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PDA PAPER

Currently Available Recombinant Alternatives to Horseshoe Crab Blood Lysates: Are They Comparable for the Detection of Environmental Bacterial Endotoxins? A Review

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ABSTRACT: Endotoxin testing by recombinant factor C (rFC) is increasing with the addition of new suppliers of reagents. By use of a recombinantly produced factor C, based on the sequence of a coagulation enzyme present in horseshoe crab amebocyte lysates, the rFC tests are designed as substitutes for the traditional *Limulus* amebocyte lysate (LAL)/*Tachypleus* amebocyte lysate tests based on horseshoe crab blood. Comparative testing of samples with both the LAL and recombinant reagents has shown a high degree of correlation, suggesting that use of rFC is comparable to the more traditional LAL tests and may be technologically superior. Recombinant factor C does not recognize the factor G pathway, the alternate coagulation pathway that the lysate reagents detect. This feature allows rFC to detect endotoxin more selectively. As a recombinantly produced material, it avoids the use of the horseshoe crabs required for lysate production, thereby protecting this species, which is at risk in some parts of the world. Recombinant factor C is expected to further benefit from a more sustainable supply chain based upon a robust biotechnological production process. We summarize here the results of many studies that evaluated the use of recombinant technology for the detection of environmental endotoxin. Additionally, we include a review of the current compendia and regulatory status of the recombinant technologies for use in the quality control of pharmaceutical manufacturing. Our analysis confirms that the recombinant technologies are comparable in protecting patient safety.

KEYWORDS: Endotoxin testing, Recombinant factor C, Amebocyte lysates, LAL, rFC, Horseshoe crabs, Environmental endotoxin, Recombinant technologies.

Introduction

The demand for endotoxin testing has grown along with the increasing demand for biological drugs. Because biologicals are parenteral medicines injected directly into patients, the absence of pyrogens is an essential requirement of the final drug products. Pyrogens are adventitious substances that may cause febrile responses at very low levels. They are carefully avoided by manufacturers through monitoring of the

quality of incoming raw materials and production reagents, personnel training and gowning, and facility environmental controls such as cleaning and testing. Lipopolysaccharides (LPS, bacterial endotoxins) are by far the most prevalent and potent pyrogens in biological processes and are a heterogeneous species of molecules shed from Gram-negative bacteria. Endotoxins vary in molecular size and structure and may be small enough to evade the 0.2 µm filtration steps in place to remove bioburden organisms. The rabbit pyrogen test (RPT) was developed to detect pyrogens and calibrated with preparations of endotoxin that had been characterized, stabilized, quantified, and made available globally to industry. The RPT fell out of routine use once the bacterial endotoxin test (BET), based on *Limulus* amebocyte lysate (LAL), was developed and proven to be a suitable replacement that was practically superior and much more sensitive and quantitative for

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confirmation of the amounts of endotoxin below the defined thresholds in production batches (as well as having the advantage of sparing animal use). Under current U.S. law, for new medicines submitted under the Biologics License Applications process, the FDA requires justification of alternatives to a pyrogen test. Typically, RPT testing on three lots of drug product is sufficient to confirm the BET is suitable to detect endotoxin in a given product, thereby minimizing animal use and the greater cost of the RPT as a routine lot release test. BET technology, based on the use of LAL from *Limulus polyphemus* or, less commonly, from *Tachypleus amebocyte* lysate (TAL) from *Tachypleus tridentatus* made using blood obtained from horseshoe crabs, has revolutionized pyrogen testing for biological medicine development. The efficiency and relative low cost of the BET has resulted in a very high volume of testing, the results of which have informed manufacturing control strategies for decades and driven measurable endotoxin levels to nearly zero for most traditional pharmaceutical manufacturing. Several vendors provide LAL endotoxin reagents, equipment, and detection services to pharmaceutical manufacturers and other customers. Existing, harmonized compendial procedures can be verified with minimal effort, in contrast to the full method validation required for noncompendial, internally developed test methods. Currently the compendia do not specify particular LAL bacterial endotoxin test methods for given pharmaceutical products in detail, as long as official reference materials are used as the basis for quantitative results. Thus, a variety of reagents and laboratory practices are allowed, so long as certain requirements specified in the compendia are met. Nearly all manufacturers follow the compendial methods because regulatory acceptance is straightforward, training of analysts is a lesser burden, and they can rely on the expertise of the LAL suppliers for technical support. Global pharmacopoeias have been considering flexibility to the use of LAL alternatives after reviewing requests to add recombinant factor C (rFC) to the scope of their compendia.

The inclusion in July 2020 of General Chapter 2.6.32: "Test for bacterial endotoxins using recombinant factor C" to the European Pharmacopoeia and ongoing activities at the U.S. and Japanese Pharmacopoeias are reflective of these recent compendial steps to add additional method and reagent choices.

Since 1997, the patent on rFC has resulted in its exclusivity to a single vendor. This delayed its acceptance in

industry, as pharmaceutical manufacturers consider single-sourced reagents risky for their supply chains, and pharmacopoeias will typically not publish single-source technologies in general test chapters. The availability of the reagent was limited to a single supplier until 2013, after which two additional recombinant reagents became available. The U.S. patent expired in 2017, and new vendors are now entering the market. At least one vendor has developed recombinant proteins for the complete cascade of the coagulation factors in TAL, which also do not require the horseshoe crab blood as a source.

Our review evaluated peer-reviewed literature comparing the results obtained with recombinant reagents to those obtained with LAL/TAL. In particular, the authors sought to establish if results using rFC were equivalent to or better than those with LAL/TAL. This was determined after a critical review of available data, with special consideration for studies in which naturally contaminated samples were tested head to head (the same samples, the only difference being the test method).

The primary objective was therefore to evaluate the use of recombinant technologies that may expand and potentially improve the quality of endotoxin testing. These recombinant technologies may also bring additional ethical and ecological benefits. Other objectives included a review of the current compendia and regulatory status of the recombinant technologies for use in the quality control of pharmaceutical manufacturing. However, our main concern was to evaluate whether currently available recombinant technologies are comparable to LAL in assuring patient safety.

Suppliers and Techniques

Because the rFC reaction consists of the activation of a single enzyme compared to the three-enzyme reaction of natural LAL, it is necessary to use a more sensitive detection method compared to the absorbance techniques described in the current pharmacopoeial chapters, which are enabled by the amplification of the signal produced by the enzymatic cascade. Although the fluorescence detection methods are more sensitive than absorbance detection methods, this comes at the cost of greater background noise and higher coefficients of variation. These effects can be mitigated by sensitivity tuning, careful attention to technique, pipetting, and choice of lab consumables. Besides the detection system and reagent, the assay is otherwise prepared and

TABLE I
Amebocyte Lysate-based Kits

LAL Kit Vendor	Kit Name	Assay Format	Amebocyte Lysate Source
Lonza	Kinetic QCL	Chromogenic, onset time	<i>Limulus polyphemus</i>
	QCL-100	Chromogenic, end point	
	PYROGENT	Gel clot, end point	
	PYROGENT Plus	Gel clot, end point	
	PYROGENT-5000	Turbidimetric, kinetic	
FujiFilm-Wako ^a	PYROSTAR ES-F	Turbidimetric, onset time <i>or</i> Gel clot, end point	<i>Limulus polyphemus</i>
	Limulus Color KY Test	Chromogenic, onset time	<i>Limulus polyphemus</i>
Charles River Laboratories	Endochrome-K	Chromogenic, kinetic	
	KTA and KTA ^{2b}	Turbidimetric, kinetic	
	Gel-Clot Endotoxin Test	Gel clot, end point	
	Endosafe Rapid LAL	Chromogenic, end point	
Seikagaku/ACC	Pyrochrome	Chromogenic, onset time	<i>Limulus polyphemus</i>
	Pyrosate	Gel clot, end point	
	Chromo-LAL	Chromogenic, kinetic	
	Pyrotell	Gel clot, end point	
	Pyrotell-T	Turbidimetric, end point	
	Endospecy ES-50M	Chromogenic, rate	<i>Tachypleus tridentatus</i>

^aLAL is co-lyophilized with a beta-glucan blocker.

^bKTA² is a rapid test.

executed in the same way as the kinetic techniques using horseshoe crab amebocyte lysate. A summary of the current endotoxin detection kits available is presented in Tables I and II.

There are currently two suppliers for rFC-based detection systems.

Hyglos-bioMérieux offers two rFC detection systems: ENDOZYME II Recombinant Factor C Test and ENDOZYME II GO, both of which rely on rFC binding to endotoxins in liquid phase and a fluorogenic substrate. In addition, the company also provides an enzyme-linked immunosorbent assay-based assay format called EndoLISA. The EndoLISA assay uses a bacteriophage-derived protein to bind the endotoxins to a solid phase. Immobilization of the endotoxins allows the washing steps to remove inhibiting sample components. Endotoxin detection occurs via rFC and a fluorogenic substrate.

The EndoLISA technology is not part of this review, as there is only limited literature available and concern has been raised regarding the specificity of the bacteriophage protein capture step, which may lead to false-negative test results.

Hyglos-bioMérieux's rFC sequence is derived from *Tachypleus tridentatus*. A parasitic protozoan host (*Leishmania tarentolae*) is used to express the factor C protein.

Lonza provides the PyroGene rFC Assay, which relies on rFC binding to endotoxins in liquid phase and a fluorogenic substrate. Lonza's rFC sequence is derived from *Carcinoscorpius rotundicauda* (1). A diverse range of different hosts can express the factor C protein.

Seikagaku provides the PyroSmart assay, which relies on binding to endotoxins in liquid phase and a chromogenic substrate with Factor C, Factor B, and proclotting enzyme sequences derived from *T. tridentatus* (2).

TABLE II
Recombinant Protein-Based Kits

rFC Kit Vendor	Kit Name	Assay Format	rFC Sequence Source	rFC Expression System
Hyglos-bioMérieux	ENDOZYME II rFC Test and ENDOZYME II GO	Liquid phase fluorogenic, end point	<i>Tachypleus</i> <i>tridentatus</i>	<i>Leishmania</i> <i>tarentolae</i> expression system
	EndoLISA	Solid phase fluorogenic ^a , end point		
Lonza	PyroGene rFC Assay	Liquid phase fluorogenic, end point	<i>Carcinoscorpius</i> <i>rotundicauda</i>	Not specified
Seikagaku	Pyrosmart ^b	Chromogenic, onset time/rate	<i>Tachypleus</i> <i>tridentatus</i>	Not specified

^aBacteriophage-derived protein binds endotoxins to the solid-phase.

^bIncludes recombinant factor C, factor B, and proclotting enzyme from *T. tridentatus*.

Literature Review

The recombinant reagents (generally referred to as rFC) are principally, and by design, comparable in the sense that they contain cloned coagulation factor C, which is present in LAL/TAL. This factor is responsible for the mechanism of action that initiates the coagulation cascade in the presence of bacterial endotoxins. Based on this principle, and the fact that rFC is made in a controllable bioprocess and therefore more purified than in LAL reagents, rFC would be expected to have some advantages, including better lot-to-lot comparability, greater specificity toward bacterial endotoxins, and a more secure supply chain. The lack of the alternative factor G pathway in rFC reagents avoids the problem of the specificity of LAL, which also detects (1→3)-β-D-glucans (hereafter referred to as beta glucans), which are also detected unless beta glucan-blocking reagents are used, as discussed in the following sections.

Several published peer-reviewed studies support the claim that rFC is comparable or superior to LAL, including the lack of sensitivity to beta glucans. Comparing the results with rFC and LAL requires careful study design to reach accurate conclusions. Studies of this nature are inherently difficult to conduct, because the variation in results within any type of endotoxin testing is relatively high because of the very low (sub-picogram) level tested and the tremendous sensitivity of the assay. As a consequence, large dilutions of

standards and samples are necessary to obtain results in range, and minor differences in procedure (temperature, mixing time, and operators) result in significant variation even when the same LAL method is used on the same samples at the same time in the same laboratory. In part, this is why the routine recovery allowed in the BET is fourfold (50%–200%). For our review, we evaluated comparative studies (LAL vs. rFC) in peer-reviewed journals and put more weight on those that controlled for these well-known variables.

The first peer-reviewed comparison (Alwis and Milton et al.) (3) tested house dust samples resuspended and diluted in triethylamine using BioWhittaker's LAL (kinetic chromogenic) and rFC (kinetic fluorogenic) reagents. Sixty samples showed a high correlation between the rFC and LAL assays ($r = 0.86$, Spearman correlation), although most samples gave higher concentrations of endotoxin with LAL. Samples were prepared differently for glucan testing (centrifuged/autoclaved) and tested by immunoassay; however, these data were not provided. Glucan-blocking buffers were not evaluated in this study, in contrast to the studies described next.

In 2010, Thorne et al. (4) compared rFC to LAL using a very large sample size (>900 airborne samples from both livestock and simulated operations) that were tested in duplicate of which nearly all 1800 analyses were quantifiable. These samples represented a wide spectrum of endotoxins collected over time from

different facilities and a broad range of levels in the environment. The endotoxin testing was carefully controlled, using multiple sample dilutions for each sample, and the same control standard endotoxin standard in both rFC and LAL tests. Lonza's kinetic chromogenic and rFC reagents and protocols were applied. Overall, the rFC test reported higher (13%) endotoxin concentrations than the LAL test. However, for the laboratory samples, LAL was slightly higher (5%). These are remarkably similar, considering 50%–200% of spiked endotoxin is acceptable for endotoxin control testing in which the exact concentration is known. Correlation analysis of the data set indicated the rFC and LAL results were close to linear (slope of 0.91), and that the head-to-head rFC to LAL comparability results were statistically significant ($P < 0.0001$) over the entire paired dataset ($n = 912$) across a very wide range (4–5 logs) of endotoxin concentrations. Remarkably, only 5 values (of 912) deviated from this linear relationship, and these outliers were approximately randomly distributed (three had higher values by LAL and two had higher values by rFC). These data were further divided for statistical evaluations comparing rFC and LAL between sampling devices, the facilities, and types of animals. Differences were evenly distributed (rFC higher in two cases and LAL higher in two cases). This study was by far the most comprehensive of all found in the peer-reviewed literature, in that it had the most head-to-head sample testing and used several lots of reagents (seven lots of rFC and six lots of LAL). Taken together, these data demonstrated that endotoxin from diverse sources can be tested by either LAL or rFC with nearly indistinguishable results. The authors of this study concluded that there was no difference between these testing methodologies.

McKenzie et al. (2011) (5) evaluated 20 household airborne samples (containing approximately 10^2 EU/mL) from a continuing birth cohort study of childhood asthma using three different rFC lots in four different diluents. The authors compared the rFC results to data generated from the same samples using LAL and orthogonally by gas chromatography-mass spectrometry (GC-MS) for quantitation of 3-hydroxy fatty acids. Although they do not provide the raw data, the authors report no bias and a high correlation of the rFC results with the LAL (Spearman coefficients of 0.94–0.95) and GC-MS results. Furthermore, they demonstrated improved reproducibility using rFC results compared to LAL and speculated that it was because of the more robust recombinant technology used to manufacture the rFC reagent.

Chen and Mozier (2013) (6) reported testing of biological products in a study of 13 samples using four different LAL techniques described in the global harmonized pharmacopoeias (gel clot, kinetic chromogenic, kinetic turbidimetric, and end point chromogenic) and end point fluorescence using rFC. The study found all methods to be generally comparable; variation within LAL methods was no different than that between LAL and rFC. The exception was two samples with higher LAL endotoxin concentrations, but in both cases, this was shown unambiguously to be caused by beta glucans based on (1) lower LAL endotoxin concentrations when glucan blocker was used, and (2) direct testing for beta glucans using the GlucateLL assay (Associates of Cape Cod). In summary, this study showed comparable results between all modes of LAL and rFC (Lonza) using a variety of samples from biotechnological processes, excepting the false signal from LAL attributable to beta glucans. The benefit of rFC was this higher specificity for the intended analyte.

Bolden and Smith (2017) (7) reported the validation results of 19 different pharmaceutical products using rFC and found rFC to be slightly more accurate (spike recovery) and precise than LAL. Additionally, an experiment evaluated the detection of three different Gram-negative bacteria and their associated endotoxins in three pharmaceutical solutions with both LAL (kinetic chromogenic technique) and rFC. The authors observed that endotoxin concentrations were comparable, with one organism (*Ralstonia pickettii*) showing slightly higher levels with rFC (vs. LAL). Another experiment demonstrated the insensitivity of rFC to beta glucan in two different in-process drug substance solutions.

Kikuchi et al. (2017) (8) compared three different LAL assays and vendors with three different rFC preparations and tested 18 purified LPS preparations, five laboratory-prepared endotoxins, and six different natural water sources, including a lake, two rivers, domestic wastewater, mineral water, and municipal tap water. The purified endotoxins varied greatly in potency (from ~ 1 EU/ μ g to $\sim 10,000$ EU/ μ g for *Porphyromonas gingivalis* ATCC 33,277 and *Burkholderia cepacia*, respectively). Wako's kinetic turbidimetric LAL assay (PYROSTAR ES-F) produced the highest value (EU/ μ g) in eight of 18 samples, whereas Seikagaku's kinetic chromogenic LAL assay (Endospecy ES-50M) produced the lowest endotoxin concentrations in 10 of 18 samples. This variation in LAL results across

vendors caused slightly higher variance in LAL vs. rFC (for $n=3$, LAL had a higher percent relative standard deviation in 11 samples whereas rFC had higher in seven). Four samples had discrepancies greater than fourfold (*Escherichia coli* J5, *E. coli* O150, *Pseudomonas aeruginosa* 10, and *Salmonella minnesota* 1114), and two of these demonstrated greater values by LAL (Wako Kinetic Turbidimetric) and two had greater values by rFC (Lonza PyroGene and Hyglos EndoZyme). Taken together, these results with purified endotoxin show that variation within LAL methods is greater than variation within rFC methods. Overall, the agreement between the class of reagent (LAL and rFC) is comparable.

Kikuchi et al. (2017) (9) also studied cruder, minimally purified (0.4 μm filtered) preparations of bacterial endotoxin, described as naturally occurring endotoxin (NOE), from five different types of bacterial cultures and six samples from various natural water sources (pond, lake, river, and tap). The NOEs, like the purified LPS samples described earlier, were detectable with all six test methods: three LAL techniques (two chromogenic and one turbidimetric) and three rFC techniques (two end point fluorescence and one chromogenic). There were no trends in rFC vs. LAL, and the results were comparable. However, the natural water source samples showed the LAL trended to higher endotoxin concentrations. But these authors did not test these samples for beta glucans, which are ubiquitous in nature and derived from a wide range of prokaryotic and eukaryotic organisms, including yeast, fungi, and seaweed (Kaur et al., 2019) (10). Endotoxin was detected in all water samples with all six methods. Overall, the authors concluded that rFC and LAL gave comparable results. And that none of the six methods gave false-negative results across all 29 samples tested. The higher concentrations of endotoxin detected by LAL in a few samples were likely because of the concomitant presence of beta glucans.

In 2014 and 2017, while studying endotoxin “masking” effects in biological systems, Schwarz et al. (11, 12) compared the measurement of LPS concentrations in recombinant protein preparations from *E. coli* hosts using LAL (Lonza Chromogenic) and rFC (Hyglos/bioMérieux). Although the primary purpose of these studies was not the comparison of LAL to rFC, they provided limited data. The 2014 study showed 1.1 vs. 1.4 EU/mL for rFC and LAL, respectively, and the 2017 study showed 1.2 and 0.5 EU/mL for rFC and

LAL, respectively. Overall, there was no significant difference or bias between the test methods. Most recently, rFC was compared with several LAL methods in a Low Endotoxin Recovery (LER) hold-time study (13). This study showed testing with Lonza’s PyroGene rFC kit detected endotoxin concentrations very similar to those of four different LAL methods (Kinetic Turbidimetric kits from Lonza and Charles River; Chromogenic kits from Lonza and Charles River Laboratories).

Other studies have demonstrated comparability of rFC and LAL when using purified LPS, including primary and secondary international standards, and lab-derived endotoxin preparations when challenged in relevant pharmaceutical products, or in specificity comparison experiments. These include Loverock et al. (2010) (14), Abate et al. (2017) (15), Bolden et al. (2017) (16), Kikuchi et al. (2018) (9), and Piehler et al. (2020) (17). Even though the purpose of this review was to evaluate the detection of environmental endotoxins with rFC and LAL, data from Piehler et al. are expanded upon to compare the performance of the assays with purified endotoxin.

In 2020, Piehler et al. (17) reported the results of a 5-year study in which 13 samples containing endotoxin were tested by three methods: LAL (Endochrome-K, Charles River Laboratories), ENDOZYME rFC, and EndoLISA (both from Hyglos GmbH/bioMérieux). These samples were derived from a single purified *E. coli* LPS source and prepared to contain a range of nominal LPS concentrations. In this investigation, the samples were tested over several years, and thus a diversity of lots of reagents are incorporated in the results. The samples were provided with nominal endotoxin concentrations. The method for assignment of nominal concentrations was not provided. Data obtained from 11 samples, when both LAL and rFC were used, demonstrated comparable results and there was no bias for one test method over the other; six samples had higher endotoxin concentrations with LAL and five had higher levels with rFC. The number of samples having higher measured endotoxin vs. the nominal value was similar: nine of 13 by LAL and seven of 11 for rFC. When comparing the results to nominal in percent recovery, the broadest range of results was by the LAL method (86%–180%) and the narrowest by rFC (83%–138%). In addition, the variation in percent recovery was slightly better for rFC (19% c.v.) compared to LAL (24% c.v.). This study shows that for purified LPS made from *E. coli*, rFC is

not inferior to LAL and is in some respects superior. Furthermore, in 2019, Muroi et al. (18) demonstrated no significant difference in results when testing three purified endotoxins using three recombinant assays (rFC and recombinant TAL) and three LAL assays.

Marius et al. (2020) (19) compared four different vaccines using two LAL and two rFC-based assays. Assay performance (precision, recovery of positive product control spikes) was similar across all samples for both LAL and rFC. Two of the unspiked samples produced a signal, one with LAL and the other with rFC. Heat treatment was required to reduce the LAL signal in the protease vaccine sample (sample A), whereas the rFC signal was not reduced with heat or serine protease inhibitors. In this case, the rFC assay reported a measurable signal whereas the LAL assay did not. In contrast, a higher signal was shown for sample C with LAL than with rFC. The authors demonstrated that this signal could be reduced by the addition of glucan blockers and concluded that beta glucans were present in the sample. Overall, this study indicated that both LAL and rFC were suitable for the detection of bacterial endotoxin in four vaccine samples prepared in complex matrices.

Summary of the Literature Review

The reviewed studies published in the peer-reviewed literature include a substantial dataset comparing LAL and rFC to each other as a class, and within each type as a technology. The types of samples represent a broad and very diverse set. Most of these studies were conducted head to head with the same samples, and therefore the known variation inherent in all endotoxin testing was minimized. Further studies are likely to arrive at the same conclusion that rFC and LAL produce comparable results, as long as the study accounts for the specificity of LAL with respect to beta glucans. Although the false-positive pathway activation of LAL can be mitigated with glucan-blocking buffers, these buffers may not block all of the glucan interference, or all types of glucans, which may account for some data in which LAL has detected higher concentrations of endotoxin than rFC, for example in natural waters. Other differences in results between LAL vendor tests are likely owing to other causes, such as the proprietary formulation of the lysates from the manufacturers. The causes of differences in results between LAL vendors cannot be fully explained without this information.

Compendial Approaches

One of the most important benefits of the current compendial methods in the European, U.S., and Japanese Pharmacopoeias is that they are harmonized. Although they leave room for some specific reagent choices, they are also supported by harmonized reference materials that are in turn linked to an International Standard provided by the World Health Organization. This makes them reliable enough for use by the international regulatory community. Achieving the same level of harmonization for alternative approaches may take some time to accomplish. Although the Pharmacopoeial Discussion Group (PDG) Pharmacopoeias (Japan, Europe, U.S.) all have introduced recent revisions that open the door to the use of alternative technologies and methods, they have done so independently. As stated in the comparative literature discussion earlier, there are significant challenges to endotoxin testing in terms of dilution, sensitivity, operator technique, reagents, and even data analysis, necessitating large collaborative studies to establish reliable reference materials. With that in mind, exchanges and cooperation between pharmacopoeias active in this field to explore harmonization including the new approaches would most certainly be beneficial as new methods are introduced.

European Pharmacopoeia

The European Pharmacopoeia (Ph. Eur.) included rFC assays directly as alternative assays in the 2016 Ph. Eur. 5.1.10. BET Guideline. (20) A stand-alone, general chapter for rFC, outside the context of the harmonized endotoxin texts of Ph. Eur. General Chapter 2.6.32. "Test for bacterial endotoxins using recombinant factor C", was published for public comments in December 2018. The European Pharmacopoeia Commission adopted the chapter in November 2019 for publication in Ph. Eur. Supplement 10.3 in July 2020. It is in force from January 1, 2021. From that date, when a rFC assay is used in place of a BET method prescribed in a monograph, it can be used after a product-specific validation in line with use of alternative methods as per the Ph. Eur. general notices; however, the method itself would not need to be validated again. The requirements for introducing rFC assays are clarified in a revised version of General Chapter 5.1.10 "Guidelines for using the test for bacterial endotoxins" published in Ph. Eur. Supplement 10.3 (July 2020).

Full cascade recombinant BET assays still have the status of alternative methods, because their use is not described in Ph. Eur. 2.6.32. The use of this technology requires full method validation.

United States Pharmacopeia

In 2018, the United States Pharmacopeia (USP) published a draft of General Chapter <1085> Guidelines on the Endotoxin Test (21), which referenced rFC (as 5.1.10. of Ph. Eur.) as an “alternative” method. In July 2019, the USP announced a stand-alone rFC chapter, but in September 2019 followed up with a draft that departed from the harmonized text of General Chapter <85> Bacterial Endotoxins Test (22) for public comment in *Pharmacopeial Forum* 45 (5). This proposal was withdrawn in April 2020 based on public comments received and additional stakeholder input collected in addition to the *Pharmacopeial Forum* consultation. A stand-alone chapter has been proposed for publication for public comment in the *Pharmacopeial Forum* with a target for early 2021.

Japanese Pharmacopoeia

In September 2019, the Japanese Pharmacopoeia (JP) published a draft of “Bacterial Endotoxins Test and alternative methods using recombinant protein-reagents for endotoxin assay” (23), which is expected to be a guidance chapter in the next revision of the JP.

Chinese Pharmacopoeia

The Chinese Pharmacopoeia (ChP) proposed a draft guidance chapter in early 2019 for publication in the 2020 revision of the ChP. This guidance chapter is expected to be similar to the other compendia guidance chapters, recognizing the recombinant reagents as suitable alternatives when appropriately validated.

Regulatory Review

In 2012, the U.S. FDA published the *Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers* (24), which recognizes rFC as an alternate endotoxin assay. The 2012 Guidance states that an alternate method such as rFC may be used for testing if the method provides advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability. Further, the 2012 Guidance requires method validation to be performed for rFC in accordance with USP <85>

and USP <1225> (25), in which the alternate method should achieve equivalent or better results.

In September 2018, the FDA licensed Eli Lilly to market Emgality, which is the first medicine to be released using recombinant reagents for the detection of bacterial endotoxins. Lilly received approval from Europe in November 2018 and subsequently received approval in Australia, Brazil, Canada, Israel, Kuwait, Lebanon, South Korea, Switzerland, Taiwan, and the United Arab Emirates.

Without a General Chapter on rFC or the binding request for rFC in a monograph (i.e., compendial), the rFC-based assays were seen as “alternative,” thus requiring a full validation of the method and were thus seen as a barrier to implementation by industry owing to the cost of regulatory change for marketed medicines. Indeed, this was the case for Eli Lilly and their product Emgality. In Europe, the implementation of Ph. Eur. 2.6.32. in Supplement 10.3 (July 2020) will change the situation, as the rFC-based assay according to the Ph. Eur. will not have to be validated in general. The product-specific verification will still have to be performed.

Conclusion

The benefits of switching to a recombinant reagent include greater specificity for endotoxin; a more reliable supply chain in the event of a market shortage because of horseshoe crab population issues; ethical benefits of using a nonanimal sourced test reagent; and potentially lower cost. However, any change must be data driven.

Inter-assay variability is evident when using recombinant reagents, just as within-assay variability is observed using LAL, even within the same suppliers. In some cases, rFC overreported concentrations of endotoxin compared to LAL; in other cases, it underreported. However, in the majority of instances, the data from the two assay systems were strongly correlated. Caution should be used when classifying differences of small magnitudes in the context of what is practically significant with respect to patient safety. Most importantly, there has been no evidence of a false-negative result using recombinant reagents. Based on this review by experts and practitioners in the field of pharmaceutical bacterial endotoxins detection, we have concluded that the currently available recombinant rFC reagents are comparable to those of the LAL assay.

LAL reagents across suppliers are not identical, but compendial LAL tests allow interchangeability based on production controls and calibration to a harmonized international standard. The comparability of rFC results to LAL results described herein are, in part, because rFC assays are also calibrated to the international standard.

In our opinion, there are sufficient data available in the existing peer-reviewed literature to adequately address questions about the comparability of currently available recombinant reagents to LAL in their ability to detect bacterial endotoxins and ensure patient safety.

This review includes papers published before April 2020.

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The PDA Endotoxin Leadership Forum was formed in 2019 to act as a strategic think tank/advisory group to the Parenteral Drug Association, whose members comprise industry, academic, compendial, and regulatory experts. Their goal is to establish an ongoing dialog regarding endotoxin-related issues and topics as they relate to the manufacturing and testing of pharmaceutical and biopharmaceutical products. Thank you to the following additional PDA Endotoxin Leadership Forum members (nonauthoring) for their invaluable critique, insights, and perspectives in crafting this effort.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

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