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Enhancing the Accuracy of Semi-quantitative Endotoxin Evaluation in Biopharmaceuticals and Vaccines R&D

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ABSTRACT

Introduction: The detection of endotoxins is crucial in the research and development of new drugs and vaccines, as it ensures the safety of these products. The quantitative Limulus Amebocyte Lysate (LAL) endotoxin tests provide sensitive and accurate results. Quantitative endotoxin tests may be substituted by LAL gel clot test with enough narrow dilution range as a semi-quantitative method. However, the accuracy and reliability of the assay can be affected by the dilution factor used. **Methods:** The endotoxin concentration of different samples of a bench top purification process of recombinant streptokinase, including inclusion body, washed inclusion body, semi-purified and purified streptokinase was determined by semi-quantitative LAL gel clot and quantitative LAL chromogenic test and the effects of narrow-downing the dilution range of the samples on the accuracy of the results was evaluated. **Results:** The statistical analysis revealed that performing duplicate LAL gel clot tests and consecutively narrowing the dilution range of the sample until at least a positive and a negative results were seen, offers a good estimation of the endotoxin concentration. The relative errors of these results were less than 12%, compared to accurate results of quantitative methods. However, conducting gel clot test at the wide dilution range for the inclusion body samples resulted in approximately 200% overestimation. **Conclusion:** the results suggest that the semi-quantitative LAL gel clot test with a narrow dilution range can be a valuable tool for relatively accurate estimation of endotoxin in biopharmaceutical products including vaccines.

INTRODUCTION

Endotoxin is the lipopolysaccharide of the outer membrane of Gram-negative bacteria and one of the most significant potential contaminants of medicinal products. The introduction of this biomolecule into the bloodstream can induce threatening inflammatory reactions that cause fever, shock, deleterious multi-organ failures, and even death [1]. Considering the severe side-effects mentioned above, the bacterial endotoxin test (BET) is one of the most crucial safety tests for research, development, and production of parenteral vaccines, injectable pharmaceuticals, and medical devices that come in contact with cerebrospinal fluid or cardiovascular system.

Various assays have been developed for the qualitative and quantitative determination of endotoxin. The first *in-vitro* BET was approved by the Federal Food and Drug Administration (FDA) in the 1970s using the Limulus amebocyte lysate (LAL) gel clot method [2, 3]. The LAL gel clot test is based on coagulation of the horseshoe crab hemolymph, exposed to bacterial endotoxin [2, 4]. In this qualitative test, the lysate has been formulated with pre-defined sensitivities and the formation of the gel after one-hour incubation of the sample with lysate indicates the presence of endotoxin at concentrations higher than the labeled sensitivity. Commercial gel clot LAL kits are available at various sensitivity levels of 0.03, 0.06, 1.25, or 0.25 EU/ml [5]. The quantitative BET tests, including the turbidimetric, chromogenic, and fluorometric tests in kinetic and endpoint modes were later developed based on natural or recombinant forms of Fc for initiating this LAL enzymatic cascade [6, 7].

All officially-approved LAL-based assays have almost the same efficiency and some validation aspects like maximum valid dilution (MVD) and recovery are crucial for their correct performance. However, they differ according to their measuring limits and sensitivities. The choice of appropriate LAL assays, especially during the research and development stages of new biopharmaceuticals and vaccines, depends on several factors, including the type and the cost of samples, availability of required equipment, and cost of experiments. Since there are no single test commercial kits for LAL quantitative assays and considering the limited storage time of reconstituted kit reagents, carefully managing the number of required tests and samples is crucial for balancing the cost of experiments. Usually, endpoint chromogenic LAL kits are preferred due to the availability of simple microplate readers. However, if only a few tests are

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required, outsourcing is recommended. It is possible to use qualitative LAL gel clot assay in a semi-quantitative mode by diluting the positive sample to obtain a more narrow range of positive and negative endpoint results. Then, the endotoxin concentration of the sample can be roughly determined by multiplying the dilution rate and the test sensitivity [8].
Here, the application of the gel clot LAL method in a semi-quantitative manner for estimation of the endotoxin concentration of different samples during recombinant proteins benchtop downstream processes is evaluated and the compatibility and benefits of this method in comparison to the LAL chromogenic assay are demonstrated.

MATERIAL AND METHODS

Samples

The samples were taken from different stages of typical benchtop downstream processes of recombinant protein streptokinase as a model of intracellular insoluble proteins expressed in Gram-negative bacterial hosts. The inclusion bodies (IBs) of streptokinase (SK) were obtained by cell disrupting of fermented *Escherichia coli* W3110 (ATCC 27325), containing a recombinant plasmid for expression of streptokinase. The harvested cells were suspended in a 1:4 w/v ratio in 20 mM Tris-HCl buffer pH 7.2, containing 1 mM EDTA [9]. The cells were disrupted using an ultrasonic system (MISONIX, USA). Following centrifugation at 9000 rpm for 20 min, the pellets of inclusion bodies were separated and suspended in 1:9 w/v ratio in 20 mM Tris buffer pH 7.2 (sample name: SK-IBs).

The inclusion body pellets were washed three times with Tris buffer containing different additives: the first wash contained 1 mM EDTA and 1% Triton X-100, the second wash contained 1 mM EDTA and 1 M urea, and the third wash contained 1 mM EDTA alone. The protein was solubilized by 4 M urea, and was refolded by eliminating the 4 M urea, using a gel-filtration chromatography with Sephadex G-25 resin (GE Healthcare, Sweden), packed in a PD10 column. The target protein was further purified using anion exchange chromatography in a gravity flow mode with diethylaminoethanol (DEAE) Sepharose Fast Flow resin (GE Healthcare, Life Sciences, Sweden), then hydrophobic interaction chromatography using Fractogel TSK-Butyl 650 S (Tosoh Bioscience, Japan), and finally a PD10 gel filtration column for buffer exchanging. The semi-purified streptokinase sample (named SP-SK) was taken from at the end of this step. To further purify and eliminate endotoxin, the protein solution was applied to a DEAE Sepharose Fast Flow column and was then incubated with 0.1% sodium deoxycholate for 1 h and was eluted from column by 0.25 M NaCl[9]. The purified streptokinase sample was buffer-exchanged using gel filtration chromatography (sample named purified SK).

Endotoxin Determination using the LAL Gel Clot Assay

A 0.25 EU/ml sensitive (λ) LAL gel clot kit (Bioendo, China) was used to determine the endotoxin concentration of the samples. The labeled sensitivity of the LAL gel clot kit was evaluated and confirmed by making duplicate serial dilutions of Control Standard Endotoxin (CSE, Bioendo, China) using LAL reagent water (LRW, Lonza, USA), resulting in final endotoxin concentrations of 0.5, 0.25, and 0.125 EU/ml. The prepared control standards (200 µl) were added to single tests lysate vials and incubated for 1 h at 37 ± 1 °C. A result was reported positive if a firm gel had formed that remained in place upon inversion.

The last positive result of the serially-diluted control standard was considered as endpoint result and the logarithmic mean of endpoint concentrations was calculated. The labeled sensitivity (λ) was confirmed if the antilogarithm of the mean value was within the open range of 0.5λ and 2λ [10].

To determine the appropriate dilution fold of samples, samples were diluted with LRW in 3 serial dilutions according to the suggested values in Table 1. The primary dilution folds for the inclusion body and the washed inclusion body were chosen based on the expected endotoxin concentrations of the inclusion bodies of any recombinant protein. For example, the endotoxin contamination of the inclusion bodies of a fusion antimicrobial peptide expressed in *E. coli* was reported to be between 1×10^{3} - 8×10^{5} EU/ml [11]. To establish the endotoxin limit of purified and semi-purified streptokinase samples, the maximum valid dilution (MVD) was considered as end and almost middle points of the suggesting dilution ranges, respectively. The maximum valid dilution for endotoxin detection was calculated according to the following equation [10]:

$$MVD = (\text{endotoxin limit} \times \text{concentration of the sample})/\lambda$$

Where λ is the labeled sensitivity of the kit and the endotoxin limit of streptokinase injection was 0.02 EU/ml per 100 IU/ml of streptokinase [12]. The maximum biological activity of streptokinase sample was 750’000 IU/ml, thus the Streptokinase MVD was determined as 0.02 EU/ml per 100 IU/ml of streptokinase×750’000 IU/ml/ 0.25 EU/ml= 600.

Table 1. Suggested dilution folds for estimation of endotoxin concentration of under-study samples.

Sample type	Sample name	Sample dilutions		
Inclusion bodies of streptokinase	SK-IBs	$1:10^{5}$	$1:10^{6}$	$1:10^{7}$
Washed inclusion bodies of streptokinase	SK-WIBs	$1:10^{5}$	$1:10^{6}$	$1:10^{7}$
Semi-purified streptokinase	SK-SP	1:10	1:600	1:1000
Purified streptokinase	SK-P	--	1:100	1:600

Two repeats of each suggested dilution were prepared using LRW. According to the endpoint results of the test, the ranges between two corresponding dilutions with positive and negative results were further narrowed and the test was repeated with the new dilution range. The endotoxin concentration of each sample was calculated according to the following equation [10]:

$$\text{Endotoxin concentration} = \frac{\text{sensitive } (\lambda)}{\exp\left(\frac{\sum_{k=1}^{n} Log\left(\frac{1}{\text{dilution factor}}\right)}{n}\right)}$$

Endotoxin Determination using LAL Endpoint Chromogenic Assay

To determine the concentration of endotoxin in each sample, an endpoint LAL chromogenic kit (QCL-1000, Lonza, USA) was used. A standard curve was obtained by plotting the

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absorbance of serial dilutions (0.1, 0.25, 0.5, and 1 EU/ml) of a control standard endotoxin, provided in the kit [13].

Statistical Analysis

To compare the results of quantitative (LAL endpoint chromogenic) and semi-quantitative (LAL gel clot) tests, paired T-test with a 95% confidence interval was conducted for the overall data, using SPSS 16 software (SPSS Inc., USA).

RESULTS

LAL Gel Clot Assay

A bench-top purification batch of recombinant streptokinase was performed and duplicate samples of inclusion bodies (SK-IBs), washed inclusion bodies (SK-WIBs), semi-purified (SK-SP), and purified streptokinase (SK-P) were taken for quantifying the endotoxin concentrations. Each sample was firstly diluted, according to the first-guess dilution folds of Table 1 and duplicate LAL gel colt assay was performed for each sample. The gel formation of each repeat of tests, is represented by "+" and negative results is shown by "-". The interval range (positive and negative results) of two serial dilution of each sample was further narrowed until a "+/-" result was detected for the test repeats of diluted samples or until the range was narrowed after 3 or 4 runs. The diluted samples of the last run of the gel clot assay in which the gel formation was observed, were considered as endpoints. Table 2 shows the consecutive gel clot runs performed to reach enough narrow dilution ranges for each sample.

Table 2. The results of LAL gel clot assay for consequence guesses of dilution ranges of samples.

Dilution/Sample												
Run	**(X)***	**SK-IB$_1$**	**SK-IB$_2$**	**(X)**	**SK-WIB$_1$**	**SK-WIB$_2$**	**(X)**	**SK-SP$_1$**	**SK-SP$_2$**	**(X)**	**SK-P$_1$**	**SK-P$_2$**
	10^5	++	++	10^5	+-	--	10	++	++	10	++	++
1st	10^6	++	++	10^6	--	--	600	++	++	100	++	++
	10^7	--	--	10^7	--	--	1000	--	--	600	--	--
2nd	5×10^6	--	--	8×10^4	++	++	700	+-	--	20	++	++
				1.2×10^5	--		800	--	--	40	++	++
							900	--	--	60	++	++
										80	--	+-
3rd	2×10^6	--	--									
	3×10^6	--	--									
	4×10^6	--	--									
4th	1.2×10^6	+-	++									
	1.4×10^6	--	--									
	1.6×10^6	--	--									
	1.8×10^6	--	--									

*: (X) is the dilution factor. For example, when a sample was diluted 1:1200000, (X) would be 1.2×10^6.

For estimating the endotoxin concentration of each sample, it was assumed that the real endotoxin concentration would be in a range between the lowest concentration of each sample that still produced a positive result (last positive results among all estimations of each sample) and the highest concentration of last run which did not produce a positive result (first negative result of last gel clot run of each sample). The dilution range corresponding to the last positive results and first negative result of last gel clot run of each sample are shown in Table 3. The coefficients of variation (%CV) of the estimated results were approximately ≤ 10%, indicating that the test and estimation approach were highly repeatable.

Table 3. Endotoxin concentration of samples estimated by endpoint gel clot assay.

Sample	Repeats	X of Positive Result *	X of Negative Result **	Log (1/X)	Mean	Antilog of Mean	Endotoxin Concentration (Eu/ml)	Average Endotoxin Concentration (EU/ml)	%CV
SK-IB$_1$	R1	10^6	1.2×10^6	-6	-6.0396	9.129×10^{-7}	273861.3	286930.65	6.44
	R2	1.2×10^6	1.4×10^6	-6.0792					
SK-IB$_2$	R1	1.2×10^6	1.4×10^6	-6.0792	-6.0792	8.333×10^{-7}	300000		
	R2	1.2×10^6	1.4×10^6	-6.0792					
SK-WIB$_1$	R1	10^5	1.2×10^5	-5	-4.9515	1.118×10^{-5}	22360.7	21180.35	7.88
	R2	8×10^4	1.0×10^5	-4.9031					
SK-WIB$_2$	R1	8×10^4	1.0×10^5	-4.9031	-4.9031	1.25×10^{-5}	20000		
	R2	8×10^4	1.0×10^5	-4.9031					
SK-SP$_1$	R1	700	800	-2.8451	-2.8116	1.543×10^{-3}	162.0	156.01	5.45
	R2	600	700	-2.7782					
SK-SP$_2$	R1	600	700	-2.7782	-2.7782	1.667×10^{-3}	150.0		
	R2	600	700	-2.7782					
SK-P$_1$	R1	60	80	-1.7782	-1.7782	1.667×10^{-2}	15	16.15	10.07
	R2	60	80	-1.7782					
SK-P$_2$	R1	80	100	-1.9031	-1.8406	1.443×10^{-3}	17.3		
	R2	60	80	-1.7782					

*: Last positive results among all estimations
**: First negative result of last estimation

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LAL Endpoint Chromogenic Assay

The LAL endpoint chromogenic kit with a detection range of 0.1 and 1 EU/ ml was applied to determine the endotoxin concentration of samples more accurately and to estimate the relative error encountered by using the qualitative gel clot method as a semi-quantitative method. The control standard endotoxin was serially diluted with LAL reagent water to final concentrations of 1, 0.5, 0.25 and 0.125 EU/ml and a triple point standard curve with the equation of OD - OD blank= 1.2042 x+ 0.0488, R2: 0.9972 was obtained (Fig. 1). The LAL reagent water was used as a blank and OD blank was 0.074. The diluted samples corresponding to the endpoint results of the gel clot method were applied for performing chromogenic test.

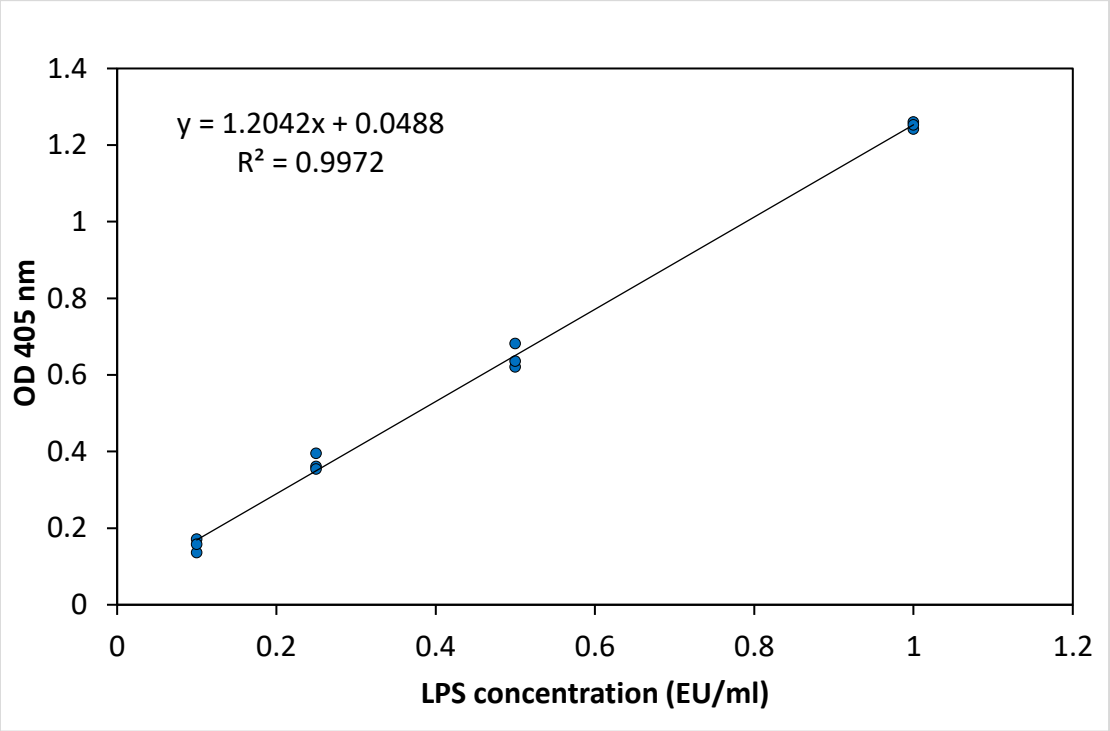

Fig. 1. The standard curve of endpoint chromogenic LAL test.

The endotoxin concentration of each sample was calculated my multiplying the concentration of the diluted sample by the dilution fold. The accurate endotoxin concentration of samples obtained by chromogenic test is shown in Table 4.

Table 4. Endotoxin concentration determined by LAL endpoint chromogenic assay.

Sample	OD_{sample}	OD_{405}*	Endotoxin Concentration in Diluted Sample (EU/ml)	Dilution Factor	Endotoxin Concentration (EU/ml)	Average Endotoxin Concentration (EU/ml)	%CV
SK-IB_1	0.441	0.367	0.264242	1.0×10^6	264242.0	266251.6	1.07
SK-IB_2	0.392	0.318	0.223551	1.2×10^6	268261.2		
SK-WIB_1	0.405	0.331	0.234346	1.0×10^5	23434.6	22253.75	7.5
SK-WIB_2	0.4	0.366	0.263411	8.0×10^4	21072.9		
SK-SP_1	0.442	0.368	0.265072	600	159	152.3	6.22
SK-SP_2	0.415	0.341	0.242651	600	145.6		
SK-P_1	0.461	0.387	0.28085	60	16.9	17.75	6.77
SK-P_2	0.497	0.423	0.310746	60	18.6		

*: OD sample - OD blank at 405 nm wavelength, and OD blank was 0.074

Comparison of Quantitative and Semi-Quantitative Method

A paired T- test was conducted for comparing the results of semi-quantitative LAL- gel clot results with those of LAL-endpoint chromogenic as a reference method (Table 5). The results of semi-quantitative gel clot and quantitative chromogenic methods had a 0.999 correlation with a significant *p*- value of 0.0001.

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Table 5. Paired Samples Test for comparing the chromogenic and gel clot results.

	Paired Differences					t	df	Sig. (2-tailed)
				90% Confidence Interval of the Difference				
	Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
chromogenic – gel clot	-7185	15351.66	5427.63	-20019.58	5649.05	-1.324	7	.227

The *p*-value of 0.227 which was higher than 0.05, indicated that the results of quantitative and semi-quantitative gel clot were similar and the observed differences were not significant.

DISCUSSION

The quantitative bacterial endotoxin tests provide sensitive and accurate methods for detecting endotoxins, allowing researchers to identify and eliminate potential sources of contamination before the products are released to the market. Although, the bacterial endotoxin test is an essential aspect of research and development for new drugs and vaccines to ensure the safety and efficacy of these products, it is costly and inconvenient for small research teams with restricted budgets and facilities due to lack of single-test commercial kits. Furthermore, when the endotoxin concentration of a sample is more than the detection range of the quantitative assays, conducting LAL gel clot tests before a quantitative bacterial endotoxin test may provide a measure of the needed dilution factor, with a lower expense. In addition, as LAL gel clot assay is prone to the most of interfering excipients of samples, it is commonly considered as a verified method for diagnosing inconsistency between the results of different LAL test methods [14].

Most suppliers of commercial LAL gel clot kits provide instructions for using this qualitative test method in a semi-quantitative fashion [5] and some literature have used LAL gel clot test in semi-quantitative manner [15-17]. However, there are only a few references comparing the results of semi-quantitative gel clot with other quantitative LAL methods [14]. Here, the competency of this approach was investigated by comparing the rough estimation of endotoxin concentration of some different samples with a wide range of endotoxin concentrations from 10^6 to less than 100 EU/ml. As it is shown in Table 2, the accuracy and reliability of the assay can be affected by a dilution factor used. The selection of the proper dilution factor is typically based on prior knowledge of the sample's expected endotoxin levels or empirical testing to determine the optimal dilution range. However, for some samples, this could be more challenging because there are not enough references providing a clear idea about the starting point for guessing the proper dilution range. For example, the endotoxin contamination of streptokinase inclusion bodies has not reported previously. Although, several studies revealed that the released endotoxin during cell disruption may attach to the inclusion bodies [18, 19, 11], only few numerical examples are available. This lack of primary data, forced us to repeat gel colt test for SK-IB samples to reach an adequate narrow range. If the endotoxin concentration had been estimated from first dilution guess which had positive and negative results at 10^6 and 10^7 dilution factors, a high concentration of 790569 EU/ml had been estimated. However, by using an adequate narrow dilution range (i.e., 1.2×10^6 – 1.4×10^6), a concentration of 273861 EU/ml was estimated. Comparing these two estimations of endotoxin concentrations revealed that an inappropriate wide dilution range may result in approximately 200% overestimation.

A more accurate result of endotoxin contamination of SK-IB was determined as 255938 EU/ml and even the absolute error was about 17923 EU/ml. This high error value was only 7% of the correct result. This finding indicated that an enough narrow dilution range of sample may result in a good approximation of true endotoxin concentration.

However, the high differences between the endotoxin concentration of streptokinase inclusion body and the reported 7.9×10^5 EU/ml for Cry4AaCter-induced inclusion bodies containing insect-derived antimicrobial peptides produced *in E. coli* [11] seems to be related to the differences between the nature of the proteins. Streptokinase is an acidic protein with an isoelectric point (PI) of 4.7 [9] while the antimicrobial peptides are cationic peptides with high PI which usually have high binding affinity to the endotoxin [20]. Thus, it could be assumed that their inclusion body may have absorbed higher amounts of LPS. For Other samples, the first guess of dilution range provided a closed estimation to the true values and merely two runs were enough to provide a narrow dilution range, resulting in a positive and a negative result for two repeats of a sample which commonly is considered as endpoint results for gel clot test [5]. However, the endotoxin contamination of purified streptokinase (18 EU/ml) didn't match to those of similar processes which had reported 2 EU/ml [9]. As no stark deviations between the results of chromogenic and gel clot methods were detected, it seems that the results inconsistency was related to the isolation circumstances between the large scale and the benchtop scale processes. Moreover, the large scale process had been performed in a clean area using validated pyrogen-free materials and reagents [9].

Considering the paired T-test results which showed good correlation between the results of chromogenic and gel clot methods, the observed differences was not significant. Although the gel clot method had a negative bias (since the point-to-point comparisons showed some positive absolute error), it could not be concluded that the gel clot test underestimated the endotoxin concentration. In conclusion, regardless the underestimation or the overestimation points of view, the maximum relative error was less than 12%, indicating that semi-quantitative LAL gel clot method with adequate narrowing of the dilution range may provide a reasonable estimation of endotoxin concentration in comparison to the quantitative LAL chromogenic method.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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