A Summary of USP's Workshop on "The Future of Endotoxins and Pyrogen Testing: Reference Standards and Procedures"

Radhakrishna S. Tirumalai, PhD

Principal Scientific Liaison, General Chapters
USP

Karen Z. McCullough, MS

Member USP General Chapters - Microbiology Expert Committee

***Dr. Tirumalai** has been at the USP since 2003 and is currently a Principal Scientific Liaison-General Chapters in the Science Division. He is the Staff Liaison to the USP General Chapters- Microbiology Expert Committee. He works with the industry, regulatory agencies and other external science based organizations in the development and revision of General Chapters. Dr. Tirumalai represents USP on PDA expert task forces and conference organizing committees related to Microbiology and Sterility Assurance, on AAMI expert Working groups related to Microbiology, Sterilization, Sterility Assurance and Biocompatibility and on the editorial board of FDA's Pharmaceutical Microbiology Manual. Dr. Tirumalai's prior industry experience encompasses process and product research and development, transfer, and product manufacturing. He has a Ph.D. degree in Biochemistry. He has authored numerous publications, review articles and several book chapters. He is a frequent speaker at conferences and has taught Pharmacopeial Microbiology courses at numerous locations globally.*

***Karen Zink McCullough** is principal consultant at MMI Associates, a consulting firm focusing on Quality System development and pharmaceutical microbiology. Ms McCullough is nationally and internationally known for her work in the Bacterial Endotoxins Test (BET), and is a frequent speaker, instructor author on such topics as BET, GMP, Metrics, Risk, and pharmaceutical microbiology. Her credits include editing two books on Microbiology and BET, authoring 26 book chapters and 19 published articles. Ms. McCullough received her BA degree in Bacteriology from Rutgers University and her MS in Molecular Biology from the University of Oregon*

Background

All parenteral articles and medical devices that contact body tissue or fluids have to be sterile and pyrogen free. A standard method that utilizes increase in body temperature of rabbits to detect acceptable level of pyrogens, USP Chapter <151>, "Pyrogen Test" was first included in USP XII (1942). However, this test has several disadvantages in addition to being an animal based test. It is non-quantitative, very sensitive to animal strain, physiological state of test animal and the stress level of test animal.

The Gram-negative bacterial endotoxins are the most prevalent and quantifiable pyrogens in parenteral preparations, therefore the *in vitro* Bacterial Endotoxins Test was developed as a surrogate to the Pyrogen Test in the late 1970's. The majority of parenteral products now require testing for Bacterial Endotoxins.

The original chapter <85>, "Bacterial Endotoxins Test" (introduced into USP 20-NF 15 in 1980) and the current harmonized version introduced in 2002 have remained essentially unchanged for the last 39 years, despite advances in technology and a better understanding of endotoxins as contaminants in parenteral products.

On June 10-11, 2019, the United States Pharmacopeia (USP) held a workshop entitled, "Future of Endotoxins and Pyrogen Testing: Reference Standards and Procedures." Hosted and moderated by members of the USP General Chapters Microbiology Expert Committee, the purpose of the workshop was to provide a forum:

a. To discuss current knowledge and newly available technologies

b. To accelerate adoption of new standards and tests based on that knowledge

c. To establish appropriate standards and tests that assure product quality and protect patient safety

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Workshop

More than 100 attendees including representatives of the three major global pharmacopeias (United States, Europe and Japan), government regulators, industry experts and reagent manufacturers from various countries met to discuss three major topics: a) Endotoxin Standards: Current Test, Reference Standards, Intended Use, Need for New Standards", b) Alternative Methods: Is an Alternative Source of Reagent Desirable?" and "Pyrogen Tests – Rabbits and MAT. Non Animal Alternatives and Standards."

Welcome remarks were made by Jaap Venema, PhD, Executive VP and Chief Science Officer for USP and David Hussong, PhD, the current Chair of the USP General Chapters – Microbiology Expert Committee. Participants heard perspectives on the future of testing from Gwenael Cirefice, Ph.D and Yukari Nakagawa, Ph.D representing the European Pharmacopeia and Japanese Pharmacopeia respectively. These four speakers provided an international perspective on the current assays and set the expectations for the rest of the conference.

Session 1 – Endotoxin Standards: Current Test, Reference Standards, Intended Use, Need for New Standards

The first session was an examination of the current BET Assays as described in USP <85>. Goals for the session included:

1. What was the original intended use of the current test? Are we asking the test to do something that it was not designed to do?
2. What is the history/evolution of the current USP reference standard? What was its intended use? Are we asking the standard to do something that it was not designed to do?
3. Is there a need for an alternate/additional reference standard that is more reflective of the types of endotoxins that would be found in a pharmaceutical manufacturing environment?

The content of this session was based on current concerns regarding the product-specific interference patterns of the current test methods, and their impact on the accuracy of the data generated. Speakers including Terry Munson (former FDA and Technical VP Parexel), Edward Tidswell (Executive Director, Sterile QA at Merck and Co. and USP Expert Committee member), Karen Zink McCullough (consultant and USP Expert Committee member) and John Dubczak, (General Manager, Microbial Solutions at Charles River Laboratories) reviewed the 40+ year history of the assays including the development of the current compendial assays, reasons for the choice of the current purified lipopolysaccharide (LPS) reference standard (RSE), the limitations of the current test, and the utility of an alternative reference standard to the RSE.

It was emphasized that the BET detects activity of one particular pyrogen (endotoxin) that is a component of the outer leaflet of the outer membrane of Gram negative bacteria. Mr. Munsons' presentation reiterated the intent of the USP Endotoxin RS was solely a) Establish the sensitivity of the endotoxin tests/ LAL reagents and b) Calibrate control standard endotoxin preparations. A lively discussion ensued and focused on the definition of a "standard" and how that definition might need to be modified in order to reflect the best science of the day including current peer reviewed research on the adaptation of microorganisms to their environments and subsequent remodeling (new molecular structure) of LPS. While no conclusion was reached, it became clear that the question of the use of the purified "calibration" standard may be of limited analytical value for spike/hold time studies in parenteral products, and perhaps a standard preparation that is more representative of real-life contaminants, namely, well characterized Native or Naturally Occurring Endotoxin (NOE), would be a better choice. The discussion will continue, but the workshop enabled participants to define concerns with the current standards and to set up future discussions on specifications for a possible alternate analytical standard.

Session 2 – Alternative Methods: Is an Alternative Source of Reagent Desirable?

The second session was prompted by the introduction of a number of products into the market that utilizes recombinant technology to produce reagents that don't require the bleeding of horseshoe crabs. The goals for the session included:

1. User experience with validation of the rFC assay.
2. What are the differences and similarities in current reagents with respect to source proteins, culture conditions, gelation cascade components, formulation and signal detection and are they comparable in terms of their detection and quantification of endotoxins?
3. The Need for an Alternative Method is driven by a) replacement of animal based/reagents or tests whenever pssible and b) Sustainability of the Horseshoe Crab (HSC) as the sole raw material source for the Bacterial Endotoxins Test (BET). It was noted that the HSC are distributed along the east coast of the United States and along the east coasts of Japan, China, India and Indonesia and that the Asian HSC species are becoming depleted.

In vivo and *in vitro*, the LAL reaction is really a series of cascading and signal amplifying reactions. However, a number of formulations are either currently marketed or are in development that utilize one or more cloned proteins in the cascade as the basis for the formulation of "recombinant" reagents. Because the current harmonized chapter focuses on the reagents as produced from horseshoe crab blood, these recombinant reagents and the test methods that accompany them are seen as "alternate tests" to the BET. In this session, Jay Bolden, Senior Consultant Biologist at Eli Lilly and Co. presented his company's data

on the comparability of the recombinant test (recombinant Factor C) and the current compendial test. Manufacturers of recombinant reagents were provided the opportunity to present non-commercial descriptions of their products, and any data that they have regarding comparability. The roundtable following the presentations sparked considerable discussion on comparability, given that most of the data presented were on samples that contained no detectable endotoxins with either the current BET methods utilizing LAL or alternate methods utilizing recombinantly derived reagents. In the end, is comparability defined as "non inferior" or is it defined as "better than or equal to"? The three pharmacopeia as well as developers of these recombinant assays are working on independent studies and study designs to provide data that will ultimately determine the new assays' inclusion in the harmonized chapter.

Session 3 – Pyrogen Tests – Rabbits and Monocyte Activation Test (MAT), Non-animal Alternatives and Standards

There are many substances and organisms that can elicit a fever in mammals. USP <151>, "Pyrogen Test" has been used for many years to screen products for all pyrogens (not just Gram-negative bacterial endotoxins).Currently, the Monocyte Activation Test (MAT) which detects or quantifies substances that activate human monocytes or monocytic cells to release endogenous mediators which have a role in the human fever response, looks to replace or supplement the compendial Pyrogen Test.

Goals for the session included the following:

1. What is the history and variability of the rabbit Pyrogen test?
2. What non-endotoxin standards are appropriate and available for the routine use of the Monocyte Activation Test?
3. What are users' experiences with the MAT?

One of the reminders in the third session of the Workshop is that the BET is not a Pyrogen test, but rather it is a test for the presence of endotoxin activity. The substitution of the LAL test for the USP Rabbit Pyrogen Test (USP <151>) was justified 40 years ago because endotoxins from Gram negative bacteria are the most potent and prevalent pyrogens found in the manufacture of parenteral products. However, an understanding of the possible presence of pyrogens other than endotoxins has a place particularly in the development of a new drug product or potentially as a replacement for the LAL test. The drawback, of course, of the current Pyrogen test is an *in vivo* assay that requires the use of rabbits. Animal rights concerns and regulatory directives to limit the use of live animals in pharmaceutical testing have rightly been adopted by the industry. Speakers in the third session of the workshop included Marlys Weary (retired, Baxter Laboratories), Thomas Hartung, PhD (Professor of Evidence Based Toxicology at The Johns Hopkins University) and Ned Mozier, PhD (VP Biotherapeutics Pharmaceutical Sciences at Pfizer). All provided context and insights into the history of the rabbit Pyrogen test as well as the development and use of the *in vitro* Monocyte Activation Test and non-endotoxin (Gram positive) pyrogen reference materials. It was noted that while Lipoteichoic Acid is a good candidate for a non-LPS Gram positive pyrogen standard, it is extremely difficult to purify it and retain activity. As with the recombinant reagents, manufacturers of *in vitro* Pyrogen tests were each invited to give a short non-commercial presentation of their data. It was noted that despite the low sensitivity, costs and variability, the use of rabbit pyrogen testing is growing rather than shrinking, and the test remains unchanged in international compendia. While the use of *in vitro* Monocyte Activation Test has advantages over the Rabbit Pyrogen Test, particularly in quantification of pyrogens and detection of non-LPS (endotoxin) pyrogens, currently it is seen more as a research tool and a screen rather than a release test procedure.

Summary

In summary, the attendees learned a) that some of the uses of the current methods and reference standard were not in alignment with their intended applications, b) the need for standards that closely resemble product contaminants is desirable c) recombinant reagents may be the choice in the future once their comparability to the horse shoe crab based LAL reagent is established clearly, d) Use of In vitro pyrogen tests such as MAT for replacement of the Pyrogen Tests / BET is still a work in progress, and e) a non LPS reference material is not yet available. This workshop helped re-emphasize the purpose and appropriate use of these tests and standards.

United States Pharmacopeial Convention (USP), a nonprofit scientific organization, develops standards for the identity, strength, quality and purity of drugs and their ingredients, which are published in the United States Pharmacopeia and National Formulary (USP–NF). Written or documentary standards for drugs or drug ingredients are expressed in USP–NF monographs, general chapters, and General Notices. A monograph is developed for a single article (e.g., drug substance, drug product, excipient), while a general chapter can apply across multiple articles. Above-1000 general chapters are informational, and contain no mandatory requirements unless specifically referenced in a monograph, General Notices, or a general chapter numbered below 1000. General chapters designated as below-1000 contain tests that may apply to items recognized in USP or NF, and may also be required by the Food and Drug Administration (FDA) to demonstrate conformance to a specification. While USP's standards are applicable under U.S. law to drug substances and products marketed in the U.S., they also are used in more than 140 countries throughout the world.