A Demonstration of Patient Safety and the Importance of the Detection of Natural Environmental Endotoxins for Recombinant Cascade Reagents

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1 Introduction

Since the discovery that the clotting of amoebocytes found in Horseshoe crab (*Limulus polyphemus*) blood is primarily driven by bacterial endotoxins[1], Limulus amoebocyte lysate (LAL) has predominately replaced the rabbit pyrogen test (RPT) to become the benchmark pyrogen testing method for the release of parenteral products, medical devices, raw materials, and in-process quality control. Having been recognized as a compendial test method for more than 40 years without a single FDA-confirmed false negative, the efficacy and patient safety associated with LAL testing is without question.

Recently, alternative methods have been developed for use in the bacterial endotoxin test, including recombinant Cascade Reagent's (rCR), products that utilize three recombinant proteins involved directly in the detection of bacterial endotoxins that are naturally found in *Limulus polyphemus*. To confirm the efficacy of rCR in the detection of bacterial endotoxins, a direct comparison between recombinant and natural reagents following Bacterial Endotoxin Test (USP<85>) was performed using 4 different formulations of rCR by testing natural contaminated pharmaceutical water samples and carbohydrates. The aim of the study was to gain a better understanding of natural and recombinant patient safety factors, defined as the ratio of endotoxin recovered by each reagent and the USP endotoxin limit of 0.5 EU/mL. A higher patient safety factor indicates a reagents ability to successfully indicate a positive sample. This study also served to obtain an understanding of how minor formulation differences can affect the recovery of NEEs.

Results show that rCR has a patient safety factor equal to or greater than natural LAL and demonstrate how slight variations in formulation can negatively affect NEE detection while still recovering RSE within BET acceptance criteria.

2 Methods

There were two types of samples included in this study; pharmaceutical water samples that had been pre-treated through deionization or carbon filtration, and food grade carbohydrates. All samples were naturally contaminated, and prior to testing, were heat-treated at 65˚C to arrest any growth of bacteria that may still have been viable. The starting EU/mL concentrations of each sample were confirmed chromogenically after heat-treatment to allow for targeted concentrations during study execution.

All testing was performed at the cGMP certified Charles River Labs facility in Ballina, Ireland. A total of 10 samples were included, 7 pharmaceutical waters and 3 carbohydrates, with water samples at targeted EU/mL concentrations upon dilution of 7.0 EU/mL, 4.0 EU/mL, <1.0 EU/mL, and carbohydrates at a final concentration of 5%. Pyrogenicity was confirmed following compendial pyrogen test methods[2] . To calculate the patient safety factor, the EU/mL result for all pyrogenic samples were divided by the BET threshold dose of 0.5 EU/mL at 10mL / kg.

Reagents used in this study were Endochrome-K™, a natural chromogenic LAL reagent that was used as a control, and 4 formulations of Trillium™, a recombinant cascade reagent. To mitigate any potential interference from (1,3)-β-Glucans, Endochrome-K™ was reconstituted with an endotoxin specific glucan blocking buffer. Formulation variants of Trillium™ employed were changes in recombinant protein concentrations, concentrations of excipients, and changes in formulation components. All testing was performed as close to contemporaneous as possible, with kinetic chromogenic assays being measured on a BioTek ELx808 incubating absorbance reader at 405nm using Charles River Endosafe® Endoscan-V endotoxin measuring software.

3 Results

Recombinant cascade reagent formulations 1 – 3 were formulated using different recombinant protein concentrations and formulation excipient concentrations. Formulation 4 was prepared with an adjusted concentration of detergent. Results show that, for endotoxin recovery, the adjustment of recombinant protein as well as salt concentration has the largest effect on the increase of endotoxin recovery compared to natural LAL and thus an increase in the calculated patient safety factor.

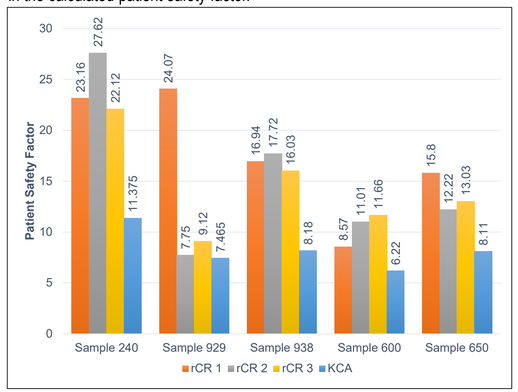

Figure 1. Calculated Patient Safety Factor
Calculated safety factor of each reagent. Only pyrogenic samples are shown

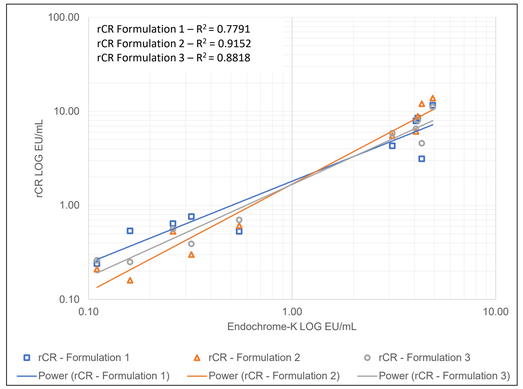

Figure 2. Comparison of rCR Formulations 1, 2, and 3 to Endochrome-K
Linear correlation of the LOG EU/mL results of rCR formulations 1, 2, and 3 compared to Endochrome-K™. Formulation 4 excluded due to its inability to detect NEE.

Table 1. A summary of EU/mL results for naturally contaminated samples. Values shown in parentheses are the ratio of EU/mL of recombinant reagents and Endochrome-K. The ratio of rCR Formulation 4 was calculated using the highest possible value if reported value was less than lambda.

	Detected Endotoxin (EU/mL) / Ratio of rCR to Endochrome-K				
Sample	**Endochrome-K**	**rCR (#1)**	**rCR (#2)**	**rCR (#3)**	**rCR (#4)**
Sucrose - 1	0.11	0.24 / (218%)	0.21 / (191%)	0.26 / (236%)	0.0068 / (4.5%)
Lactose	0.16	0.54 / (335%)	0.16 / (156%)	0.25 / (156%)	<0.005 / (3.1%)
Sample 706	0.26	0.64 / (246%)	0.53 / (204%)	0.56 / (215%)	<0.005 / (1.9%)
Sample 698	0.32	0.76 / (238%)	0.3 / (94%)	0.39 / (122%)	<0.05 / (1.6%)
Sucrose - 2	0.55	0.53 / (96%)	0.61 / (111%)	0.7 / (127%)	<0.05 / (1.2%)
Sample 600	3.11	4.29 / (138%)	5.51 / (177%)	5.83 / (187%)	0.06 / (8.3%)
Sample 929	4.06	7.9 / (195%)	6.11 / (150%)	6.52 / (161%)	0.26 / (1.2%)
Sample 650	4.15	8.47 / (204%)	8.86 / (213%)	8.02 / (193%)	<0.05 / (1.5%)
Sample 938	4.34	3.12 / (72%)	12.04 / (277%)	4.56 / (105%)	<0.005 / (1.2%)
Sample 240	4.91	11.58 / (236%)	13.81 / (281%)	11.06 / (225%)	<0.005 / (1.0%)

4 Discussion

This study shows the importance of focusing on endotoxins found in naturally contaminated products, in addition to purified reference standard endotoxins, when developing the formulation of recombinant reagents. All reagents used were able to detect RSE used in a standard curve within BET guidelines, however rCR formulation 4 was unable to detect NEE (Table 1). The main variations in formulation used in this study were based on altering the ratios of recombinant proteins, the increase of salt concentrations, and a change in detergents. A higher risk of false negatives was observed with formulation 4, indicating that designing a formula solely based on purified endotoxin could lead to a higher probability of patent safety risk.

Figure 2 shows the linear relationship between rCR formulations 1, 2, and 3 compared to Endochrome-K. The formulations with the strongest linear regression was formulation 2 and 3, with an R^2 value of 0.9153 and 0.8818 respectively. Formulation 1 had the weakest linear correlation with an R^2 value of 0.7791, which can also be seen in its over-prediction of NEE samples (Table 1). Hence, the formulations 2 and 3 were better candidates for further development of recombinant reagents.

Further, Figure 1 shows that for all samples tested in this study, rCR has a patient safety factor higher than that of natural LAL. This overprediction provided more assurance when developing an alternative recombinant detection method for endotoxins. The use of the patient safety factor was important to demonstrate that recombinant reagents can be utilized by the users for product testing and release in their environment with the same confidence as LAL reagents.

5 Conclusion

These results demonstrate that recombinant cascade reagents can detect endotoxins in naturally contaminated samples with efficacy comparable to natural LAL reagents. Furthermore, the findings underscore the necessity of formulating recombinant reagents to be both sensitive to RSE and reactive to NEE, ensuring patient safety in parenteral drug product testing using alternative methods.

References
1) Levin, J. and Bang, F.B. "Clottable protein in Limulus: Its Localization and Kinetics of Its Coagulation by Endotoxin." Thromb. Diath. Haemorrh., 19, p.186 (1968).
2) United States Pharmacopoeia "Pyrogen Test" <151>