumococci. The effect of the shaking rate during the opsonization step (i.e., the 30-min incubation step with diluted test serum, complement, and bacteria) was examined. The results from the analysis of three test serum specimens in the serotype 14 OPA are shown as an example in Table 7. The specimens were tested at different shaking rates during the opsonization incubation step, followed by the addition of PMNs as effector cells (at 400:1 with bacteria) before further phagocytic step incubation and plating, in accordance with the standard protocol. A 30% or greater reduction of CFU (i.e., the percent difference between the numbers of CFU at T_0 and the numbers of CFU at T_{75} , which indicates nonspecific killing) was observed when bacteria were shaken at 100 or 250 rpm, while lower levels of nonspecific killing were observed when the shaking rate was ≤ 50 rpm. Experiments similar to this were performed with the other serotypes to identify the optimal shaking rates for each strain. For example, for pneumococcus serotype 14, a shaking rate of 50 rpm was selected to maximize the interaction between bacterial cells and antibodies (i.e., opsonization) while maintaining the viability of the pneumococci. Although there was no effect on the precision of the OPA (i.e., when the three serum specimens were used) at the different shaking speeds in

the example with serotype 14, high levels of nonspecific killing, as indicated above, may result in CFU counts below the acceptable range.

The effector cell is another major component that plays a pivotal role in OPA performance. Genetic and functional polymorphisms of the cell surface IgG receptors in human PMNs, especially the biallelic Fc γ RIIa (CD32) receptor, which is important for an effective interaction with IgG2, affect the opsonophagocytic potential of opsonized pneumococci (7, 28). Additionally, the polymorphism of complement receptor 1 has also been reported (16). Thus, PMNs from different donors may have a substantial effect on interassay OPA precision. Furthermore, since it is unrealistic that PMNs from the same human donor will be available on a day-to-day basis, OPA variability due to PMN effector cells can be reduced by using pools of PMNs from a number of healthy donors rather than PMNs from a single and different donor on each assay day. An alternative to the use of pooled PMNs in the OPA is the use of tissue-cultured, differentiated HL60 cells as effector cells, as suggested previously (30). Although these are immortalized cultured cells and may, therefore, be advantageous for use for interlaboratory OPA standardization, a number of quality control criteria must be met to ensure the adequate performance of the HL60 cells in the assay. For example, after DMF-induced differentiation of cultured HL60 cells, cell viability, as measured by annexin V-propidium iodide staining of nonfunctional apoptotic or necrotic cells, should be $\geq 65\%$ for subsequent use in the OPA. Furthermore, since only differentiated HL60 effector cells are functional in the OPA, there are criteria that can be used to assess and ensure the use of properly differentiated cells. These criteria include the up-regulation of the CD35 marker of differentiation (complement receptor 1) and the down-regulation of the CD71 marker of proliferation (transferrin receptor). Our acceptance criteria are that CD35 must be detected on at least 55% of the total cells and that CD71 must be detected in no more than 15% of the total cells. Another important performance parameter of HL60 effector cells is their tissue culture passage number. High-passage-number HL60 cells have a reduced opsonophagocytic potential compared with that of lower-passage-number cells. A typical example of this is shown in Fig. 4 for the pneumococcal serotype 6B assay with HL60 cells derived from undifferentiated cells at either passage 51 or 111 tested at 3 days postdifferentiation. These data indicate incomplete opsonophagocytosis at low serum dilutions with cells from passage 111 (i.e., >20 CFU

TABLE 7. Effects of changes in horizontal shaking rate during opsonization step on pneumococcal viability in serotype 14 OPA^a

Shaking rate (rpm)	Relative no. of CFU (%) ^b	Titer		
		Serum specimen 1	Serum specimen 2	Serum specimen 3
0	82	4,096	2,048	256
50	83	4,096	2,048	128
100	70	4,096	2,048	128
250	31	4,096	2,048	128

^a Effector cells were PMNs used at a PMN/bacterium ratio of 400:1. Viability (i.e., the relative number of CFU) was derived from the comparison of the numbers of CFU for the T_0 and T_{75} controls for each experiment.

^b Percentage of CFU for the T_{75} control relative to that for the T_0 control.

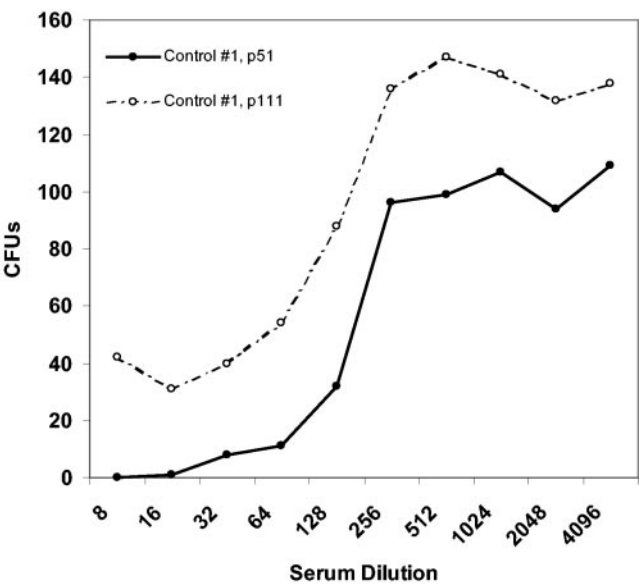


FIG. 4. Effect of HL60 cell age (expressed in cell passage) on opsonophagocytic activity. Dilutions of a serum specimen from a non-vaccinated adult were tested in the pneumococcal serotype 6B OPA with day 3 HL60 cells (400:1) from postdifferentiated passage 51 (p51; solid line) and 111 (p111; dotted line). Incomplete opsonophagocytic activity (i.e., high baseline numbers of CFU [>20]) was observed when high-passage-number (>80) differentiated HL60 cells were used. However, the OPA titer assigned to the serum specimen remained the same when qualified HL60 cells from a different passage were used.

at a 1:8 dilution of serum) compared to that with cells from passage 51. To maintain the consistent performance of the pneumococcal OPA in our laboratory, HL60 cells are used only to passage 80; beyond passage 80, a reduction in opsonophagocytic activity occurs.

The ratio of the effector cell to the target bacterium is another key element to be considered for optimal and consistent OPA performance. Various ratios (from 50:1 to 400:1) of either PMNs or HL60 cells (effector cells) to bacteria were examined. An important acceptance criterion is that there is nearly complete opsonophagocytic activity at low dilutions of a known OPA-positive serum specimen. The results obtained with PMNs and HL60 cells at effector cell/target bacterium ratios of 50:1 and 400:1 in the pneumococcal serotype 9V assay are shown in Fig. 5. At a ratio of 50:1, both PMNs and HL60 cells displayed incomplete opsonophagocytic activity at low serum dilutions. However, at ratio of 400:1, both effector cell types performed in a similar fashion. Both demonstrated nearly complete opsonophagocytosis at low serum dilutions and nearly identical OPA titers and phagocytic curves (in terms of the numbers of CFU) at high serum dilutions. Our observations of the similar reactivities of HL60 cells and PMNs as effector cells in the pneumococcal OPA are consistent with those presented in a previous report (30).

DISCUSSION

The performance of the pneumococcal OPA method has been evaluated and validated in accordance with ICH and FDA guidelines, including its specificity, precision, linearity, accuracy, and robustness (9, 10, 11). The optimization and

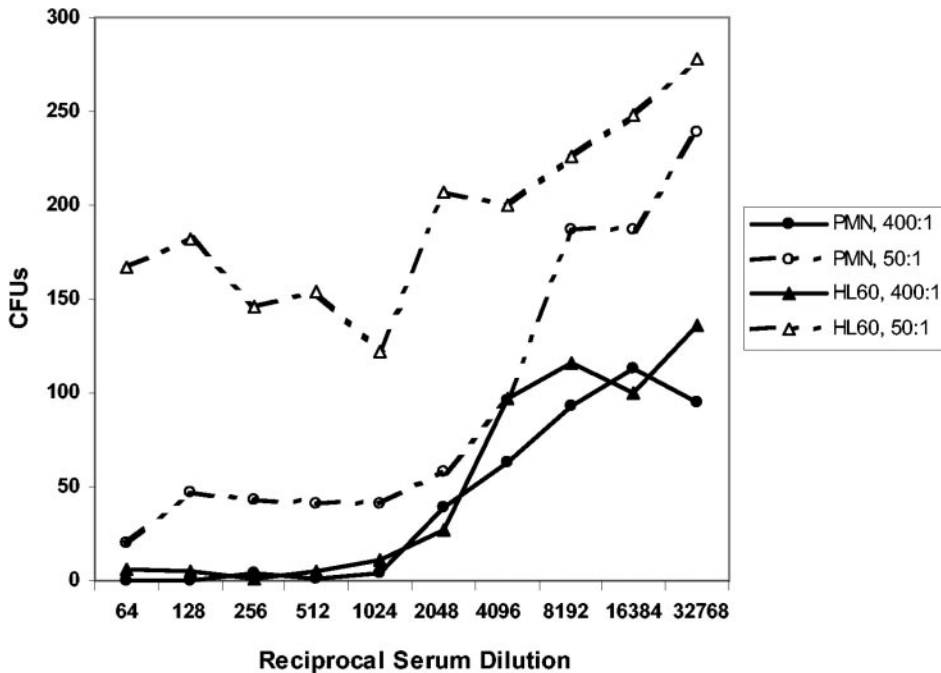


FIG. 5. Changes in effector cell/target bacterium ratio on pneumococcal serotype 9V OPA performance. The numbers of CFU obtained in the pneumococcal serotype 9V OPA with one serum specimen are shown by using PMNs or HL60 cells (passage 19) at day 4 postdifferentiation at 400:1 (solid lines) and 50:1 (dotted lines). A single analyst performed the comparison study on the same day using the same bacterial culture to minimize variation.

standardization of the OPA to obtain favorable performance characteristics are particularly challenging because of the use of multiple labile components in the assay, including viable bacteria, freshly isolated or cultured phagocytic (effector) cells, and complement. However, the establishment and rigorous attention to extensive validation parameters, as described in this study, results in consistent and acceptable OPA performance. Despite the diligent control of these labile components, the assay remains semiquantitative, with discontinuous titer assignments made as result of the serial dilution of the test sera. In addition, the lack of an established reference standard for accurate assignment of OPA values between laboratories remains an obstacle to further standardization.

The degree of PnPs-specific opsonophagocytic activity or nonspecific killing associated with OPA can be mediated by controllable conditions, such as the age (i.e., passage number) of the HL60 cells and the selection of and the growth conditions for the target bacterial strain. Regarding the former, the use of DMF-differentiated HL60 cells, which possess phagocytic functions similar to those of PMNs, rather than the use of actual PMN pools, will help with assay standardization. Our results suggest that (i) differentiated HL60 cells and PMNs react similarly in the OPA at a 400:1 (effector cell/target bacterium) ratio (30) and (ii) aged HL60 effector cells do not perform effectively, as indicated by the lack of complete opsonophagocytic activity in the presence of abundant opsonizing antibodies. The selection and growth state of the target bacteria are also important: pneumococci with capsules of moderate size and in the late log phase of growth are used in our OPA. Pneumococci with small capsules may have enhanced sensitivity to complement-mediated killing, similar to rough strains (30). On the other hand, pneumococci with large capsules may be refractory to opsonophagocytosis, even though moderate levels of opsonizing antibodies are present (17). Stationary-phase cells may be at the verge of autolysis and have increased sensitivity to nonspecific mediators of killing. In some cases, non-PnPs-specific killing can occur due to the presence of components peculiar to certain specimens or groups of specimens. These components include opsonizing antibodies to other pneumococcal proteins (i.e., other than antibodies specific for PnPs) and collectins (30, 35). This non-PnPs-specific OPA killing activity is sometimes observed with sera from nonvaccinated (naturally infected) adults, as evidenced by less than 80% inhibition by homologous PnPs (even at concentrations $>125 \mu\text{g/ml}$ [data not shown]) when a few serum specimens were assessed in the serotype 1 and 23F OPAs (Fig. 2). Thus, specificity must be monitored within a given population.

A recent international interlaboratory study (29) in which HL60 cells, seven different serotypes of bacterial strains, and two control serum samples were shared showed a wide range of variability in pneumococcal OPA titer assignments for 12 pairs of pre- and postimmune adult serum specimens among five different testing laboratories. The OPA titers assigned to a given serum specimen by different testing laboratories may vary up to 256-fold difference (the titers varied from 4 to 1,024). This large disparity in titer assignments suggests that assay standardization requirements for the pneumococcal OPA extend beyond the universal reagents that were provided by a single source. Our experience shows that, in addition to bacterial strain selection and growth state, the conditions of

use of the bacteria in the OPA (e.g., the shaking rate of each serotype during incubation) is an important determinant affecting viability in the absence of opsonizing antibodies. Thus, the optimization and standardization of all parameters surrounding the preparation and use of bacteria throughout the assay should be addressed. For example, if the bacteria in the OPA are sloughing polysaccharides into the reaction well, these polysaccharides may compete for the binding of opsonophagocytic antibodies (which act as homologous competitors) and may adversely affect the resulting OPA titer. Similarly, the presence of nonviable pneumococci would have the same effect. Moreover, quality control criteria for the monitoring of viability and the differentiation status of HL60 cells prior to a performance of OPA, as we have described, should be in place for method standardization purposes to enhance consistency.

In addition to standardizing the preparation and use of bacteria and effector cells, qualification of complement lots to ensure equivalent reactivities and the use of daily system suitability tests and control sera are needed for a complete package to ensure excellent OPA performance in each run. The system suitability tests monitor for problems within an assay run, while control sera (with low, medium, and high levels of opsonizing antibodies) can be used to monitor the performance within a run and to identify more subtle interassay deviations and trends.

The two common assays used to measure immune responses to pneumococci as a result of either natural infection or vaccination are EIA and OPA. Consensus guidance for the standardized pneumococcal EIA was recently published (38). One advantage of EIA is that it does not use live biological entities, thus eliminating a major source of variability that is present in OPA. On the other hand, EIA measures the total levels of antibodies to PnPs, with no discrimination between functional and nonfunctional antibodies. In some cases, there is no direct correlation between the EIA titer and the OPA titer, especially for sera from nonvaccinated individuals (1, 30, 31, 36, 37). In contrast, OPA measures only functional antibodies, which are those believed to correlate with protection against invasive pneumococcal disease (1, 3, 14, 30, 36). However, these functional antibodies may be elicited by molecules other than PnPs, such as pneumococcal proteins or opsonins (30). Hence, a correlation between these two assays may be difficult to demonstrate, especially for specimens from the nonvaccinated (naturally infected) population. While the standardization and validation of OPA are more complex than those of EIA, the approach to OPA validation described here ensures the reliability and reproducibility of OPA because the multiple biological components are carefully controlled for quality and assay performance is monitored for system suitability in each assay run. In summary, standardization and application of this validation approach for OPA enable a reliable assessment of the immune status of individuals to multiple serotypes of *S. pneumoniae*. Furthermore, widespread adoption of this strategy will likely facilitate the enhanced interlaboratory reproducibility of OPA.

REFERENCES

1. Anttila, M., M. Voutilainen, V. Jaantti, J. Eskola, and H. Kayhty. 1999. Contribution of serotype-specific IgG concentration, IgG subclasses and relative antibody avidity to opsonophagocytic activity against *Streptococcus pneumoniae*. Clin. Exp. Immunol. 118:402-407.

2. Black, S. B., H. R. Shinefield, J. Hanse, L. Elvin, D. Laufer, and F. Malinoski. 2001. Postlicensure evaluation of the effectiveness of seven valent pneumococcal conjugate vaccine. *Pediatr. Infect. Dis. J.* **20**:1105–1107.
3. Bruyn, G. A. W., B. J. M. Zegers, and R. van Furth. 1992. Mechanisms of host defense against infection with *Streptococcus pneumoniae*. *Clin. Infect. Dis.* **14**:251–262.
4. Butler, J. C., R. F. Breiman, J. F. Campbell, H. B. Lipman, C. V. Broome, and R. R. Facklam. 1993. Pneumococcal polysaccharide vaccine efficacy. An evaluation of current recommendations. *JAMA* **270**:1826–1831.
5. Committee on Infectious Diseases. 2000. Policy statement: recommendations for the prevention of pneumococcal infections, including the use of pneumococcal conjugate vaccine (Pneumovax), pneumococcal polysaccharide vaccine, and antibiotic prophylaxis. *Pediatrics* **106**:362–366.
6. Concepcion, N., and C. E. Frasch. 2001. Pneumococcal type 22F polysaccharide absorption improves the specificity of a pneumococcal-polysaccharide enzyme-linked immunosorbent assay. *Clin. Diagn. Lab. Immunol.* **8**:266–272.
7. de Haas, M. 2001. IgG-Fc receptors and the clinical relevance of their polymorphisms. *Wien. Klin. Wochenschr.* **113**:825–831.
8. Fedson, D. S., and J. A. Scott. 1999. The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. *Vaccine* **17**:S11–S18.
9. Food and Drug Administration. 2001. Guidance for industry: bioanalytical method validation. [Online.] <http://www.fda.gov/cder/guidance/4252fnl.pdf>.
10. International Conference on Harmonization. 1995. Guidance for industry: validation of analytical procedures. Document Q2A. [Online.] <http://www.fda.gov/cder/guidance/ichq2a.pdf>.
11. International Conference on Harmonization. 1996. Guidance for industry: validation of analytical procedures. Document Q2B. [Online.] <http://www.fda.gov/cder/guidance/ichq2b.pdf>.
12. Jansen, W. T. M., M. Vakevainen-Anttila, H. Kayhty, M. Nahm, N. Bakker, J. Verhoef, H. Snippe, and A. F. M. Verheul. 2001. Comparison of a classical phagocytosis assay and a flow cytometry assay for assessment of the phagocytic capacity of sera from adults vaccinated with a pneumococcal conjugate vaccine. *Clin. Diagn. Lab. Immunol.* **8**:245–250.
13. Jodar, L., J. Butler, G. Carlone, R. Dagan, D. Goldblatt, H. Kayhty, K. Klugman, B. Plikaytis, G. Siber, R. Kohberger, I. Chang, and T. Cherian. 2003. Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. *Vaccine* **21**:3265–3272.
14. Johnson, S. E., L. Rubin, S. Romero-Steiner, J. K. Dykes, L. B. Pais, A. Rizvi, E. Ades, and G. M. Carlone. 1999. Correlation of opsonophagocytosis and passive protection assays using human anticapsular antibodies in an infant mouse model of bacteremia for *Streptococcus pneumoniae*. *J. Infect. Dis.* **180**:133–140.
15. Kanayasu-Toyoda, T., T. Yamaguchi, E. Uchida, and T. Hayakawa. 1999. Commitment of neutrophilic differentiation and proliferation of HL-60 cells coincides with expression of transferrin receptor. *J. Biol. Chem.* **274**:25471–25480.
16. Katyal, M., B. Sivasankar, S. Ayub, and N. Das. 2003. Genetic and structural polymorphism of complement receptor 1 in normal Indian subjects. *Immunol. Lett.* **89**:93–98.
17. Kim, J. O., S. Romero-Steiner, U. B. Sorensen, J. Blom, M. Carvalho, S. Barnard, G. Carlone, and J. N. Weiser. 1999. Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of *Streptococcus pneumoniae*. *Infect. Immun.* **67**:2327–2333.
18. Kyaw, M. H., I. G. Jones, and H. Campbell. 2001. Prevention of pneumococcal disease in children. Pneumococcal conjugate vaccines: their use globally could have a major impact on public health. *Acta Paediatr.* **90**:473–476.
19. Marston, B. J., J. F. Plouffe, Jr., T. M. File, B. A. Hackman, S.-J. Salstrom, and H. B. Lipman. 1997. Incidence of community-acquired pneumonia requiring hospitalization. Results of a population-based active surveillance study in Ohio. *Arch. Intern. Med.* **157**:1709–1718.
20. Nahm, M. H., D. E. Briles, and X. Yu. 2000. Development of a multi-specificity opsonophagocytic killing assay. *Vaccine* **18**:2768–2771.
21. Ortqvist, A. 2001. Pneumococcal vaccination: current and future issues. *Eur. Respir. J.* **17**:1282–1293.
22. Overturf, G. D., and The Committee on Infectious Diseases. 2000. Technical report: prevention of pneumococcal infections, including the use of pneumococcal conjugate and polysaccharide vaccines and antibiotic prophylaxis. *Pediatrics* **106**:367–376.
23. Peters, T. M., and K. M. Edwards. 2002. Pneumococcal vaccines: present and future. *Pediatr. Ann.* **31**:261–268.
24. Puumalainen, T., N. Ekstrom, R. Zeta-Capeding, J. Ollgren, K. Jousimies, M. Lucero, H. Nohynek, and J. Kayhty. 2003. Functional antibodies elicited by an 11-valent diphtheria-tetanus toxoid-conjugated pneumococcal vaccine. *J. Infect. Dis.* **187**:1704–1708.
25. Quataert, S. A., C. S. Kirch, L. J. Quackenbush-Wiedl, D. C. Phipps, S. Strohmeier, C. O. Cimino, J. Skuse, and D. V. Madore. 1995. Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. *Clin. Diagn. Lab. Immunol.* **2**:590–597.
26. Rennels, M. B., K. M. Edwards, H. L. Keyserling, K. S. Reisinger, D. A. Hogerman, D. V. Madore, I. Chang, P. R. Paradiso, F. J. Malinoski, and A. Kimura. 1998. Safety and immunogenicity of heptavalent pneumococcal vaccine conjugated to CRM197 in United States infants. *Pediatrics* **101**:604–610.
27. Robinson, K. A., W. Baughman, G. Rothrock, N. L. Barrett, M. Pass, C. Lexau, B. Damaske, K. Stefonek, B. Barnes, J. Patterson, E. R. Zell, A. Schuchat, C. G. Whitney, and the Active Bacterial Core Surveillance (ABCs)/Emerging Infections Program Network. 2001. Epidemiology of invasive *Streptococcus pneumoniae* infections in the United States, 1995–1998. Opportunities for prevention in the conjugate vaccine era. *JAMA* **285**:1729–1735.
28. Rodriguez, M. E., W.-L. van der Pol, L. A. M. Sanders, and J. G. J. van de Winkel. 1999. Crucial role of Fcγ RIIa (CD32) in assessment of functional anti-*Streptococcus pneumoniae* antibody activity in human sera. *J. Infect. Dis.* **179**:423–433.
29. Romero-Steiner, S., C. Frasch, N. Concepcion, D. Goldblatt, H. Kayhty, M. Vakevainen, C. Laferriere, D. Wauters, M. H. Nahm, M. F. Schinsky, B. D. Plikaytis, and G. M. Carlone. 2003. Multilaboratory evaluation of a viability assay for measurement of opsonophagocytic antibodies specific to the capsular polysaccharides of *Streptococcus pneumoniae*. *Clin. Diagn. Lab. Immunol.* **10**:1019–1024.
30. Romero-Steiner, S., D. Libutti, L. B. Pais, J. Dykes, P. Anderson, J. C. Whitin, H. L. Keyserling, and G. M. Carlone. 1997. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. *Clin. Diagn. Lab. Immunol.* **4**:415–422.
31. Soininen, A., M. Karpala, S. L. Wahlman, H. Lehtonen, and H. Kayhty. 2002. Specificities and opsonophagocytic activities of antibodies to pneumococcal capsular polysaccharides in sera of unimmunized young children. *Clin. Diagn. Lab. Immunol.* **9**:1032–1038.
32. Stansfield, S. K. 1987. Acute respiratory infections in the developing world: strategies for prevention, treatment and control. *Pediatr. Infect. Dis. J.* **6**:622–629.
33. Trayner, I. D., T. Bustorff, A. E. Etches, G. J. Mufti, Y. Foss, and F. Farzaneh. 1998. Changes in antigen expression on differentiating HL60 cells treated with dimethylsulphoxide, all-*trans* retinoic acid, a 1,25-dihydroxyvitamin D₃ or 12-*O*-tetradecanoyl phorbol-13-acetate. *Leukemia Res.* **22**:537–547.
34. Tuomanen, E. I., R. Austrian, and H. R. Masure. 1995. Pathogenesis of pneumococcal infection. *N. Engl. J. Med.* **332**:1280–1284.
35. van de Wetering, J. K., L. M. van Golde, and J. J. Batenburg. 2004. Collectins: players of the innate immune system. *Eur. J. Biochem.* **271**:1229–1249.
36. Vidarsson, G., I. Jonsdottir, S. Jonsson, and H. Valdimarsson. 1994. Opsonization and antibodies to capsular and cell wall polysaccharides of *Streptococcus pneumoniae*. *J. Infect. Dis.* **170**:592–599.
37. Vidarsson, G., S. T. Sigurdardottir, T. Gudnason, S. Kjartansson, K. G. Kristinsson, G. Ingolfsson, S. Jonsson, H. Valdimarsson, G. Schiffman, R. Schneerson, and I. Jonsdottir. 1998. Isotypes and opsonophagocytosis of pneumococcus type 6B antibodies elicited in infants and adults by an experimental pneumococcus type 6B-tetanus toxoid vaccine. *Infect. Immun.* **66**:2866–2870.
38. Wernette, C. M., C. E. Frasch, D. V. Madore, G. Carlone, D. Goldblatt, B. Plikaytis, W. Benjamin, S. A. Quataert, S. Hildreth, D. J. Sikkema, H. Kayhty, I. Jonsdottir, and M. H. Nahm. 2003. Enzyme-linked immunosorbent assay for quantitation of human antibodies to pneumococcal polysaccharides. *Clin. Diagn. Lab. Immunol.* **10**:514–519.
39. World Health Organization. 1999. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. [Online.] <http://www.who.int/emc-documents/meningitis/whocdscsredc997c.html>.
40. World Health Organization. 2004. State of the art of new vaccines: research and development. [Online.] http://www.who.int/vaccine_research/documents/new_vaccines/en/print.html.
41. World Health Organization Young Infants Study Group. 1999. Bacterial etiology of serious infections in young infants in developing countries: results of a multicenter study. *Pediatr. Infect. Dis. J.* **18**:S17–S22.