LAL/TAL and Animal-Free rFC-based Endotoxin Tests: Their Characteristics and Impact on the Horseshoe Crab Populations in the United States and Asia

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Abstract

The current global demand for endotoxin testing is projected to double by 2024. In light of the serious decline of the Asian horseshoe crab species, and regulated harvesting restrictions on the American horseshoe crab, it is unlikely that *Limulus* and *Tachypleus* Amebocyte Lysate (LAL/TAL) reagents alone can meet the markets' demand. Validated animal-free endotoxin tests are available, such as assays based on recombinant horseshoe crab Factor C (rFC). While data on their efficacy and reliability is available, the pharmaceutical industry and regulatory authorities have only started to implement and make routine use of rFC assays in the past 5 years. Although the pharmaceutical, medical device industries and healthcare facilities' reliance on the horseshoe crab for endotoxin testing does not pose the greatest threat to the species' survival, they are the only user groups with the power to transcend geographic boundaries, government malaise, linguistics, social, cultural and corporate indifference. For these industries have the ability through supply chain management, to augment their endotoxin test requirements with rFC assays and in doing so, put an end to the use of TAL, and reduce the harvesting pressure on LAL production. In this paper we discuss the status and threats posed to the world's four extant horseshoe crab species. Furthermore, we review the state of the global endotoxin test market, the characteristics and misconceptions surrounding the use of the animal-free endotoxin test rFC and the role the pharmaceutical, medical device industries and healthcare facilities can play in the survival of the world's four horseshoe crab species.

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Introduction and History of Bacterial Endotoxin Testing

The human immune system is able to fend off a variety of organisms potentially harmful to the body. Even its comparably primordial innate immune system can detect a number of microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) without prior exposure and may initiate an appropriate immune response, including the recruitment of the adaptive immune system (Mogensen 2009). Among these MAMPs, bacterial endotoxin, a major constituent of the outer membrane of Gram-negative bacteria, acts as one of the most potent immunological stimulants (Raetz and Whitfield 2002). Even minute amounts can cause a wide spectrum of immune reactions ranging from fever – leading to the categorization of endotoxin as "pyrogen" (fever-inducing substance) – to organ dysfunction and septic shock, if they enter the blood circulation (Wolff 1973). Due to the severe physiological reactions to this potential contaminant and the abundance of Gram-negative bacteria (Gorbet and Sefton 2005), parenterally administered pharmaceuticals, i.e. ones injected into the body, and medical devices exposed to the blood circulation are required to be tested for endotoxin (United States Pharmacopeia USP 2016a, European Pharmacopoeia Ph.Eur. 2016a, Japanese Pharmacopoeia JP 2016). Only concentrations considered harmless are permitted, most commonly 5 endotoxin units (EU) per kilogram bodyweight (USP 2016a, Ph.Eur. 2016a, JP 2016).

The first authorized method to test for endotoxin and, indistinctively, other pyrogens, was the Rabbit Pyrogen Test (RPT), established in the 1920s (Seibert 1923) and introduced into the United States Pharmacopeia in 1942 (USP 1942). As part of this procedure, a pharmaceutical product under investigation is injected into the ear veins of several rabbits. If the product contains elevated levels of endotoxin, the rabbits display a body temperature increase beyond a pre-defined threshold. Consequently, said product will be rejected and disposed. However, this method is only qualitative, as the exact pyrogen concentration remains unknown.

In the 1950s, Frederik Bang and Jack Levin found that the hemolymph of *Limulus polyphemus* clotted upon exposure to components of Gram-negative bacteria, later revealed as endotoxin (Bang 1953, Levin 1964). Specifically, the horseshoe crab's immune cells, called amebocytes, secreted coagulating agents. This discovery was decisive for the development of the *Limulus* amebocyte lysate (LAL) bacterial endotoxin test (BET). In the mid-1970s, horseshoe crabs began to be harvested for their hemolymph used in the production of LAL. Within a few years, this animal extract came into global use by the pharmaceutical and medical device industries to test for the presence of bacterial endotoxins in parenteral drugs and implantable medical devices for both humans and animals. The LAL test proved to be a simpler, more reliable, more specific, cheaper, quantitative and more humane alternative to the RPT it replaced (Cooper et al. 1971). By the late 1970s, the Asian *Tachypleus* species began to be harvested for same purpose, the production of equivalent *Tachypleus* amebocyte lysate (TAL) (Xiamen Bioendo Technology 2019).

In parallel to the spreading use of LAL/TAL, recombinant deoxyribonucleic acid (DNA) technology emerged, i.e. the creation of DNA sequences not found in nature (Cohen 1973, Hughes 2001, Cohen 2013). This allowed the deliberate transfer of genes between organisms and their expression into proteins in easily cultivated organisms of choice, such as bacteria, yeast or animal cells (Brondyk 2009). Thus, a wide range of biological products began to be manufactured, starting with insulin (Goeddel et al. 1979) and expanding to many different proteins and other biomolecules.

While said biomolecules could be processed into products such as pharmaceuticals, recombinant DNA technology also allowed for their thorough analysis, greatly facilitating research into biochemistry. As part of this development, the molecular machinery in LAL/TAL was explored and unraveled in the late 1980s and early 1990s (Nakamura et al. 1986, Muta et al. 1991, Iwanaga and Lee 2005). Based on this knowledge, the endotoxin detector protein of the lysate, Factor C, was successfully synthesized in an organism other than horseshoe crabs, namely yeast, insect and mammalian cells (Roopashree et al. 1996,

1997, Ding and Ho 2003, Ding et al. 1997, Pui et al. 1997). Since this recombinant horseshoe crab Factor C (rFC) fulfilled the same function as its native equivalent, it was incorporated into a bacterial endotoxin test equivalent to LAL/TAL tests, i.e. the first rFC assay from 2003 (Ding and Ho 2001, 2003, 2010, Lonza 2018).

Characteristics of LAL/TAL and rFC tests

LAL/TAL contains a multitude of proteins produced and secreted by amebocytes. Many of the functionally notable ones include so-called zymogens or pro-enzymes. These are proteins that require activation before they become enzymatically active. Here, such activity is reflected in the cleavage of other proteins at specific sites, i.e. the activation of other zymogens and otherwise functional proteins.

Upon exposure to endotoxin, the zymogen Factor C changes its structural conformation and excises a part of itself. Thus, active Factor C cleaves Factor B which will in turn cut proclotting enzyme, whose active form clotting enzyme truncates coagulogen into coagulin. Ultimately, several coagulin molecules crosslink each other into a solid network; the hemolymph or lysate coagulates (Fig. 1a). At each step of the enzymatic cascade, the reaction is amplified. Consequently, minute amounts of endotoxin can elicit a strong response, reflected in the paramount sensitivity of LAL/TAL. On the other hand, the additionally present zymogen Factor G can be activated by beta-glucans (Mikami et al. 1982, Roslansky et al. 1991, Muta et al. 1995), e.g. from fungi or water filters made of cellulose (Pearson et al. 1984). Factor G cleaves proclotting enzyme equivalent to Factor B. Moreover, LAL/TAL has been shown to be reactive to substances such as thrombin, thromboplastin, nucleic acids (Elin and Wolff 1973), peptidoglycans and exotoxins from Gram-positive bacteria (Wildfeuer et al. 1974, Baek et al. 1985, Brunson and Watson 1976) and dithiothreitol (DTT; Platica et al. 1978). Accordingly, LAL/TAL can also react in the absence of endotoxin.

In its simplest and most classical format, the so-called gel clot test, LAL/TAL is simply subjected to a sample inside of a glass vial. An amount of endotoxin equal to or greater than the lysate's sensitivity will lead to coagulation within one hour at 37°C, visible as turbid solid clot that stays at the bottom of the vial, when inverted. In the course of coagulation, turbidity gradually increases, a process that can be measured in instruments reading absorbance. The more endotoxin, the quicker the turbidity rises. As samples and defined amounts of standard endotoxin can be compared with regard to turbidity development upon addition of LAL/TAL, the amount of endotoxin in the samples can be deduced (Urbascheck et al. 1987). This quantitative BET is known as turbidimetric LAL/TAL test. To enhance the rise in absorbance, a synthetic color-generating substrate of clotting enzyme can be added to LAL/TAL, which is the test principle of the so-called chromogenic test (Berzofsky 1994), a second quantitative BET. For convenience in handling and measurement as well as for saving lysate and sample, both quantitative test formats are usually performed in microtiter plates These are plastic vessels commonly equipped with 96 wells, i.e. small separate reaction spaces functionally equivalent to tiny test tubes.

In contrast to LAL/TAL, rFC assays solely rely on the enzymatic function of Factor C. Instead of Factor B, a synthetic fluorescence-generating molecule is processed by rFC after activation by endotoxin (Fig. 1b). In correspondence to the chromogenic LAL/TAL test, this rFC reagent is added to samples and control standard endotoxin (CSE) inside of microtiter plates. The rise in fluorescence intensity, measured by a respective instrument, is proportional to the amount of endotoxin. While the response-amplifying enzymes of LAL/TAL are missing, even fluorescence light invisible to the human eye can be measured. Therefore, these fluorogenic rFC assays display sensitivity to endotoxin which is comparable to LAL/TAL tests. Since 2015, the entire line of Factor C, Factor B and proclotting enzyme has been produced recombinantly and integrated into a commercially available chromogenic assay (Mizumura et al. 2016). All of these rFC-based assays do not react to beta-glucans for lack of Factor G (Bolden 2019). Accordingly, they do not show false-positive signals in testing these polysaccharides and potentially other LAL-reactive substances (see above), i.e. signals that do not come from endotoxin.

Comparing BET, i.e. LAL/TAL and rFC tests in different formats (Williams 2007), you will not find that "one size fits all", meaning a perfect combination of robustness, speed, sensitivity, easy handling and low price. Gel clot LAL/TAL tests are very simple, low-priced and require only modest equipment, namely for dilution and heating. Furthermore, the high amount of lysate compared to sample lends the test rather high robustness against interference (Hughes et al. 2015). However, a variety of substances commonly interfere with the enzymes in BET (Twohy et al. 1984). The relatively high amount of lysate required for gel clot tests, means that more horseshoe crabs need to be bled for a defined number of BET trials. On the downside of simplicity, the incubation time needs to be controlled manually and clot identification is not perfectly unambiguous, especially since coagulation can be hampered by mechanical shock. Moreover, automation of handling and result interpretation is hardly feasible. These aspects appear particularly unfavorable considering ever tighter regulations on Data Integrity, i.e. the strive for preventing data manipulation to guarantee patient safety (United States Food and Drug Administration FDA 2018). Consequently, the gel clot test is primarily used by manufacturers with low budget and long-standing products whose change of endotoxin control would ask for considerable efforts. While the gel clot test is qualitative or semi-quantitative at best – it returns ranges of endotoxin concentrations ('between', 'greater than', 'lower than') – all other test formats yield accurate, numeric values and are accordingly defined as quantitative BET procedures.

Turbidimetric tests are their simplest variants in the sense of only using the lysate with a few stabilizers, just as gel clot tests. Hence, they are comparably inexpensive and particularly useful for non-challenging samples such as purified water which could account for up to 70% of all bacterial endotoxin testing. Since the increase of absorbance from rising turbidity however lags behind color formation in the presence of a respective substrate, chromogenic tests tend to be more robust than their turbidimetric counterparts. In this regard, rFC assays are comparable to chromogenic LAL/TAL tests, but largely remain unaffected by color in samples.

LAL/TAL is provided in a lyophilized, solid form and a suitable solution is added to reconstitute and prepare the reagent for a test. Reconstituted LAL/TAL should be used immediately, as it starts coagulating, even if endotoxin is not added. Potentially similar to human blood (Hurley et al. 2015), horseshoe crab hemolymph might harbor a low level of inherent endotoxin that causes this phenomenon. On the other hand, rFC reagents are supplied in a liquid form and remain inactive for hours after reagent preparation for a test, i.e. the mixing of rFC, its fluorogenic substrate and its activity-promoting buffer. This flexibility enhances test robustness and facilitates automation, as the reagents can be provided to a pipetting machine with less respect for its speed of action. Thus, the rate of errors and manual labor can be reduced.

Each test format from a specific manufacturer shows a unique level of tolerance or susceptibility to interference from a range of substances. To elaborate, whereas one BET reagent may yield accurate results in testing high amounts of a certain compound which interferes with another reagent, the relationship may be inverted in case of analyzing another compound for endotoxin (McCullough et al. 1992). Likewise, several reagents may systematically return different results from a single sample (Kikuchi et al. 2017, 2018). As the amebocyte extracts and rFC preparations are not expected to show tremendous quality differences between manufacturers – the former come from large pools of horseshoe crabs alike and the latter are structurally highly similar proteins independent from the species donating the Factor C gene –, most of these peculiarities might be associated with the choice of stabilizers for both the enzymatic reagents and the co-supplied control standard endotoxin (Parenteral Drug Association 2019).

Regulation of Bacterial Endotoxin Tests

Ever since its first commercialization in 2003, rFC has faced an uphill battle to gain the regulatory and customer acceptance of LAL/TAL, independent from manufacturer and test format. When LAL emerged as alternative reagent for bacterial endotoxin testing, the advantages over the rabbit pyrogen test (RPT) were obvious (Federal Register 1977). LAL was specific to the predominantly found pyrogen endotoxin and, as only revealed later, beta-glucans. In this respect, the LAL test was more sensitive (van Noordwijk

et al. 1976, Wachtel et al. 1977, Mascoli et al. 1979) and, after the introduction of endotoxin standards, also more quantitative (Rudbach et al. 1976).

Generally, the LAL test's convenience was striking, as it could simply be bought from reagent vendors, whereas the rabbits most commonly needed to be bred and kept in-house, resulting in considerable maintenance expenses. Likewise, the test procedure was shortened from several hours to one, allowing for quicker results. Accordingly, LAL tests offered substantial cost savings and the ability to test radiopharmaceuticals whose short half-life requires rapid results (Hartung 2015, Liebsch 1995). Meanwhile, the influence of biological variability was reduced, as lysate produced from numerous horseshoe crabs yielded more reproducible results than the typical RPT which could only involve up to eight rabbits. Under these circumstances, the LAL test quickly gained approval by the FDA (Federal Register 1973) and adoption as compendial method into USP XX Chapter <85> (USP 1980). The European (Ph.Eur.) and Japanese Pharmacopoeias (JP) followed with their harmonized chapters 2.6.14. and 4.01 respectively.

Upon their introduction, rFC assays generally improved on specificity, reproducibility and animal protection. rFC is not activated by LAL-active beta-glucans (see section above), providing a more specific bacterial endotoxin test. The biological variability was diminished even further (McKenzie et al. 2011), since rFC always bears the very same primary structure (amino acid sequence of the protein), is synthesized by genetically identical cells, and is purified in a tightly controlled biochemical environment. To compare, LAL comes from genetically different populations of horseshoe crabs that may additionally be subject to seasonal environmental influences and varying states of health (Jorgensen and Smith 1973). Particularly in relationship to different chemical environments as found in the wide spectrum of pharmaceutical samples, the enzymatic activity of rFC can thus be reproduced more easily than in case of the animal extract LAL. Consequently, preparation procedures established for specific samples can be applied more reliably (Microcoat 2019).

rFC can help to take pressure off the global horseshoe crab populations that are harvested for the manufacturing of LAL/TAL. Even if *Limulus polyphemus'* mortality due to respective best-practice handling is comparably low (ASMF 2018), any horseshoe crab that can avoid bleeding maintains a higher chance of survival and reproduction. In a single 30L production run (Fig. 2), the amount of rFC produced is equivalent to the bleeding on average of 6,000 horseshoe crabs without the associated collection, transportation, bleeding, husbandry and release associated with the production of LAL.

Whereas environmental protection – and wildlife conservation in particular – have gained momentum in the past decades, the manufacturing industry has often only contributed to this trend upon pressure from regulatory bodies, the public and governmental incentives. Accordingly, the adoption of rFC for ethical reasons has mostly been the effort of dedicated individuals such as Jay Bolden of Eli Lilly, who in 2013 began leading his company away from traditional TAL/LAL BET and towards the animal-free rFC. In his 2018 progress report to the United Nations Global Compact (UNGC; Eli Lilly and Company 2018), Eli Lilly and Company CEO David A. Ricks indicated that, by 2020, ninety percent of the company's endotoxin test will be conducted via synthetic compounds.

From its introduction in 2003, it took nine years until the FDA explicitly mentioned rFC assays as acceptable reagents for BET, followed by the European Pharmacopoeia in 2016 (FDA 2012, Ph.Eur. 2016b). The primary validation of the first rFC assay by its manufacturer Lonza (Loverock et al. 2009) and the introduction of an rFC assay by the manufacturer Hyglos (now bioMérieux, Grallert et al. 2011), had provided rFC users with crucial validation support and the opportunity to switch between reagent suppliers. Thus, they certainly helped in driving this first step in gaining regulatory acceptance. Nonetheless, rFC assays were officially regulated as alternative BET methods, at least until chapter 2.6.32. Test for Bacterial Endotoxins using Recombinant Factor C of the European Pharmacopoeia becomes effective (EDQM 2019) in 2021 This potentially stimulates the chapter's adoption into USP and JP as well.

If users currently decide to use an rFC assay for release of pharmaceutical products or medical devices, they need to follow a validation adhering to USP Chapter <1225> Validation of Compendial Procedures (USP 2016b) according to the FDA (Fig. 3, FDA 2012). Namely, they show that the method is working in their laboratory as intended by demonstrating acceptable accuracy, precision, specificity, detection limit, quantitation limit, linearity, range and robustness. While specificity is established for each kind of sample individually in the test for interfering factors, the other aspects can be analyzed within a week (Williams 2018). Using comparative data between rFC assay and several LAL tests, e.g. from the manufacturer-sponsored primary validation of the method (Microcoat 2019), users can even omit repeating the direct test comparison that is required by the FDA. Although rFC manufacturers have thus attempted to ease the validation, it remains a hurdle that does not apply to users of compendial LAL/TAL; some preparatory testing and the test for interfering factors are sufficient for establishing routine LAL/TAL testing. Given high financial pressure and regulatory scrutiny, many healthcare companies have therefore kept choosing LAL/TAL over rFC.

In terms of equivalence to LAL/TAL tests, rFC assays face a particular challenge. They share the biochemistry of the decisive Factor C, the handling is highly similar to quantitative LAL tests and regulatory requirements for routine testing are the same (USP 2016a, Ph.Eur. 2016a, JP 2016). Ultimately, even the read-out, namely Endotoxin or International Units (EU/IU), is identical. While these matches ease the shift from LAL/TAL to rFC in laboratory practice, they have been exploited by LAL manufacturers to compare these reagents' results and question the validity of rFC (Dubczak 2018), especially since users rarely compare different LAL/TAL reagents on samples containing endotoxin.

As indicated before, each reagent features a unique composition of enzymes, stabilizers, standard endotoxin and additional laboratory equipment such as microtiter plates or glass vials. Hence, every BET returns individual values, i.e. each LAL/TAL and rFC test yields a specific value (Wachtel et al. 1977, Kikuchi et al. 2017, 2018). At least for all LAL/TAL tests independent from the format, they are nevertheless assumed to be acceptable approximations to true endotoxin concentrations. In contrast to this categorical acceptance, differences of any rFC assay to any LAL/TAL test have been highlighted by a few LAL manufacturers and dedicated LAL users as supposedly unacceptable, particularly if an rFC assay returned lower values (Bolden et al. 2015, Dubczak 2018). Then again, the latter has also been found vice versa (Sorrentino 2011, Kikuchi et al. 2017, 2018, Williams 2019).

Ideally, equivalence is studied on large multi-center datasets that are robust against outliers. Most notably, the Pharmaceuticals and Medical Devices Agency of Japan (PMDA) compared three chromogenic LAL tests and three rFC-based assays in an extensive study involving up to five laboratories and purified endotoxin samples from 18 different bacterial strains, crude endotoxin preparations from 5 strains and 6 environmental endotoxin samples (Kikuchi et al. 2017, 2018). They concluded that LAL and rFC detected endotoxin in all investigated samples, did not demonstrate any clear principle-specific difference and could therefore be considered equivalent (Fig. 4).

Despite studies from the PMDA (Kikuchi et al. 2017, 2018), pharmaceutical manufacturers (Chen and Mozier 2013, Bolden 2019), academic institutions (Sorrentino 2011) and rFC manufacturers (Loverock 2009) as well as contract research organizations (Microcoat 2019), common criticism of rFC assays has focused on an ostensible lack of data proving the equivalence to LAL tests. For comparison, a Baxter Travenol study featuring thousands of parallel RPT and LAL tests has been cited (Mascoli et al. 1979, Dubczak 2018). Then again, only 37 LAL tests and 4 RPT returned positive results at the time. The study indeed indicated that endotoxin was the most prevalent pyrogen and that the absence of endotoxin might be equated with the absence of pyrogens. However, the equivalence of both tests with regard to endotoxin from different bacterial strains (see PMDA study above) was not studied. Moreover, despite other LAL-RPT comparison studies (Wachtel et al. 1977), it is unclear, if this data can be extrapolated from gel clot to other LAL test formats. Finally, the impartiality of the authors might be questioned, as Baxter Travenol manufactured their own LAL and therefore had vital commercial interest in promoting LAL.

Another argument against rFC has been the FDA license that is granted to each LAL reagent's manufacturing since 1977 (Federal Register 1977). When the first rFC assay was commercialized, the manufacturer submitted a request for designation in order to learn about the FDA center responsible for granting such premarket approval. Since rFC was synthetically produced, neither an animal product with its inherent variability nor intended for diagnosing diseases in humans or animals, the FDA concluded that premarket approval was not required (Berzofsky 2004). At this point, it should be noted that the FDA license is not a general indicator for the quality of LAL tests and their capability to detect endotoxin; it is simply intended to regulate and control the manufacturing process of LAL (Burgenson 2019). Whereas rFC manufacturers lack respective FDA oversight, they nonetheless adhere to stringent guidelines such as Good Manufacturing Practice (GMP) and International Organization for Standardization (ISO) requirements (Burgenson 2019). Accordingly, any change of the reagent impacting assay results must be disclosed and rFC users may audit these manufacturers, thus encouraging the adherence to strict quality systems.

Due to their different reagent sources, the manufacturing processes of LAL/TAL and rFC actually differ substantially (Jorgensen and Smith 1973, Armstrong et al. 2008). For LAL/TAL, hemolypmh is drawn from the hearts of horseshoe crabs, pooled and supplemented with anticoagulant. Via centrifugation, the amebocytes are separated from the rest of the hemolymph. Water is added to induce cell bursting (osmotic pressure) and thereby release of the enzymes relevant for the reagent. Another centrifugation strips them from cell debris and, after addition of stabilizing substances, the enzymes are freeze-dried to yield solid and stable LAL/TAL. rFC in contrast, is produced in cells that secrete the enzyme into their growth medium and that are subsequently removed using centrifugation or filtration. To purify rFC, the medium flows through chromatographic resins that selectively bind rFC or its impurities. Similarly to LAL/TAL, stabilizing substances are added to allow for years of shelf lives. On the other hand, freeze-drying (lyophilization) is not necessary, as rFC does not display LAL/TAL's previously mentioned unspecific activity in the absence of endotoxin.

Effects of Horseshoe Crab Harvesting and Habitat Loss

Distributed along the Atlantic coast of the United States and the Southeast Gulf of Mexico and throughout coastal South and Southeast Asia (Fig. 5), each of the world's four extant horseshoe crab species exhibit genetic variation throughout their spawning range and are at risk of local extinction primarily due to anthropogenic activities that vary between the countries they inhabit. The primary stressors are loss of coastal habitat, both marine and terrestrial, from land reclamation, infrastructure development, coastline armoring, erosion, pollution and unsustainable harvesting for bait, human consumption, amebocyte lysate production made from the hemolymph of the *Limulus* and *Tachypleus* species, by-catch, chitin, fertilizer, and traditional Chinese medicine (Smith et al. 2017, Akbar et al. 2018, IUCN 2019). The future survival of the four horseshoe crab species will ultimately depend upon the preservation of spawning and nursery habitat and overcoming social, cultural and corporate indifference to unsustainable harvesting practices. This is a challenging prospect in light of the ever-increasing human density along the same beaches and nearshore areas horseshoe crabs rely upon for propagation and growth (Gauvry 2015).

In the US, from the late 1800s to the early 1900s the American horseshoe crab were harvested unsustainably for fertilizer and livestock feed, depleting the resource by the 1940s (ASMFC 1998). As the fertilizer and livestock feed user groups moved away from horseshoe crabs to alternative and synthetic products, the population began to recover. By the 1960s, all commercial harvest of horseshoe crabs had ceased and, by the late 1970s, the population had substantially recovered (Shuster 1996, ASMFC 1998). Although the use of horseshoe crabs in medical research began in the early 1900s (Shuster 1962), it was not until the commercialization of LAL and the emergence of this new user group in the mid-1970s that horseshoe crabs were once again harvested in greater numbers. In the mid-1990s, yet another commercial user group emerged, who began harvesting the resource for bait in the American eel (*Anguilla rostrata*) and whelk pot fisheries *(Busycon spp.)*.

Although the majority of the American horseshoe crabs harvested for LAL are returned-to-sea with a relatively low mortality of 15% (ASMFC 2018), the combined unregulated harvest from these two user groups again resulted in the population's decline (ASMFC 2018). In 1997, the States of Delaware and New Jersey along with a ground swell of support from birding organizations, horseshoe crab advocacy groups and the scientific community, urged the Atlantic States Marine Fisheries Commission a United States Federal Governmental body with broad fisheries management authority, to develop a horseshoe crab fisheries management plan (FMP) to monitor and regulate the harvest of the American horseshoe crab coast wide (ASMFC 1997). The purpose of the horseshoe crab FMP is to ensure a sustainable population throughout its spawning range, so as to ensure the continued role of the horseshoe crab resource in the ecology of coastal ecosystems, food resource for migratory birds and other dependent wildlife and the fishing and non-fishing public (ASMFC 2018). In March of 2001, to protect essential marine habitat in the mid-Atlantic region, home to the largest concentration of the American horseshoe crab, the National Marine Fisheries Service (NMFS) at the recommendation of the ASMFC, established the Carl N. Shuster, Jr. Horseshoe Crab Reserve. It prohibited the harvest and transfer of horseshoe crabs in federal waters within a 30 nautical mile radius of the mouth of the Delaware Bay (ASMFC 2001).

Presently, the American horseshoe crab species *Limulus polyphemus* is only marginally stable throughout its United States range, with an improving population in the North East region (Maine to Rhode Island), still declining in the New York region (Connecticut to New Jersey), remaining stable in the Delaware Bay region (New Jersey to Virgina), and increasing in abundance in the Southeast region (North Carolina to Florida; ASMFC 2019).

The Mexican horseshoe crab population on the species extreme southern range is poorly understood and does not fall under the jurisdiction of the ASMFC. There is insufficient data to confirm population stability (Zaldivar-Rae et al. 2009, Smith et al. 2017). Although under Mexican law, the harvest, sale and purchase of horseshoe crabs are illegal, there is no active management of the species. Consequently, there is a growing illegal harvest of horseshoe crabs for bait in the octopus fishery with a preference for males which are most compatible with traditional drift fishing techniques (Smith et al. 2017).

While the regulated harvest of the American horseshoe crab used for bait in the American eel (*Anguilla rostrata*) and whelk pot fisheries *(Busycon spp.)* continues to decline from a high of 2.6 million in 1999 to an averaged low of 753,000 between 2004-2017 (ASMFC 2019), a 245% decrease, the harvest for LAL production continues to increase from a low of 130,000 crabs in 1989 (FDA 1998, ASMFC 1998) to an averaged high of 485,197 between 2004-2017 (ASMFC 2019), a 273% increase. Although a recent study indicates no adverse impact on the American horseshoe crab population from current LAL production harvesting levels (Smith 2019, ASMFC 2019), it is uncertain whether LAL production, which is expected to double by 2024 (Fig.6) (Zion Market Research 2019), can continue to increase to meet its projected growth, much less absorb the Asian TAL market as it begins to decline from unsustainable harvesting practices. In an effort not to exceed their current harvesting footprint, some LAL producers have partnered with the American eel (*Anguilla rostrata*) and whelk pot fisheries *(Busycon spp.)*, bleeding animals before they are used as bait (ASMF 2018). This practice may expand as these two user groups seek to share a finite resource managed for sustainability within a growing market. For as long as the harvest of the American horseshoe crab can be managed for sustainability and the availability of viable animal-free BET are available, it will be unlikely that the American eel and whelk pot fisheries will be asked to reduce their harvest in favor of biomedical use. Although often viewed as the villains in this debate by LAL producers and conservationists, who move within a different social economic class, it is important to remember that commercial fishing is one of the oldest vocations in the United States and helped provide the financial means to launch the American Revolution, thus offering the enduring status of a heritage activity (Kurlansky 1997).

Throughout South and Southeast Asia, home to three of the world's four horseshoe crab species (*Tachypleus tridentatus, Tachypleus gigas, Carcinoscorpius rotundicauda*) horseshoe crab conservation advocacy is only beginning to take root after centuries of exploiting this marine life primarily for human consumption, and since the late 1970's, the production of the bacterial endotoxin test TAL. When human

considerations overshadow environmental concerns, ignorance and indifference are often at the core of a species decline. In 2019, the IUCN Horseshoe Crab, Specialist Group, in collaboration with its members throughout South and Southeast Asia, released their IUCN Red List assessment of *Tachypleus tridentatus*. The assessment classified the species as endangered throughout its spawning range and at risk of local extinction (IUCN 2019). It is believed that once the RED List assessments for the two other Asian horseshoe crab species have been completed, they will also be listed at risk. Although there is a growing trend throughout Asia to breed and release horseshoe crab larvae into areas of population decline, currently there is no matrix to assess the efficacy of these programs.

Horseshoe crabs are eaten in many South and Southeast Asian countries, with China being the largest consumer and the primary supplier of TAL (Akbar et al. 2018, IUCN 2019, Zion Market Research 2019). The byproducts of these user groups supply secondary markets with chitin, fertilizer and traditional Chinese medicine (Novitsky 2017, pers. comm). The consumption of horseshoe crabs throughout South and Southeast Asia is driven by poverty, livelihood and social status, intertwined with broad historical and cultural trends, linked to economic growth and social stratification (Fabinyi 2011). Without action on the consumption end of the commodity trade, either through public awareness campaigns and or cost that exceeds market viability, coupled with government intervention, it is unlikely the trade in horseshoe crabs will abate. Although there are some indications that human consumption of wildlife in China is declining, consumers with higher income and educational backgrounds are consuming wildlife at a higher rate (Zhang, et al. 2014) and there is no indication the consumption of horseshoe crabs in China has declined (Fu et al. 2019).

Some mangrove species (*Carcinoscorpius rotundicauda)* contain a tetrodotoxin that can be fatal if eaten (Kanchanapongkul et.al 2008, Suleiman et al. 2017). Consequently, harvest of this species for human consumption remains local. It is also small and difficult to harvest in quantity to be of value in the production of amebocyte lysate. Therefore, the largest threat to this species and the primary reason for its decline is the loss of habitat throughout its range (Akbar et al. 2018). South Asia, Southeast Asia and Asia-Pacific contain approximately 46% of the world's mangrove ecosystems, including the most biodiverse mangrove forests. On the other hand, this region also exhibits the highest global rates of mangrove loss and deforestation for alternative land uses (Gandhi et al, 2019).

Currently, there are only a few South and Southeast Asian countries with laws in place to protect their horseshoe crab resource and its habitat, and fewer still who enforce them, which encourages a substantial illegal trade (Akbar et al. 2018, IUCN 2019). The world's four horseshoe crab species are not protected by the Convention on International Trade in Endangered Species (CITES). Consequently, cooperation between countries that import horseshoe crabs from countries that ban and or restrict their export is poorly regulated and enforced (Akbar et al. 2018, IUCN 2019). NGOs with the support of the scientific community alone cannot reverse deeply ingrained social, cultural and corporate indifference to the decline of these species, without a government coordinated conservation and management effort, coupled with a robust law enforcement mechanism within and between countries where horseshoe crabs live and spawn.

TAL producers in China, some of whom are LAL producers in the United States, circumvent the application of sustainable harvesting practices used in the United States for *Limulus,* by implying they are beholden to poorly enforced or nonexistent government harvesting regulations and the pervasive social, cultural and corporate indifference towards the sustainability of the Asian horseshoe crab species (Dubczak 2019). However, it is the ever-increasing distance that harvesters incur to secure the horseshoe crab resource and the need to recoup associated cost that make the bleed-and-release model used in the United States untenable in Asia. This in turn strengthens the symbiotic relationship between the harvesters or importers for human consumption and TAL production. TAL producers rent their horseshoe crabs from harvesters or importers only long enough to drain their hemolymph, before returning them to be butchered for human consumption and secondary markets i.e. chitin, fertilizer and traditional Chinese medicine (Novitsky 2017, pers. comm). These secondary markets ebb and flow with the demand for human consumption and TAL. With Asia being one of the largest and fastest growing healthcare markets (IMS

2017), the demand for TAL is expected to double by 2024 (Zion Market Research 2019), potentially offsetting decreases in human consumption through public awareness campaigns.

Since the Chinese horseshoe crab population, which was once robust, is no longer viable for this scale of enterprise, animals from countries with poorly enforced or nonexistent harvesting and or export laws will continue to be exploited. The report that TAL only represents 20% of the world's BET market (Dubczak 2019), should not be taken as an insignificant number, for the affected species is already Red-Listed as Endangered and on the Verge of Extinction (IUCN 2019). Can an ethical argument to be made for why producers of TAL should continue to exploit this diminishing resources or why the pharmaceutical, medical device and health care providers should continue to use TAL when there are other equally viable BET?

Absence of Clear Ethical Directives

First introduced in 1959 by Russell and Burch the concept of replace, reduce and refine (3Rs) was intended to promote the humane treatment of animals used in scientific procedures (Russell and Burch, 1959). Even though the 3Rs are now widely accepted and practiced as ethical standards in Western societies (Orlans et al, 1998, NHMRC 2013, CCAC Guidelines 2017), the scope and scales of 3Rs still remain to be clarified. For example, the majority of invertebrate species, which include the four extant horseshoe crab species, are excluded from legislation regulating scientific research on animals, with the exception of cephalopods (Berry et al, 2015). However, as was the case for the four suborders of cephalopods, growing global support behind the conservation of the horseshoe crab species, has begun to deepen our understanding of what constitutes pain and suffering in other living beings as a result of human activities. There is a growing animal-free endotoxin detection trend developing in Europe (Council of Europe, 2010). It already manifested in the introduction of the Monocyte Activation Tests (MAT), a whole-pyrogen assay based on human immune cells, as a replacement for the rabbit pyrogen test (RPT) by the Ph.Eur. into chapter 2.6.30 (2010). Regarding BET, Ph.Eur. chapters 5.1.10 (2016b) and 2.6.32 (EDQM 2019) continue in advancing the 3Rs. The former already included rFC assays as alternative BET and the latter will make them compendial. This will eliminate the necessity for additional method validation and thereby present them as fully accepted BET replacements of LAL/TAL (Fig. 3).

In the absence of clear ethical directives, leadership is required. There is only one user group who has the ability to transcend the geographic boundaries, government malaise, linguistics, social, cultural and corporate indifference that hamper the conservation of two of the three Asian horseshoe crab species (*Tachypleus tridentatus, Tachypleus. gigas)* and that is the pharmaceutical, medical device industries and healthcare facilities around the world who are reliant upon TAL for bacterial endotoxin test. Most are unaware that their TAL supply chain will not meet future demands and by their continued use of TAL, are complicit in the decline of the *Tachypleus* species. By implementing a supply chain management policy, that replaces their use of TAL with LAL and augments their endotoxin test requirements with rFC assays where applicable, they can reduce harvesting pressure on the two Asian Tachypleus species, and at the same time reduce transferred harvesting pressure on the American horseshoe crab species (*Limulus polyphemus)* used in the production of LAL, which is also expected to double by 2024 (Zion Market Research 2019).

Conclusion

By excluding the presence of harmful amounts of endotoxin in pharmaceuticals and medical devices, LAL and TAL have helped in guaranteeing patient safety for decades. Established as they are, these animal extracts will likely stay in operation for the foreseeable future. Nonetheless, ample evidence has demonstrated that rFC-based assays fulfill the function of LAL/TAL equivalently, with the added advantage of providing viable animal-free BET. Presently, replacing the LAL/TAL lysate with rFC may require additional validation procedures. However, this relatively minor effort can provide a modern,

reliable analysis method that can also make a significant contribution to the conservation of the horseshoe crab species.

Until there is a viable alternative bait for the American eel (*Anguilla rostrata*) and whelk pot fisheries *(Busycon spp.)* and a migration towards rFC assays or other animal-free bacterial endotoxin tests, the American horseshoe crab *(Limulus polyphemus)* will continue to be managed for the migratory shorebirds, other dependent wildlife and the fishing and non-fishing public, regardless of the bacterial endotoxin test industry's reliance upon this species. The social elitism and distain currently shown towards the horseshoe crab bait fishery by the bacterial endotoxin test producers in the United States need to be reconciled, as both user groups must find ways to work together to share this finite and sustainably managed resource, within a growing marketplace.

In Asia, the three horseshoe crab species (*Tachypleus tridentatus, Tachypleus gigas, Carcinoscorpius rotundicauda*) will continue to decline until there is a coordinated conservation effort on the part of the governments where these animals live and spawn and a change in the social, cultural and corporate indifference to unsustainable harvesting practices. Until then, these animals will keep losing essential habitat and genetic diversity, and be harvested unsustainably for human consumption, the production of TAL and secondary markets. The pharmaceutical, medical device industries and healthcare facilities have the ability to make a significant contribution to the conservation of the Asian horseshoe crab species by eliminating the purchase of TAL from their supply chain. Additionally, they would be wise to make BET supply chain management decisions based upon the collective knowledge of the IUCN, ASMFC and the vast global network of NGO's and scientists around the world who are dedicated to the biology and conservation of these ancient mariners. These organizations are working independent of the marketplace and stakeholders to understand and protect the world's four extant horseshoe crab species and have proven themselves to be honest brokers, versus the often biased opinions of some user groups who harvest and or exploit them.

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References

Akbar B. John · B. R. Nelson · Hassan I. Sheikh · S. G. Cheung · Yusli Wardiatno · Bisnu Prasad Dash · Keiji Tsuchiya · Yumiko Iwasaki · Siddhartha Pati "A review on fisheries and conservation status of Asian horseshoe crabs " Biodiversity and Conservation, Springer Nature B.V. 2018

Armstrong P., Conrad M. (2008). Blood Collection from the American Horseshoe Crab, Limulus Polyphemus. JoVE. 20.

Atlantic States Marine Fisheries Commission (ASMFC) (2019), Horseshoe Crab Benchmark Stock Assessment and Peer Review Report, Washington, DC

Atlantic States Marine Fisheries Commission (ASMFC) (2018), Review of the Atlantic States Marine Fisheries Commission Fisheries Management Plan for Horseshoe Crab, Washington, DC

Atlantic States Marine Fisheries Commission (ASMFC) (2013), Horseshoe Crab Stock Assessment Update, Washington, DC

Atlantic States Marine Fisheries Commission (ASMFC) (2001), Review of the Fishery Management Plan for Horseshoe Crab (Limulus polyphemus), Washington, DC

Atlantic States Marine Fisheries Commission (ASMFC) (1998), Interstate Fishery Management Plan for Horseshoe Crab, Fishery Management Report No 32 of the Atlantic States Marine Fisheries Commission, Washington, DC

Atlantic States Marine Fisheries Commission (ASMFC) (1997), Proceedings of the Atlantic States Marines Fisheries Commission 56th Annual Meeting, American Eel Management Board Meeting, Hershey, Pennsylvania

Baek, L., Høiby, N., Hertz, J.B., Espersen, F. 1985. Interaction between *Limulus* Amoebocyte Lysate and Soluble Antigens from *Pseudomonas aeruginosa* and *Staphylococcus aureus* Studied by Quantitative Immunoelectrophoresis. Journal of Clinical Microbiology. 22(2): 229-237.

Berry, A., Vitale, A., Carere, C., & Alleva, E. (2015). EU guidelines for the care and welfare of an "exceptional invertebrate class" in scientific research. Annali Istituto Superiore di Sanita, 51, 267e269.

Berzofsky, R.N. 1994. Kinetic assay for endotoxin using limulus amebocyte lysate and chromogenic substrate. Patent US5310657. Priority October 30, 1989, Granted May 5, 1994.

Berzofsky, R.N. 2004. Does Endotoxin Testing = FDA Licensing? LAL Review. 1004(1), 1-3.

Bolden, J., Platco, C., Dubczak, J., Cooper, J.F., McCullough, K.Z. (2015). The Use of Endotoxin as an Analyte in Biopharmaceutical Product Hold-Time Studies. Pharmacopeial Forum 41(5), Stimuli to the Revision Process.

Bolden, J. 2019. Recombinant Factor C: Progressive Endotoxin Detection One Year In. 5th Global Endotoxin Testing Summit. June 3, 2019.

Brondyk, W.H. 2009. Chapter 11 Selecting an Appropriate Method for Expressing a Recombinant Protein. Methods in Enzymology. Academic Press. Volume 463, 131-147, https://doi.org/10.1016/S0076-6879(09)63011-1.

Brunson, K.W., Watson, D.W. 1976. *Limulus* amebocyte lysate reaction with streptococcal pyrogenic exotoxin. Infection and Immunity 14(5): 1256-1258.

Burgenson, A. 2019. Another Perspective on rFC. PDA Letter. https://www.pda.org/pda-letter-portal/home/full-article/another-perspective-on-rfc. August 05, 2019. Accessed on August 30, 2019.

Canadian Council on Animal Care (2017). CCAC guidelines: Husbandry of animals in science.Ottawa, Ontario, Canada

Chen, L., Mozier, N. 2013. Comparison of *Limulus* amebocyte lysate test methods for endotoxin measurement in protein solutions. Journal of Pharmaceutical and Biomedical Analysis 80: 180– 185.

Cohen SN. 2013. DNA cloning: a personal view after 40 years. Proc Natl Acad Sci U S A. 2013 Sep 24;110(39):15521-9. doi: 10.1073/pnas.1313397110. Epub 2013 Sep 16.

Cohen SN, Chang ACY, Boyer HW, Helling RB. Construction of biologically functional bacterial plasmids in vitro. Proc Natl Acad Sci USA. 1973;70(11):3240–3244.

Cooper, JF, Levin, J, and Wagner, HN Jr. 1971. Quantitative comparison of in vitro and in vivo methods for the detection of endotoxin. J Lab Clin Med 78(1):138.

Council of Europe. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L 276, 20 October 2010.

Ding JL, Chai C, Pui AWM, et al. Expression of full length and deletion homologues of *Carcinoscorpius rotundicauda* Factor C in *Saccharomyces cerevisiae*: immunoreactivity and endotoxin binding. J Endotoxin Res 1997; 4: 33–43.

Ding, J.L., Ho, B. 2001 A new era in pyrogen testing. Trends Biotechnol. 19(8) 277–281.

Ding, J.L., Ho, B. 2003 Assays For Endotoxin. US 6645724 B1. Application April 7, 1999. Publication November 11, 2003. National University Of Singapore.

Ding, J.L., and Ho, B. 2010. Endotoxin detection - from Limulus amebocyte lysate to recombinant factor C. Subcell Biochem 53, 187-208.

Dubczak, J. 2018. Standing Guard. PDA Letter, September 26, 2018. https://www.pda.org/pda-letter-portal/archives/full-article/standing-guard. Accessed on August 30.

Dubczak,J. 2019, Expert View: Charles River, Conservation initiatives for an invaluable partner. europeanpharmaceuticalreview.com

Dubczak. J. 2019, Blue-blooded crabs at heart of pharma dilute on drug testing, Reuters Health News, Zurich

European Pharmacopoeia 6.7. 2010. 2.6.30. Monocyte activation test, EDQM Council of Europe, Strasbourg, France.

European Pharmacopoeia 8.8. 2016. 2.6.14. Bacterial endotoxins, EDQM Council of Europe, Strasbourg, France. pp. 194-198.

European Pharmacopoeia 8.8. 2016. 5.1.10. Guidelines for using the test for bacterial endotoxins, EDQM Council of Europe, Strasbourg, France. pp. 5931-5934.

EDQM Council of Europe. 2019. New general chapter on test for bacterial endotoxins using recombinant factor C. Give your feedback. https://www.edqm.eu/en/news/new-general-chapter-test-bacterial-endotoxins-using-recombinant-factor-c-give-your-feedback January 09, 2019. Accessed on August 28, 2019.

Eli Lilly and Company. 2018. United Nations Global Compact Communication on Progress Report. https://assets.ctfassets.net/hadumfdtzsru/3zPl8biFFz7pvlsjTzP9OQ/ea26dbece0a590ba58f8d7bcbb1f80f9/2018_United_Nations_Global_Compact_Communication_on_Progress_Report.pdf. Lilly Corporate Center, Indianapolis, IN (USA).

Elin, R.J. and Wolff, S.M. (1973) Nonspecificity of the *Limulus* amoebocyte lysate test: positive reactions with polynucleotides and proteins. The Journal of Infectious Diseases. 128(3): 349–352.

Federal Register. 1973. Status of Biological Substances Used for Detecting Bacterial Endotoxins. 38(8), January 12, 1973, 1404.

Federal Register. 1977. Licensing of Limulus Amebocyte Lysate, Use as an Alternative for Rabbit Pyrogen Test" 42(213), November 4, 1977, 57749.

IMS Institute for Healthcare Informatics (2017); IMS Global Use of Medicines, IMS Institute for Healthcare Informatics, Norwalk

Fabinyi, M. (2011). Historical, cultural and social perspectives on luxury seafood consumption in China. Environmental Conservation, 39, 83–92.

Fu, Y., Huang, S., Wu, Z., Wang, C. C., Su, M., Wang, X., ... & Wang, J. (2019). Socio-demographic drivers and public perceptions of consumption and conservation of Asian horseshoe crabs in northern Beibu Gulf, China. Aquatic Conservation: Marine and Freshwater Ecosystems 29(8), 1268-1277

Gauvry, G. 2015. Current horseshoe crab harvesting practices cannot support global demand for TAL/LAL: The pharmaceutical and medical device industries' role in the sustainability of horseshoe crabs. In: Carmichael, R.H., Botton, M.L., Shin, P.K.S., and Cheung, S.G. (eds), Changing Global Perspectives on Horseshoe Crab Biology, Conservation and Management, pp. 475−482. Springer International Publishing, Switzerland.

Goeddel DV, Kleid DG, Bolivar F, Heyneker HL, Yansura DG, Crea R, Hirose T, Kraszewski A, Itakura K, Riggs AD. 1979. Expression in Escherichia coli of chemically synthesized genes for human insulin. Proc Natl Acad Sci U S A. 1979 Jan;76(1):106-10.

Gorbet, M.B., Sefton, M.V. 2005. Endotoxin: The uninvited guest. Biomaterials, 266811–6817.

Grallert, H., Leopoldseder, S., Schuett, M., Kurze, P., Buchberger, B. 2011. EndoLISA: a novel and reliable method for endotoxin detection. Nature Methods, Application Notes. October 2011, iii-v

Hartung, T. 2015 The Human Whole Blood Pyrogen Test – Lessons Learned in Twenty Years. ALTEX 32(2), 79-100.

Hughes, S.S. 2001. Making dollars out of DNA. The first major patent in biotechnology and the commercialization of molecular biology, 1974-1980. Isis. 2001 Sep. 92(3) 541-75.

Hughes, P.F., Candau-Chacon, R., Thomas, C., Gomez-Broughton, C., Suvarna, K., Rani Narasimhan, L., Chi, B. 2015, Low Endotoxin Recovery: An FDA Perspective. BioPharma Asia March/April 2015, 14-25.

Hurley, J. C., Nowak, P., Ohrmalm, L., Gogos, C., Armaganidis, A., Giamarellos-Bourboulis, E.J. 2015. Endotoxemia as a Diagnostic Tool for Patients with Suspected Bacteremia Caused by Gram-Negative Organisms: a Meta-Analysis of 4 Decades of studies. J. Clin. Micro. 53(4): 1183-1191.

IUCN. 2019. The IUCN Red List of Threatened Species. Version 2019-1. Available at: www.iucnredlist.org. (Accessed: 21 March 2019).

Iwanaga, S., Lee, B.L. 2005. Recent advances in the innate immunity of invertebrate animals. J Biochem Mol Biol. 2005 Mar 31;38(2):128-50.

The Japanese Pharmacopoeia 4.01 Bacterial Endotoxins Test, Seventeenth Edition (JP17), The Ministry of Health, Labour and Welfare, Pharmaceuticals and Medical Devices Agency,Tokyo, Japan, 2016, pp. 110-114.

Kanchanapongkul J. Tetrodotoxin poisoning following ingestion of the toxic eggs of the horseshoe crab Carcinoscorpius rotundi- cauda, a case series from 1994 through 2006. Southeast Asian J Trop Med Public Health 2008; 39: 303-6.

Kikuchi, Y., Haishima, Y., Fukui, C., Murai, T., Nakagawa, Y., Ebisawa, A., Matsumura, K., Ouchi, K. , Oda, T., Mukai, M., Masuda, T., Katto, Y., Takasuga, Y., Takaoka, A. 2017. Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides," Pharmaceutical and Medical Device Regulatory Science, Bd. 48, Nr. 4, pp. 252-260.

Kikuchi, Y., Haishima, Y., Fukui, C., Nakagawa, Y., Ebisawa, A., Morioka, T., Matsumura, K., Ouchi, K. , Uchida, K., Martinez, O., Oda, T., Mukai, M., Masuda, T., Tsukihashi, Y., Takasuga, Y., Takaoka, A. 2018 „Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides, Part 2," Pharmaceutical and Medical Device Regulatory Science, Nr. 10, pp. 708-719.

Kurlansky M. 1997. Cod: A Biography of the Fish that Changed the World.
New York: Penguin Press.

Kwan, B. K. Y., Cheung, J. H. Y., Law, A. C. K., Cheung, S. G., & Shin, P.K.S. (2017). Conservation education program for threatened Asian horseshoe crabs: A step towards reducing community apathy to environmental conservation. Journal for Nature Conservation, 35, 53–65

Liebsch, M. 1995. Die Geschichte der Validierung des LAL-Tests. Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch, Berlin (Germany). ALTEX 12(2), 76-80.

Lonza. 2018. FDA Approves First Drug Using the Recombinant Factor C Assay for Endotoxin Testing. News Release. Walkersville, MD (USA) / Basel (CH). November 8, 2018. http://e3.marco.ch/publish/lonza/551_871/181108_Product-News_PyroGene-Eli_Lilly_FINAL_FINAL.pdf

Loverock, B., Simon, B., Burgenson, A., Baines, Al. 2009. A Recombinant Factor C Procedure for the Detection of Gram-negative Bacterial Endotoxin. The United States Pharmacopeial Convention, Stimuli to the Revision Process, Pharmacopeial Forum, 35(6), 1613-1621.

Mascoli, CC., Weary, ME. 1979. Applications and advantages of the Limulus amebocyte lysate (LAL) pyrogen test for parenteral injectable products. Progress in Clinical and Biological Research. 29, 387–402.

McCullough, KZ., Weidner-Loeven, C. 1992. Variability in the LAL test: comparison of three kinetic methods for the testing of pharmaceutical products. J Parenter Sci Technol. May-Jun 46(3), 69-72.

McKenzie, J.H., Alwis, K.U., Sordillo, J.E., Kalluri, K.S., Milton, D.K. 2011. Evaluation of lot-to-lot repeatability and effect of assay media choice in the recombinant Factor C assay. J Environ Monit. 2011 Jun;13(6):1739-45. doi: 10.1039/c1em10035a. Epub 2011 May 9.

Mikami, T., Nagase, T., Matsumoto, T., Suzuki, S., Suzuki, M. 1982 Gelation of *Limulus* Amoebocyte Lysate by Simple Polysaccharides. Microbiol. Immunol. 26(5): 403-409.

Mizumura, H., Ogura, N., Aketagawa, J., Aizawa, M., Kobayashi, Y., Kawabata, S. I., & Oda, T. 2017. Genetic engineering approach to develop next-generation reagents for endotoxin quantification. Innate immunity, 23(2), 136–146. doi:10.1177/1753425916681074.

Mogensen, T.H. 2009. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. Clin Microbiol Rev. 2009 Apr; 22(2): 240–273. doi: 10.1128/CMR.00046-08.

Muta T, Miyata T, Misumi Y, Tokunaga F, Nakamura T, Toh Y, Ikehara Y, Iwanaga S. 1991. Limulus factor C. An endotoxin-sensitive serine protease zymogen with a mosaic structure of complement-like, epidermal growth factor-like, and lectin-like domains. J Biol Chem. 1991 Apr 5;266(10):6554-61.

Muta T, Seki N, Takaki Y, et al. Purified horseshoe crab factor G. 1995. Reconstitution and characterization of the (1,3)-β-D-glucan-sensitive serine protease cascade. J Biol Chem 1995; 270: 892–897.

Nakamura, T. , Morita, T. and Iwanaga, S. 1986. Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in Limulus hemocytes. European Journal of Biochemistry, 154: 511-521. doi:10.1111/j.1432-1033.1986.tb09427.x

National Health and Medical Research Council, Australian Research Council, Australian code for the care and use of animals for scientific purposes, 8th edition, 2013

van Noordwijk, J., de Jong, Y. 1976. Comparison of the Limulus test for endotoxin with the rabbit test for pyrogens of the European Pharmacopoeia. Journal of Biological Standardization. 4(2), 131-139.

Novitsky 2017, personal communication

Orlans, F.B., Beauchamp, T.M., Dresser, R., Morton, D.B., Gluck, J.P. (1998). The Human Use of Animals: Case Studies in Ethical Choice. Oxford: Oxford University Press

Parenteral Drug Association. 2019. Technical Report No. 82. Low Endotoxin Recovery. Bethesda, MD (USA).

Pearson, F.C, Bohon, J., Lee, W., Bruszer, G., Sagona, M., Jakubowski, G., Dawe, R., Morrison, D., Dinarello, C. (1984) Characterization of *Limulus* Amoebocyte Lysate-Reactive Material from Hollow-Fiber Dialyzers. Appl. Environ.Microbiol. 48, 1189–1196.

Platica, M., Harding, W., Hollander, V.P. 1978. Dithiols simulate endotoxin in the *Limulus* reaction. Experientia 34(9): 1154-1155.

Pui AWM, Ho B, Ding JL. Yeast recombinant Factor C from horseshoe crab binds endotoxin and causes bacteriostasis. J Endotoxin Res 1997; 4: 391–400.

Raetz, C.R., Whitfield, C. 2002. Lipopolysaccharide endotoxins. Annu Rev Biochem 71:635–700

Roopashree SD, Ho B, Ding JL. Expression of *Carcinoscorpius rotundicauda* Factor C in *Pichia pastoris*. Mol Mar Biol Biotechnol 1996; 5: 334–343.

Roopashree SD, Ho B, Ding JL. Recombinant COS-1 cells express *Carcinoscorpius rotundicauda* Factor C. Biotech Lett 1997; 19: 357–361.

Roslansky P. F., Novitsky, T. J. 1991. Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans.," Journal of Clinical Microbiology, Bd. 29, Nr. 11, pp. 2477-2483.

Rudbach,J.A., F.I.Akiya, R.J.Elin, H.D.Hoch-stein, M.K.Luoma, E.C.B.Milner, K.C.Milner, and K.R.Thomas. 1976. Preparation and Properties of a National Reference Endotoxin.J.Clin.Microbiol.3:21-25.

Russell, W.M.S., Burch, R. L. (1959). Principles of Humane Experimental Technique, London: Methuen & Co.

Seibert, F. B. The cause of many febrile reactions following intravenous injections. Am. J. Physiol. 1923, 71 (3), 621– 651.

Shuster, C.N., Jr. “The Delaware Bay area - an ideal habitat for horseshoe crabs. Public Service Electric and Gas Company,” Hancocks Bridge, New Jersey. (1996): 26pp. + appendices.

Shuster, C. N. Jr. "Serological correspondences among horseshoe ‘crabs’ (Limulidae)." Zoologica 47, no1 (1962): 1–9.

Smith, D. R., Brockmann, H. J., Beekey, M. A., King, T. L., Millard, M. J., and Zaldívar-Rae, J. A. (2017). Conservation status of the American horseshoe crab, (*Limulus polyphemus*): a regional assessment. *Rev. Fish Biol. Fish.* 27, 135–175.

Smith DR, Newhard JJ. 2019. The long-term effect of bleeding for Limulus amebocyte lysate on annual survival: an analysis of tagging data. Fourth International Workshop on the Science and Conservation of Horseshoe Crabs, Qinzhou City, PR China

Suleiman M, Muhammad J, Jelip J, William T, Chua TH. (2017). an outbreak of tetrodotoxin poisoning from consuming horseshoe crabs in Sabah. Southeast Asian J Trop Med Public Health. 2017 Jan;48(1):197-203.

Twohy, C. W., Duran, A. P., Munson, T. E. 1984. Endotoxin contamination of parenteral drugs and radiopharmaceuticals as determined by the *Limulus* amebocyte lysate method. J.Parenter.Sci.Technol. 38, 190-201.

United States Food and Drug Administration. 2012. Guidance for Industry - Pyrogen and Endotoxins Testing: Questions and Answers,“ U.S. Department of Health and Human Services, Rockville, MD, USA.

United States Food and Drug Administration. 2018. Guidance for Industry - Data Integrity and Compliance With Drug CGMP: Questions and Answers,“ U.S. Department of Health and Human Services, Rockville, MD, USA.

United States Pharmacopeia. 1942. Pyrogen Test, in The United States Pharmacopeia XII, The United States Pharmacopeial Convention, Rockville, MD, USA.

United States Pharmacopeia. 1980. <85> Bacterial Endotoxins Test, in The United States Pharmacopeia XX and The National Formulary XV, The United States Pharmacopeial Convention, Rockville, MD, USA.

United States Pharmacopeia. 2016a. <85> Bacterial Endotoxins Test, in The United States Pharmacopeia 39 and The National Formulary 34, The United States Pharmacopeial Convention, Rockville, MD, USA, pp. 161-166.

United States Pharmacopeia. 2016b. <1225> Validation of Compendial Procedures, in The United States Pharmacopeia 39 and The National Formulary 34, The United States Pharmacopeial Convention, Rockville, MD, USA.

Urbascheck, B., Becker, K.-P., Ditter, B. 1987. Method for determining endotoxin concentrations. Patent US4663298A. Priority October 31, 1984. Granted May 5, 1987.

Wachtel, R.E., Tsuji, K. 1977. Comparison of Limulus Amebocyte Lysates and Correlation with the United States Pharmacopeial Pyrogen Test.

Wildfeuer, A., Heymer, B., Schleifer, K.H., Haferkamp, O. 1974. Investigations on the specificity of the *Limulus* test for the detection of endotoxin. Applied Microbiology 28(5): 867–891.

Williams, K.L. 2007. Limulus Amebocyte Lysate Discovery, Mechanism, and Application. In Endotoxins, Pyrogens, LAL Testing and Depyrogenation, Third Edition. 191-219.

Williams. K.L. 2018. Set-up, performance and alternative validation of recombinant Factor C assays for endotoxin testing. European Pharmaceutical Review 23(3). 53-55.

Williams, K.L. 2019. Response to “Standing Guard”. PDA Letter, February 2019, 17–19.

Wolff, S.M. 1973 Biological effects of bacterial endotoxins in man. J. Infect. Dis. 128:259–264.

Xiamen Bioendo Technology Co., Ltd. 2019. About Us. https://www.lalendotoxin.com/about-us_d1 Accessed on August 29, 2019.

Zald´ıvar-Rae J, Sapie´n-Silva RE, Rosales-Raya M, Brockmann HJ (2009) American horseshoe crabs, Limulus polyphemus, in Me´xico: open possibilities. In: Tanacredi JT, Botton ML, Smith DR (eds) Biology and conservation of horseshoe crabs. Springer, New York, pp 97–113

Zion Market Research (2019), Pyrogen Testing Market, New York, New York

Zhang, L., & Yin, F. (2014). Wildlife consumption and conservation awareness in China: A long way to go. Biodiversity and Conservation, 23, 2371–2381.

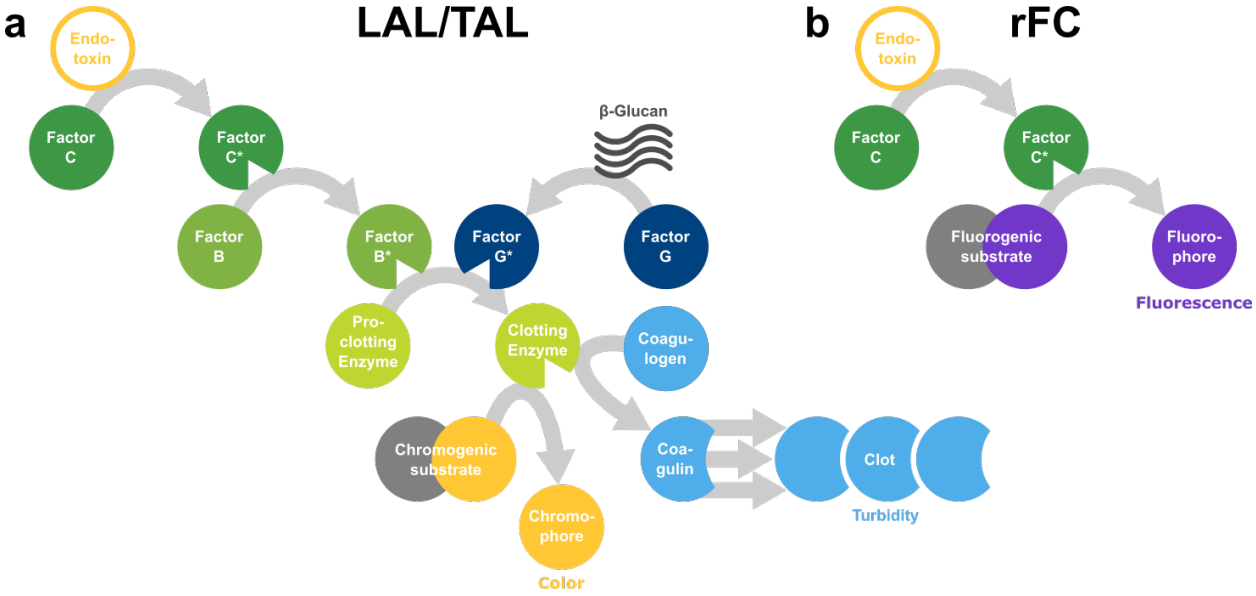

Fig. 1. Enzyme biochemistry in a. LAL/TAL and b. rFC. * denotes an activated enzyme. Color and turbidity formation can be measured using an absorbance reader, fluorescence development using a fluorescence reader.

Fig. 2. Reactor holding cell culture for manufacturing recombinant proteins such as Factor C.

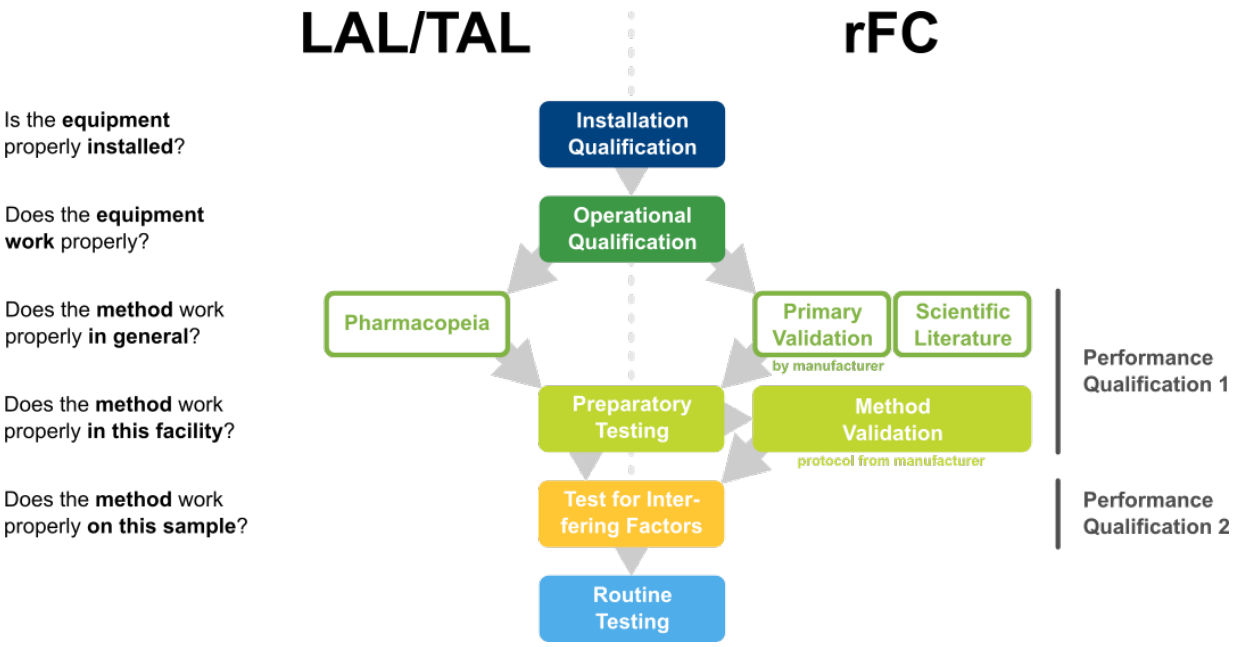

Fig. 3. Comparison of processes for establishing bacterial endotoxin testing in pharmaceuticals or medical devices using either LAL/TAL or rFC. Colored boxes indicate activities that need to be carried out in the premises of the BET user, while white boxes denote documents that are used as references.

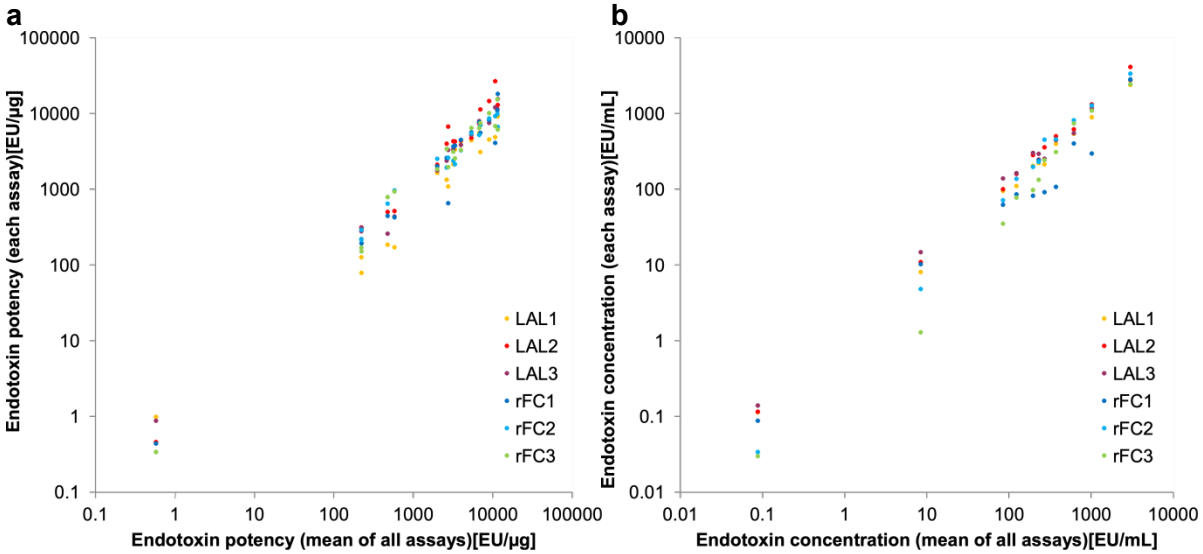

Fig. 4. Comparison of data from collaborative study to compare kinetic-chromogenic LAL tests and rFC-based assays on a. purified lipopolysaccharides from defined bacterial strains and b. crudely purified endotoxin from defined bacterial strains and naturally occurring endotoxin from environmental water samples (adapted from Kikuchi et al. 2017).

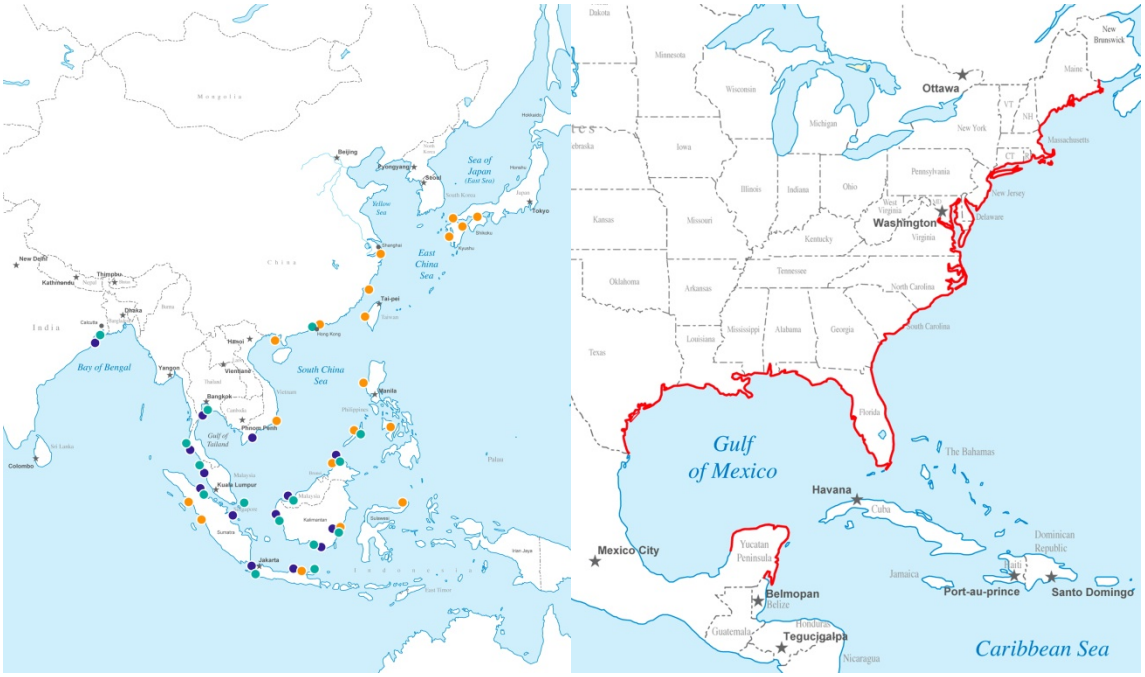

Fig. 5. Geographic ranges of *Carcinoscorpius rotundicauda* (green), *Tachypleus tridentatus* (orange), *Tachypleus gigas* (blue), and *Limulus polyphemus* (red)

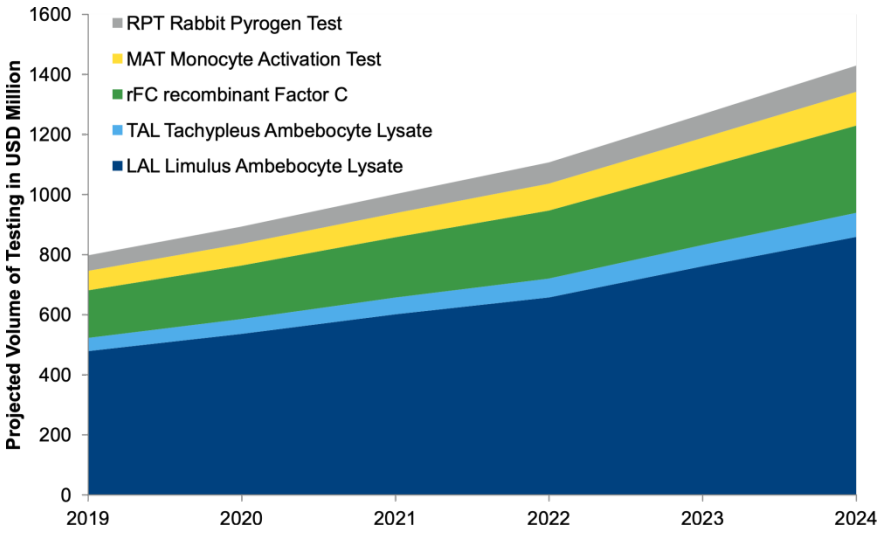

Fig. 6. Projected Volume of Pyrogen and Endotoxin Testing by type in USD Million (adapted from Zion Market Research 2019).