Functional Challenges for Alternative Bacterial Endotoxins Tests Part 2: Comparability

James Akers,[1] Dennis E. Guilfoyle,[2] David Hussong,[3] Karen McCullough,[4] Robert Mello,[5] Donald Singer,[6] Edward Tidswell,[7] and Radhakrishna Tirumalai[8]

[1]*Akers Kennedy Associates, Inc., Leawood, KS*
[2]*Johnson and Johnson, New Brunswick, NJ*
[3]*Eagle Analytical, Houston, TX*
[4]*MMI Associates, Whitehouse Station, NJ*
[5]*Mello PharmAssociates, LLC, Reisterstown, MD*
[6]*Ecolab Life Sciences, St. Paul, MN*
[7]*Merck & Co, West Point, PA*
[8]*North Potomac, MD*

Note: The views expressed in this article are solely those of the individual authors and do not necessarily reflect the opinions of organizations with which the authors may be affiliated.

Introduction

Part one of this three-part series examined the scientific basis for recombinant methods and the history and extensive studies utilized previously for the acceptance of alternate tests in the field of pyrogen and bacterial endotoxins testing. As reported in part one, we believe there are three necessary elements to a complete validation of a recombinant method's ability to assure continued product quality and patient safety (Akers, *et al*., 2020):

1. Comparability of analytical capability per USP <1225>,
2. Product specific suitability testing per USP <85> and
3. The demonstration of equivalent or better test results than the compendial method per USP <1223>

Suitability and Comparability

Some have suggested that the recombinant reagents are merely variants of the naturally sourced lysate and therefore, by extension, can be easily substituted for the natural reagent(s) with minimal evaluation beyond a suitability test as described in USP <85>. The components and formulation of naturally sourced and recombinant reagents are clearly NOT the same. The natural lysate contains many molecular entities necessary to the innate immunity of the living animal, and which are missing in recombinant reagents (Obayashi, *et al*; 1985; Ding and Navas, 1995; Ding and Ho, 2001; Iwanaga, 2002; Mizumura, *et al*, 2017; Muori *et al*, 2019; Akers, *et al*, 2020.) For these reasons, methods using reagents from recombinant sources are alternatives that are minimally related to the naturally derived reagent .

There are publications in the scientific literature that address validation of alternative recombinant methods in terms of product suitability/PPC recovery (Loverock, *et al*., 2010; Bolden and Kelly, 2017; Abate, *et al*., 2017; Bolden 2020; Marius, *et al*., 2020). The "Test for Interfering Factors" (formerly known as "Inhibition/Enhancement Testing") described in

USP <85> (USP 2020a) is a product-specific demonstration that test interference arising from a product formulation can be mitigated such that a known level of calibration analyte activity can be quantitatively recovered. This same test is also used as a system suitability test for routine bacterial endotoxin testing.

Although published studies have demonstrated suitability for recombinant methods, we believe that these data do not demonstrate **test result equivalence (comparability)** between reference compendial methods and recombinant methods as required by USP and FDA (USP 2020b; USP 2020c and FDA 2012). Most of the published studies claiming comparability include data from test articles that have no measurable autochthonous endotoxin activity in any segment of the manufacturing process. *It is not possible to claim comparability when the impurity that is being measured, in this case, endotoxins activity, is absent in the test article at quantifiable levels.* The recovery of the analyte (RSE or CSE) does not experimentally confirm the alternative method's ability to recover natural product contaminants.

Glossary

Terms and acronyms used in this publication are provided below.

Term or Acronym	Definition
Endotoxins from autochthonous sources	Endotoxins generated by microorganisms adapted to and indigenous within a specific niche or environment. In our current context, that environment is the product, manufacturing system and associated utilities which includes those endotoxins from microbial contamination of ingredients such as water.
Calibration Standards (Analytes)	Calibration standards also known as analytes include the USP Refer-ence Endotoxin Standard (RSE) and secondary Control Standard Endotoxins (CSEs). All the calibration standards purchased from USP or included in test kits are currently prepared from hot phenol (West-phal) extracted, purified and formulated lipopolysaccharide. RSE is prepared from *Escherichia coli* O113:H10:K(-) and CSEs may be prepared from any of several different species/strains of *E. coli.* Sec-ondary calibration analytes must be calibrated against the primary standard (RSE).
Recombinant Reagents	Two types of recombinant reagents are currently either commercially available or are in development. Recombinant Factor C (rFC) is a recom-binant reagent containing only the Factor C zymogen protease cloned from the horseshoe crab's natural clotting cascade. Recombinant Cas-cade Reagents (rCR) are recombinant reagents containing all three zymogen proteases cloned from the natural clotting cascade. (Akers, *et al*., 2020)
Relative recovery	Endotoxins activity in a sample quantitated by recombinant methods as a percentage of endotoxins activity quantitated in the same sample by standard compendial methods.
Reference Compendial Method(s)	The compendial methods found in the USP 2019, <85> "Bacterial Endotoxins Tests"

Approach Used for Data Reassessment

There is a lack of data in the public domain that relate assayable levels of endotoxins activity in a test article using both the standard compendial method(s) and recombinant methods. We have reviewed relevant articles and have re-assessed the reported data, where possible, to understand genuine "head to head" comparability between recombinant and reference compendial methods. We are most interested in the alternative method's ability to detect and quantify Gram negative bacterial endotoxins from the organisms likely to be found in a healthcare product manufacturing setting.

In the cited publications (below), we reassessed each sample by calculating the endotoxins activity test result for each recombinant method as a percentage of the corresponding value for the recovery of endotoxins activity determined using the referenced compendial method. We call this "relative recovery."

For example, if Alternative Method 1 detects 3 EU/mL for Sample 1 and the Reference Compendial Method used for the same sample detects 5 EU/mL, then the calculated *relative recovery* for Method 1 as applied to Sample 1 is

> (3 EU/mL ÷ 5 EU/mL) x 100 or 60%, meaning that the Alternative Method 1 recovered 60% of activity relative to the referenced USP method.

Unless otherwise noted, the reassessment parameters we applied include:

- Where multiple reference compendial methods were provided in a study, we compared the recovery of each recombinant method to the average of the reference compendial methods.
- Test results below any method's LOQ or test results accompanied by invalid Positive Product Control (PPC) results were not included in reassessments.
- After the calculation of the relative recovery for each sample, the results were divided into a series of recovery ranges and graphed. The graphs represent the number of samples in each of the defined ranges. This presentation of the data enables visualization of possible method-specific variability within the data set.
- The data are referenced to a 50-200% recovery relative to the referenced compendial method. This recovery range is illustrated in each of the figures by a gray box around the data that fall within this range. Each figure is accompanied by a table (Tables 2-5) that provides a matrix of the results sorted by method within the referenced study. The compendial BET assumes a maximum potential variability of 50-200%. We have used that range here for convenience although data within that range may not be indicative of comparability depending on Gaussian distribution of individual test results.
- Recombinant methods for each publication are labeled "Method 1, 2, 3." Although the same method may have been used in multiple studies, they are not uniformly labeled from study to study.

Reassessment of published data can be challenging as there are numerous sources of analytical variation that may not have been addressed or reported in each publication but could contribute empirically to overall variability within and between studies.

- Recombinant reagents have no glucan pathway. Therefore, valid comparability studies should require the use of glucan blockers to <85> referenced methods to reduce or eliminate the effects of the Factor G pathway on the reference test results. If not blocked, the presence of glucans could cause the measured endotoxins activity using the naturally sourced lysate to be overestimated inncomparison to the recombinant reagents thereby reducing relative recovery values.
- We cannot ascertain if the comparability tests were conducted simultaneously on the same prepared sample. The use of different prepared samples or samples held under varying conditions could impact test results.
- The use of RSE for all calibration including standard curve preparation and PPC will eliminate the lot and method-specific requirement for potency determination of CSEs, which could add to the variability of the tests.

Results

Study 1: Thorne et al. *2010*

An article published by Thorne and co-workers compared the relative recovery of endotoxins activity in air samples from livestock facilities using both the reference compendial kinetic chromogenic method and an rFC product. The Thorne study was extensive, looking at approximately 400 field samples and 500 field-derived laboratory samples.

While the Thorne data demonstrate a fairly consistent level of comparability between detection of endotoxins activity by the compendial and recombinant methods, the types of Gram-negative microorganisms and endotoxins typically found in livestock facility dust are irrelevant to the recovery of endotoxins autochthonous to parenteral manufacturing facilities, equipment and utilities (Zucker *et al*., 2000; Zhao *et al*., 2014, Reid, 2019). In addition, the methods used for airborne sample collection and preparation are inconsistent with methods used in a pharmaceutical laboratory.

Study 2: Chen and Mozier, 2013

Chen and Mozier provide one of the few published comparability studies conducted on routine parenteral product intermediates with assayable levels of endotoxins from autochthonous manufacturing sources. Their study design is complex and looks not only at a comparison of the recombinant method to four different compendial reference methods on thirteen different sample formulations and one CSE control, but also measures levels of glucan activity and examines the effect of freeze/thaw cycles on test data. Glucan blockers were not used except as noted. In addition, the study was conducted by different analysts and different laboratories. Of the thirteen product samples the original source authors eliminated three from their analysis (23%) because of loss of activity during freeze/thaw. Table 1, ordered by increasing glucan activity, is a summary of all samples tested in the study.

The data presented in Table 1 is a compilation of data from the measurement of glucan activity and relative recovery using the

Table 1. Comparison of rFC and Compendial Standard Methods, Chen and Mozier 2013

Sample	Glucan pg/mL	Relative Recovery (no glucan blocker)
1	negative	106%
2	negative	66%
11	negative	77%
13	negative	76%
141	Not tested	92%
7	20	131%
10	47	28%
3	112	90%
6	7600	41%
8	>20000	19%
1Sample 14 is the 1 EU/mL control		

recombinant Factor C method. All samples with no glucan (samples 1, 2, 11, 13) fell within the 50-200% recovery range, but generally below 100% recovery. Data for samples 7, 10, 3, 6, and 8 suggest, as expected, that lower glucan levels have less of an effect on relative recovery than high glucan levels. The outlier in this case is sample 10, identified as a protein in a lipid formulation. Although these data were generated without the use of a glucan blocker, Chen and Mozier demonstrated that adding a glucan blocker to the standard methods for the analyses of Samples 6 and 8 did reduce their reactivity to the glucans (data not shown). The blocking data suggest that the effectiveness of the blocker depends not only on the sample but also on which reference compendial method is used.

Study 3: Reich et al. *2014*

Reich and co-workers presented a study that compared the recovery of endotoxins activity in a few "natural waters" including rivers, swimming ponds, quarries, spring water, tap water, rain barrel water, mineral water and deionized water. It was unclear from the description of the experimental design if the standard method was supplemented with a glucan neutralizing buffer. These data are summarized in Figure 1 and Table 2 below.

Figure 1 indicates that 29% of all samples tested fell below 50% - 200% recovery range. Recombinant analysis of a deionized water sample recovered only 7% of the standard lysate activity using methods 2 and 3 (data not shown) and was not compared to

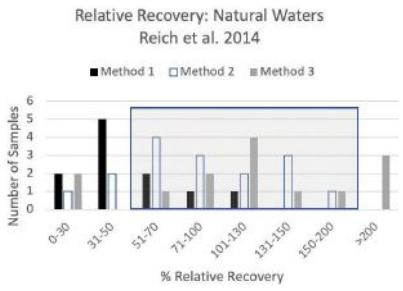

Figure 1. Relative Recovery, Natural Waters, Reich et al.

Table 2. Comparison of Three Recombinant Methods, Reich *et al.*

Recovery Range	Method 1	Method 2	Method 3
0-50%	64%	19%	14%
51-200	**36%**	**81%**	**64%**
>200	0%	0%	21%

Method 1. Figure 1 and Table 2 suggest the pattern of endotoxins detection activity recovery in this study is much lower for Method 1 than for Methods 2 and 3, and Method 3 over-estimated endotoxins activity in three cases.

Study 4: Kikuchi et al. *2017*

Kikuchi *et al*. (2017) examined recoveries of endotoxins activity from three different sources: 1) purified LPS from a variety of Gram-negative microorganisms, 2) "Naturally Occurring Endotoxin" (NOE), a suspension of outer membrane vesicles and cell wall components shed from Gram-negative microorganisms grown under laboratory conditions and 3) water drawn from various sources the authors labeled "natural waters". These natural waters were lake water, river water, household wastewater (domestic sewage), mineral water and tap water.

This study used three reference kinetic chromogenic methods: K-QCL (Lonza), ES-II (Fuji Film/Wako), an LAL reagent with a glucan blocker included in the formulation (Tsuchiya, *et al*., 1990), and Endospecy (Seikagaku), a TAL lysate where the Factor G pathway has been fractionated out of the formulation (Obayashi *et al*., 1985). Since Endospecy has no Factor G pathway, it was the most relevant comparator for re-assessment of the relative recovery calculations for the two rFC reagents and one rCR reagent. Because of the importance of the Kikuchi data to understanding both the questions of glucan involvement and detection of endotoxins from autochthonous sources, we are reporting our re-assessment only of the samples identified as "natural waters." (Note that the "household waste" data point was eliminated from our re-assessment of "natural waters" as it is irrelevant to healthcare product manufacturing.)

The re-assessed data comparing relative recovery for "natural waters" are shown in Figure 2 and Table 3.

This re-assessment indicated that one-third of all samples tested, representing all recombinant methods, fell below 50% relative

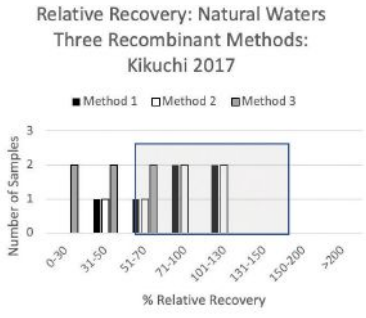

Figure 2. Relative Recovery, Natural Waters, Kikuchi *et al.*

Table 3. Comparison of Three Recombinant Methods for Natural Waters, Kikuchi *et al.*			
Recovery Range	**Method 1**	**Method 2**	**Method 3**
<50%	17%	17%	67%
51-200	**83%**	**83%**	**33%**
>200	0%	0%	0%

recovery. Because all reagents compared in this re-analysis had no glucan pathway, the data suggest the under-estimation of activity resulted from something other than glucan activity.

Summary data in Table 3 indicate that Methods 1 and 2 showed similar underestimation results. Method 3 was significantly different in that two-thirds of the samples recovered under 50% of the standard method.

Study 5: Reid, 2019

A study reported by Nicola Reid tested samples from pharmaceutical waters sampled a) post-deionization and b) post-carbon treatment, the former being a direct feed to WFI generation. In this analysis, methods evaluated included three rFC methods and one rCR method. The referenced compendial methods were supplemented with glucan blockers as instructed by the reagent manufacturers. Data from the deionized water study are presented in Figure 3 and Table 4.

Figure 3 shows that 41% samples tested using the three recombinant methods recovered less than 50% of endotoxins activity relative to a referenced kinetic chromogenic compendial method. The data in Table 4 further illustrates that, relative to the referenced method, all alternate recombinant methods had significant numbers of under-quantitated samples, with Method 4 showing 89% of all samples tested by that method being underestimated.

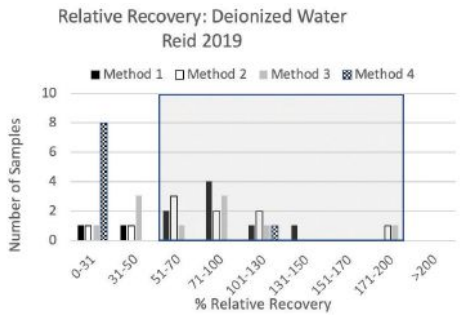

Figure 3. Relative Recovery, Pharmaceutical Deionized Waters, Reid

Table 4. Comparison of Four Recombinant Methods for Pharmaceutical Deionized Water, Reid				
Recovery Range	**Method 1**	**Method 2**	**Method 3**	**Method 4**
0-50%	20%	20%	14%	89%
51-200	**80%**	**80%**	**60%**	**11%**
>200	0%	0%	0%	0%

The Reid study also analyzed water sampled from the post-carbon treatment stage upstream of the WFI production process (Figure 4 and Table 5).

Data presented in Figure 4 and Table 5 suggest that the relative recoveries of endotoxins activity in water sampled after carbon treatment were consistently low in the four alternative methods, with a total of 82% of the all samples tested showing relative recoveries less than 50%. Method 3 did not recover activity in any sample above the 50% relative recovery mark.

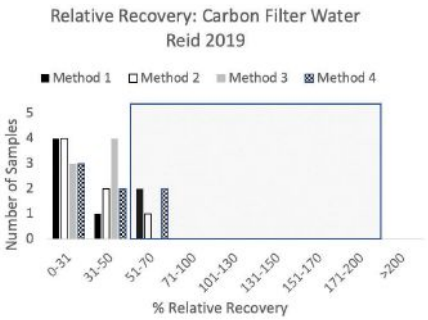

Figure 4. Relative Recovery, Pharmaceutical Carbon Treated Waters, Reid

Table 5. Comparison of Four Recombinant Methods for Pharmaceutical Carbon Treated Water, Reid				
Recovery Range	**Method 1**	**Method 2**	**Method 3**	**Method 4**
0-50%	71%	86%	100%	71%
51-200	**29%**	**14%**	**0%**	**29%**
>200	0%	0%	0%	0%

Study 6, Piehler, et al. *2020*

In 2020 Piehler and co-workers published an article on the comparison of LAL and rFC assays over the course of five years while employing commercially available proficiency test samples used for the training of laboratory analysts. While all testing results confirmed the labeled nominal value of the proficiency sample within the range of 50-200%, one sample did not meet the relative recovery requirements of this re-assessment study.

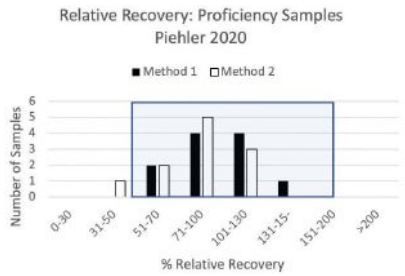

Figure 5. Relative Recovery, Pro ciency Samples, Piehler *et al.*

The authors indicated that each of these proficiency samples were of unknown composition. However, when we contacted the manufacturer of these products, we were told that they were purified, formulated and lyophilized LPS (personal communication). The source and identity of the bacterial species used to generate the purified LPS was not disclosed. Although one result fell below 50%, the overall pattern of these results clustered around 100% recovery, which would be expected by any method using only purified LPS.

Discussion

This report presents no original experimental data. It is a review in which we reassessed data available from published reports purporting to evaluate comparability among multiple reference compendial methods and available recombinant methods. In our opinion while none of these studies meets comparability requirements in the compendial sense, they do provide general trends that merit discussion.

The Thorne study is unique, but since it is undoubtedly picking up aerosolized endotoxins from enteric microorganisms autochthonous to livestock pens, we believe that the results are not relevant to the Gram-negative non-fermenting aquatic microorganisms typically found in pharmaceutical water systems (Reid, 2019). Enterobacteriaceae species are exceedingly uncommon in parenteral manufacturing or implantable device manufacturing systems, ingredients or components.

The Chen and Mozier study objectives were clearly relevant in that they included testing of pharmaceutical product intermediates and therefore the possibility of measuring autochthonous endotoxins and glucans. However, the number of experimental variables made the data difficult to interpret. The exclusion of some data and inclusion of "outliers" may question the robustness of the assays. Still, we do feel that if the experimental variables were reduced and controlled, this type of study design with sufficient replicate samples would be precisely what is needed to demonstrate comparability. However, we would expect to see such studies performed by many sponsoring laboratories on the widest possible array of ingredients, intermediates, product formulations and components. It is only with a large population of studies that the capability, ruggedness and reproducibility of an alternate method can be known with sufficient statistical weight.

Despite some authors' stated beliefs that comparability between the recombinant reagents and the standard compendial methods are established, we found that there are consistent patterns of low relative recovery among recombinant methods for testing of "natural" and pharmaceutical waters (Table 6).

Table 6. Summary Table, Average of All Samples, All Methods

Recovery Range	Reich, et al	Kikuchi, et al	Reid Deionized	Reid, Post Carbon
0-50%	29%	33%	41%	82%
51-200	**63%**	**67%**	**59%**	**18%**
>200	7%	0%	0%	0%

In our re-assessment of the data, although the Reich, Kikuchi and Reid reported analysis performed with different types of waters ("natural" and "pharmaceutical"), a consistent pattern indicates that under-estimation by the recombinant reagents emerged in all these studies. Additionally, each study appeared to reflect a level of method-specific bias, suggesting that, at this point in their development, the recombinant methods are not similar enough in formulation or performance to be considered interchangeable. The Kikuchi data are particularly insightful because that was the only study in which possible glucan interference was mitigated by the removal of the Factor G pathway in the referenced formulation. Despite this unique formulation, the data follow the same overall patterns of under-estimation as the other investigations using water sourced testing materials.

Data offered by Reid (2019) represent the only studies of true pharmaceutical waters, and do not exhibit significantly different patterns in recoveries than did the Reich and Kikuchi studies. The data consistently show a tendency toward low recovery, and overestimation by any recombinant method was rare. The proprietary nature of these different recombinant reagents and their formulations prevent us from discerning the possible cause(s) of these analytical differences.

The validation of alternative recombinant methods requires a clear and statistically supportable demonstration of the equivalence of test results compared to the compendial methods (USPa, USPb, USPc, USPd, 2020; FDA, 2012). A satisfactory outcome in positive product control (PPC), performed using RSE or CSE, must not be presumed to be sufficient to demonstrate "validation" of recombinant methods or any other category of alternative method. The purpose of the PPC is a system suitability control conducted using *existing validated test methods* to assure that no residual product interference remains in the prepared sample. Alone, the PPC test is an insufficient criterion for the establishment of equivalency or non-inferiority to the compendial methods.

We acknowledge that each of the studies that were re-assessed represents a statistically small data set and that significantly, most studies were published without a detailed explanation of their experimental designs. The small data set does not allow for a clear statistical conclusion regarding non-inferiority of any of the evaluated alternative methods to the reference compendial method. However, even with differences in experimental design, our observed consistent pattern of underestimation of autochthonous endotoxins activity by recombinant reagents can potentially represent a patient safety risk. It would be irresponsible to assume recombinant reagent comparability until a sufficient number of well-designed studies with consistent appropriate statistical assessments have been completed.

Any meaningful studies to prove equivalence, comparability or non-inferiority of a test method must be designed to generate enough data to establish statistical reproducibility. Three trials are often considered sufficient in some validation exercises but demonstrating the suitability of a new assay approach to replace an established compendial method requires indisputable confidence. A new compendial method can be accepted as validated only after rigorous testing has been conducted and peer-reviewed by suitable field experts to ensure that the method has been assessed over a full range of test conditions and with appropriate statistical validity.

Our recommendations for future comparability studies include:

1. Construct a well-controlled experimental design with a clear objective.

2. Clearly define material handling to assure homogeneity and stability of test samples.
3. Coordinate testing to assure that all analyses are done concurrently and on the same sample.
4. Use glucan blockers for reference methods to diminish the risk of standard method over-estimation of endotoxins activity.
5. Employ RSE as the calibration standard to eliminate variation associated with CSE potency determination.
6. Select test samples containing assayable levels of activity that can properly assess contamination arising from autochthonous sources.
7. Define and establish a standard algorithm to evaluate data from comparability studies.

Conclusion

While we are always hopeful that better, quicker, and less environmentally impactful analyses become available for industry application, continued product quality and patient safety require that we do not accept any alternative method under any circumstance until it has been thoroughly studied and comparability data are scientifically vetted and pass compendial, statistical and regulatory scrutiny. Given these concerns, the authors believe that the current published studies are not complete validation studies demonstrating comparability or equivalence. We believe they are best characterized as preliminary or proof of concept studies for wholly new test methods with the implication that further, much more detailed and controlled comparability studies need to be conducted.

References

1. Abate, Wondwossen, Anas A. Sattar, Jian Liu, Myra E. Conway and Simon K. Jackson. 2017. Evaluation of recombinant factor C assay for the detection of divergent lipopolysaccharide structural species and comparison with Limulus amebocyte lysate-based assays and a human monocyte activity assay. Vol.66:888-897 Journal of Medical Microbiology.
2. Akers, James, Dennis Guilfoyle, David Hussong, Karen McCullough, Robert Mello, Donald Singer, Edward Tidswell, Radhakrishna Tirumalai. 2020. Functional Challenges for Alternative Bacterial Endotoxins Tests. Part 1. Attributes for Alternative Tests. Am Pharm. Rev. 23(4): 76-81
3. Bolden, Jay. 2020. Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products and Comparability to Limulus Amebocyte Lysate. Pharmacopeial Forum, Stimuli to the Revision Process. 45(3)
4. Bolden, Jay and Kelly Smith. 2017. Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products. J Pharm Sci and Tech 71: 405-412
5. Chen, Lin and Ned Mozier. 2013. Comparison of Limulus amebocyte lysate test methods for endotoxin measurement in protein solutions. Journal of Pharmaceutical and Biomedical Analysis. 80:180–185
6. Ding, J.L. and B. Ho. 2001. A new era in pyrogen testing. TRENDS in Biotechnology (8)
7. Ding, J.L. and T. Navas. 1995. Molecular cloning and sequence analysis of Factor C cDNA from the Singapore horseshoe crab, Carcinoscorpius rotundicauda. Mol. Marine Biol. And Biotech. 4(1): 90-103
8. Iwanaga, Sadaaki. 2002. The molecular basis of innate immunity in the horseshoe crab. Current Opinion in Immunology. 14(1): 87-95.
9. Kambayashi, J., M. Yokota, M. Sakon, E. Shiba, T. Kawasaki, T. Mori, M. Tsuchiya, H. Oishi, S. Matsuura. 1991. A novel endotoxin-specific assay by turbidimetry with Limulus amoebocyte lysate containing β-glucan. Journal of Biochemical and Biophysical Methods. 22(2): 93-100
10. Kikuchi, Y., Y. Haishima, C. Fukui, T. Murai, Y. Nakagawa, A. Ebisawa. 2017. "Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides," Pharmaceutical and Medical Device Regulatory Science 48: 252-260.
11. Loverock, Bruce, Barry Simon, Allen Burgenson, and Alan Baines. 2010. A Recombinant Factor C Procedure for the Detection of Gram-negative Bacterial Endotoxin. Pharmacopeial Forum, Stimuli to the Revision Process. Vol. 36(1):321-329
12. Marius, Marine, Frederic Vacher and Thierry Bonnevay. 2020. Comparison of LAL and recombinant Factor C endotoxin testing assays in human vaccines with complex matrices. PDA Journal of Pharmaceutical Science and Technology March 2020, pdajpst.2019.010389; DOI: https://doi.org/10.5731/pdajpst.2019.010389
13. Mizumura, Hikaru, Norihiko Ogura, Jun Aketagawa, Maki Aizawa, Yuki Kobayashi, Shun-ichiro Kawabata, Toshio Oda. 2017. Genetic engineering approach to develop next-generation reagents for endotoxin quantification. Innate Immunity 23(2): 136-146
14. Muroi, Masashi, Norihiko Ogura, Hikaru Mizumura, Jun Aketagawa, Toshio Oda, Ken-ichi Tanamoto. 2019. Application of a Recombinant Three-Factor Chromogenic Reagent, PyroSmart, for Bacterial Endotoxins Test Filed in the Pharmacopeias. Biol. Pharm. Bull 42(12):2024-2037
15. Obayashi T, Tamura H, Tanaka S, Ohki M, Takahashi S, Arai M, Masuda M, Kawai T. 1985. A new chromogenic endotoxin-specific assay using recombined limulus coagulation enzymes and its clinical applications. Clin Chim Acta. 149(1):55-65.
16. Piehler M, R. Roeder, S. Blessing and J. Reich. 2020. Comparison of LAL and rFC Assays - Participation in a Proficiency Test Program between 2014 and 2019. Microorganisms 2020, 8, 418; doi:10.3390/microorganisms8030418
17. Reich J, K. Heed K, H. Grallert. Detection of naturally occurring bacterial endotoxins in water samples. European Pharmaceutical Review [Internet]. 2014 Dec 23 [cited 2018 Apr 3];19(6):67–8. https://www.europeanpharmaceuticalreview.com/whitepaper/29826/whitepaper-detection-of-naturally-occurring-bacterial-endotoxins-in-water-samples/
18. Reid, Nicola. 2019. A global perspective for quantify8ing all endotoxins within pharmaceutical water systems. Presented at PharmaLab, Dusseldorf, Germany.
19. Thorne, Peter S., Sarah S. Perry, Rena Saito, Patrick T. O'Shaughnessy, John Mehaffy, Nervana Metwali Thomas Keefe, Kelley J. Donham, Stephen J. Reynolds. 2010. Evaluation of the Limulus Amebocyte Lysate and Recombinant Factor C Assays for Assessment of Airborne Endotoxin. Appl. Env. Microbiol. 76(15): 4988-4995.
20. Tsuchiya, M., A. Takaoka, N Tokioka, S. Natsuura. 1990. Development of an endotoxin-specific Limulus amebocyte lysate test blocking beta-glucan-mediated pathway by carboxymethylated curdlan and its application. J. Journal Bacteriol. 45(6): 903-911
21. United States Food and Drug Administration. 2012. Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-pyrogen-and-endotoxins-testing-questions-and-answers
22. United States Pharmacopeia. 2020a. <85>, "Bacterial Endotoxins Test."
23. United States Pharmacopeia. 2020b. <1223>, "Validation of Alternative Microbial Methods"
24. United States Pharmacopeia. 2020c. General Notices 6.30
25. United States Pharmacopeia. 2020d <1225>, "Validation of Compendial Procedures"
26. Zhao, Yang, Andre J.A. Aarnink, Mart C.M. DeJong, Peter W.G. Groot Koerkamp. 2014. Airborne Microorganisms from Livestock Production Systems and Their Relation to Dust. Critical Reviews in Environmental Science and Technology. 44: 11071-1128
27. Zucker BA, Trojan S, Müller W. 2000 Airborne gram-negative bacterial flora in animal houses. J Vet Med B Infect Dis Vet Public Health. 47(1):37-46. doi:10.1046/j.1439-0450.2000.00308.