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https://doi.org/10.22239/2317-269X.02447

Performance of an rFC-based assay for endotoxin quantification in hyperimmune sera

Desempenho de um ensaio baseado em Fator C recombinante para a quantificação de endotoxinas em soros hiperimunes

Ellen Gameiro Hilinski[I,II]*

Daniela Dal Molim Ghisleni[II]

Carla Lilian de Agostini Utescher[III]

Wagner Quintilio[IV]

Terezinha de Jesus Andreoli Pinto[II]

[I] Núcleo de Ensaios Biológicos e de Segurança, Centro de Medicamentos, Cosméticos e Saneantes, Instituto Adolfo Lutz, São Paulo, SP, Brasil

[II] Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brasil

[III] Laboratório de Virologia, Instituto Butantan, São Paulo, SP, Brasil

[IV] Laboratório de Biotecnologia Viral, Instituto Butantan, São Paulo, SP, Brasil

* **E-mail:** ellen.hilinski@ial.sp.gov.br

Received: Jan 29, 2025
Approved: Jul 18, 2025

How to cite: Hilinski EG, Ghisleni DDM, Utescher CLA, Quintilio W, Pinto TJA. Performance of an rFC-based assay for endotoxin quantification in hyperimmune sera. Vigil Sanit Debate, Rio de Janeiro, 2025, v.13: e02447. https://doi.org/10.22239/2317-269X.02447

ABSTRACT

Introduction: Endotoxin contamination poses a significant risk to the safety of pharmaceutical products, particularly those administered parenterally. Ensuring patient safety requires strict adherence to the regulatory standards for sterile and pyrogen-free pharmaceutical formulations. **Objective:** This study aimed to evaluate the feasibility of using the Recombinant Factor C (rFC) method, specifically Endolisa®, for detecting bacterial endotoxins in hyperimmune sera (pentavalent antibothropic, anti-rabies, and tetanus antitoxin). **Method:** Samples were spiked with endotoxin solutions at concentrations ranging from 0.05 to 10 EU/mL and analyzed using the rFC kit. The key performance parameters were thoroughly evaluated, such as specificity, detection and quantification limits, precision, accuracy, and linearity. Each concentration was tested a minimum of five times across six points. **Results:** The method demonstrated precision with relative standard deviations ranging from 3.5% to 19.0%, accuracy for endotoxin recovery between 94.0% and 134.0%, and linearity across the concentration range of 0.05 to 5 EU/mL for the three sera. The detection and quantification limits were established at 0.05 EU/mL. **Conclusions:** The results confirm that the rFC method provides accurate, precise, specific, and linear quantification of endotoxins in hyperimmune sera within the range of 0.05 to 5 EU/mL at 1:100 dilution. However, for samples spiked with 10 EU/mL (1:100 dilution), the method did not meet the compendial criteria.

KEYWORDS: Recombinant Factor C; Endotoxins; Hyperimmune Serum; *In Vitro* Test

RESUMO

Introdução: A contaminação por endotoxinas representa um risco significativo à segurança de produtos farmacêuticos, especialmente aqueles administrados por via parenteral. Garantir a segurança dos pacientes exige estrita conformidade com os padrões regulatórios para formulações farmacêuticas estéreis e livres de pirogênio. **Objetivo:** Avaliar a viabilidade do uso do método baseado no Fator C Recombinante (FCr), especificamente o Endolisa®, para a detecção de endotoxinas bacterianas em soros hiperimunes (pentavalente antibotrópico, antirrábico e antitetânico). **Método:** As amostras foram contaminadas com soluções de endotoxinas em concentrações variando de 0,05 a 10 UE/mL e analisadas utilizando o kit Endolisa®. Foram avaliados parâmetros de desempenho como especificidade, limites de detecção e quantificação, precisão, exatidão e linearidade. Cada concentração foi testada no mínimo cinco vezes em seis pontos diferentes. **Resultados:** O método apresentou precisão, com desvios-padrão relativos entre 3,5% e 19,0%, e exatidão, com recuperação de endotoxinas variando de 94,0% a 134,0%. Além disso, demonstrou correlação linear para o intervalo entre 0,05 e 5 UE/mL para os três soros analisados na diluição 1:100. Os limites de detecção e quantificação foram definidos em 0,05 UE/mL. **Conclusões:** Os resultados confirmam que o método empregando FCr permite uma quantificação precisa, exata, específica e linear de endotoxinas em soros hiperimunes no intervalo de 0,05 a 5 UE/mL. No entanto, para amostras contaminadas com 10 UE/mL (diluição 1:100), o método não atendeu aos critérios compendiais.

PALAVRAS-CHAVE: Fator C Recombinante; Endotoxinas; Soro Hiperimune; Técnicas *in vitro*

INTRODUCTION

Contamination by bacterial endotoxins in pharmaceutical products for parenteral use poses a severe threat to patient safety. These lipopolysaccharides, which are components of the outer membrane of Gram-negative bacteria, can trigger fever, septic shock, and multiple organ failure, even at minimal concentrations[1]. Given this risk, precise endotoxin quantification is essential to ensure the quality and safety of parenteral drugs, aligning with regulatory requirements to mitigate the risks of contaminated intravenous products[1,2].

Historically, the *Limulus amebocyte* lysate (LAL) test became the gold standard for detecting endotoxins[3,4]. Based on the reaction of horseshoe crab hemolymph to endotoxins, this method significantly reduced the need for *in vivo* pyrogen testing using rabbits. However, ethical and ecological concerns have arisen due to the intensive exploitation of horseshoe crabs, threatening their populations and questioning the sustainability of LAL as the sole solution for laboratory testing[3,5,6,7]. In this context, Recombinant Factor C (rFC) emerges as a promising alternative. Developed using biotechnology, rFC replicates the sensitivity of factor C found in horseshoe crab hemolymph, allowing for endotoxin detection without exploiting these animals[5,6]. Furthermore, by eliminating other enzymatic cascade components that react with substances, like 1,3-ß-glucans, the rFC method avoids common LAL interferences, offering greater accuracy for complex pharmaceutical matrices[5,8,9].

The implementation of rFC in laboratory routines aligns with the 3Rs principle: replacement, reduction, and refinement of animal use in research[10,11]. As established in international regulations, such as the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines[12] and global pharmacopeias[4,13,14], alternative methods must be considered whenever technically feasible. rFC meets these requirements and reinforces the pharmaceutical industry's commitment to ethical and sustainable practices.

From a regulatory standpoint, authorities such as the United States Pharmacopeia[15] and the European Pharmacopoeia[16] have already recognized the technical feasibility of rFC for endotoxin testing in specific products. In Brazil, adopting alternative methods has gained prominence in discussions led by the Brazilian Health Regulatory Agency (Anvisa)[4], considering both efficiency and compliance with international standards. The validation and global harmonization of these methods are fundamental steps for their widespread acceptance.

Using rFC also brings significant operational advantages. The method reduces the result variability between different lots and manufacturers, a common issue with LAL[5,15,17]. Additionally, it offers greater sensitivity, making it particularly effective for products requiring strict endotoxin detection limits, such as hyperimmune sera, vaccines and parenteral solutions[8,9,18,19,20].

Despite its benefits, challenges remain in adopting rFC as a global standard.

Technical and economic barriers and cultural resistance within the industry require collaborative efforts among manufacturers, regulators, and researchers. Investments in research to expand the method's applicability, coupled with education on its benefits, are essential to overcoming these hurdles.

rFC represents a significant evolution in pharmaceutical quality control, combining analytical precision, ethics, and environmental sustainability[3,21]. Its implementation strengthens the industry's commitment to patient safety and environmental preservation, aligning with the demands of a sector increasingly focused on responsible and innovative practices[15,16].

Although the rFC assay is a valuable tool for endotoxin detection, it is not suitable for detecting all types of pyrogens. Therefore, in contexts where the presence of non-endotoxin pyrogens is a concern, additional methods, such as the Monocyte Activation Test (MAT), may be necessary to ensure the safety and efficacy of pharmaceutical products[22]. The rFC assay is highly specific for endotoxins, offering a sensitive detection limit for endotoxin testing in pharmaceuticals and medical devices[21]. It is an animal-free, 3Rs-compliant method. In contrast, the MAT utilizes human blood cells, specifically monocytes, to detect a broad range of pyrogens by incubating the sample and measuring the production of cytokines, such as interleukins, through an ELISA[22]. This broad application makes MAT particularly suited for detecting both endotoxins and non-endotoxin pyrogens, offering a comprehensive safety profile for complex formulations[23]. While the rFC assay is ideal for endotoxin-specific testing, MAT's ability to detect non-endotoxin pyrogens ensures that it can be used for a wider array of pharmaceutical products, including vaccines and biologics[24,25,26]. Both methods are 3Rs-compliant, reducing the need for animal testing; however, the choice between them should depend on the product's composition, intended use, and regulatory requirements. Ongoing validation and regulatory acceptance are crucial for their adoption in pyrogen testing across different industries.

This study aimed to evaluate the application of an innovative method based on rFC, a synthetic receptor derived from the coagulation cascade of horseshoe crab blood, combined with a fluorogenic substrate, for detecting bacterial endotoxins in hyperimmune sera. The research focused on antibothropic (SAB), anti-rabies (SAR), and tetanus antitoxin (SAT) sera, which play a crucial role in public health and are widely used in the treatment of severe medical emergencies, such as envenomation, rabies, and tetanus. Assessing the feasibility of the rFC method for endotoxin quantification in these products is of great importance, as it ensures the safety and quality of essential biological products while promoting the reduction of animal-based testing methods, in alignment with ethical principles and international regulations.

METHOD

Reagents

The pentavalent antibothropic serum (5 mg/mL), anti-rabies serum (200 IU/mL), and tetanus antitoxin (1,000 IU/mL)

hyperimmune serum, manufactured by the Butantan Institute (São Paulo, Brazil), were diluted 1:100 (v/v) with non-pyrogenic water and used as samples for testing with the Endolisa® kit (bioMérieux, Germany). The endotoxin limits (EL) for substances administered parenterally were calculated as K/M, where K is the threshold pyrogenic dose of endotoxin per kilogram (kg) of body weight in a single hour (h) period (5 EU/kg/h), and M is the maximum recommended dose of the product per kilogram of body weight in a single hour period (M = dose/kg/h)[13,27]. The maximum doses of SAT, SAB, and SAR are 25, 120, and 15 mL/70 kg/h, respectively[28,29,30]. The EL values for SAT, SAB, and SAR were determined to be 336, 70, and 23 EU/mL, respectively.

The kit includes rFC, fluorometric substrate, nonpyrogenic water, substrate buffer, pre-coated microtiter strips, binding buffer, cover foil, and endotoxin standard. All experiments were conducted using the same sample and kit batches. Endotoxin standard 500 EU/mL (bioMérieux, Germany), from *E. coli* O55:B5, was used for spiking the samples. All solutions were prepared in endotoxin-free borosilicate glass tubes (bioMérieux, Germany) with non-pyrogenic water, followed by vigorous mixing for at least 3 minutes between dilutions.

The parameters Specificity, Detection and Quantification Limits, Precision, Accuracy, and Linearity were evaluated to assess the acceptance criteria established in the United States Pharmacopeia[31].

rFC assays

Endotoxin quantification was performed using the EndoLISA® kit according to the manufacturer's instructions on a fluorescence microplate reader (excitation at 380 nm; emission at 445 nm).

Preparation of the EndoLISA® microtiter plate

The EndoLISA® microtiter plate was pre-coated with phage-derived binding proteins specific for endotoxins. For the preparation of the standard curve, 100 µL of the control standard solutions ranging from 0.05 to 50 EU/mL were added in duplicate, in decreasing order of concentration. As a blank control, 100 µL of pyrogen-free water was added in quadruplicate.

For the assay, 100 µL of hyperimmune serum samples diluted 1:100 (v/v) were added to the wells. These samples were artificially spiked with 10 µL of endotoxin standard at concentrations between 0.5 and 100 EU/mL, resulting in final concentrations ranging from 0.05 to 10 EU/mL, depending on the parameter being evaluated. For the wells corresponding to the PPC, an additional 10 µL of endotoxin standard at 50 EU/mL was added to achieve a final spike of 5 EU/mL. The plate was then mixed for 2 minutes at 800 rpm using a shaker incubator.

Binding step

Following plate preparation, 20 µL of the binding buffer was added to each well. The plate was sealed with a cover foil and incubated at 37°C for 90 minutes in a shaker incubator at 450 rpm.

Washing step

After incubation, the contents of the wells were carefully discarded into a waste container to prevent cross-contamination. Using a multichannel pipette, 150 µL of wash buffer was added to each well, followed by the removal of the liquid after inverting the plate. This washing procedure was repeated two additional times, for a total of three washes.

Preparation of the assay reagent

To prepare the assay reagent, 8 parts of assay buffer, 1 part of the fluorescent substrate, and 1 part of the enzyme solution (rFC) were mixed in a pyrogen-free container, followed by gentle homogenization. The reagent was used immediately after the preparation.

Detection step

After washing, 100 µL of the assay reagent was added to each well using a multichannel pipette, and the plate was placed into the microplate reader. After temperature stabilization for 60 seconds, the plate was incubated at 37°C for 90 minutes, without shaking, for fluorescence signal acquisition.

The assay is self-validating; for each sample dilution, a corresponding well spiked with 5 EU/mL of endotoxin must be included. The software automatically validates the results based on this spike.

Curve generation and endotoxin quantification were performed using Gen5™ software version 3.05, based on the standard curve. The test is considered valid if the endotoxin recovery in each PPC is between 50–200% of the nominal value and if the coefficient of variation (CV) between the sample and control replicates is less than 25%.

Performance evaluation

To evaluate precision, accuracy, and linearity, samples were spiked with endotoxin (*E. coli* O55:B5) at concentrations of 0.05, 0.5, 1.0, 2.0, 5.0, and 10.0 EU/mL, with each concentration tested in at least five replicates. Five replicates in duplicate were analyzed for both the sample and the PPC. The assay plates were individually prepared for each of the three hyperimmune sera evaluated.

For the evaluation of the detection and quantification limits, data from the 0.05 EU/mL concentration were used. Intermediate precision was assessed at 0.05 and 0.5 EU/mL, using 10 replicates in duplicate for both the sample and the PPC, performed by two different operators on separate days, totaling 20 replicates per concentration.

Data evaluation

The analytical step for performing the tests included the use of negative controls in quadruplicate, and the calculations for determining endotoxin levels were performed only when the

negative controls presented fluorescence values lower than the lowest point of the standard curve (0.05 EU/mL), as recommended by the kit manufacturer.

The CV between the 5 replicates of spiked samples with 1, 2, 5, and 10 EU/mL, as well as between the 10 replicates of artificially contaminated samples with 0.05 and 0.5 EU/mL, was determined to assess repeatability. Intermediate precision included the determination of the CV of the mean value of 20 replicates tested by two operators on different days, of samples at 0.05 and 0.5 EU/mL.

The method accuracy was assessed by the recovery value of the control standard endotoxin (CSE) added to the samples, that is, by comparing the endotoxin values obtained with the nominal values of the added CSE. The mean recovery values obtained for each of the 5 replicates of artificially contaminated samples with 1, 2, 5, and 10 EU/mL were calculated, as well as for the 10 replicates of artificially contaminated samples with 0.05 and 0.5 EU/mL.

The detection and quantification limits of the method were calculated based on the standard deviation of the difference in relative fluorescence units (dRFU) between the first and last readings of the assay, relative to the blank, using the equations described in the note of Table 4.

To evaluate the linearity parameter, linear regression analysis was performed using the average logarithmic values of the endotoxin concentrations obtained from multiple replicates. Specifically, the analysis considered the averages of five replicates for 1, 2, and 5 EU/mL, as well as ten replicates for 0.05 and 0.5 EU/mL. The calculated equation, coefficient of determination (R^2), and linearity graphs for each of the three sera are presented in Figure. Notably, the data for the 10 EU/mL were excluded from the linearity evaluation due to noncompliance with the acceptance criteria for precision and accuracy.

Statistical analyses were conducted using the Minitab® software version 19 (Minitab Inc., USA).

RESULTS AND DISCUSSION

The rFC assay, based on the 3Rs concept, is an alternative to the use of the LAL reagent in the bacterial endotoxin test for complex samples. The commercial EndoLISA® kit was challenged considering the specificity, detection and quantification limits, precision, accuracy, and linearity parameters for each hyperimmune sera: antibothropic, anti-rabies, and tetanus antitoxin.

Recent advancements in endotoxin detection have emphasized the need for more precise and reliable methods in the context of complex biological matrices[14,15,16,31]. This includes newer rFC assays that enhance specificity and reduce the potential for false positives, even in challenging samples.

Initially, three concentrations were tested for each of the three hyperimmune sera: undiluted (neat), diluted 1:10 (v/v), and diluted 1:100 (v/v) in pyrogenic water, to determine which of the concentrations would allow the recovery of endotoxin levels between 50% and 200% for the positive control samples of the product. As observed in Table 1, the undiluted (neat) samples presented recovery values lower than 30% for the three sera evaluated, evidencing the inhibition of endotoxin levels as a consequence of the interference of some components of the product. The greatest inhibition was evidenced in SAT, for which it was practically impossible to recover any level of endotoxin overload.

For the 1:10 and 1:100 dilutions, the three tested sera presented recovery percentages within the acceptance criterion[31] (50 to 200%). However, these results varied between sera, with a trend toward increased endotoxin levels in the SAB and SAT samples and a decrease in the SAR samples. At the 1:100 dilution, all tested sera showed average endotoxin recovery values not only within the established acceptance range but also closer to 100%, which is desirable in order to avoid values near the extreme limits of the criterion. Moreover, the CV observed at this dilution was lower than that at 1:10, except for SAB and SAT, which still showed relatively higher variability. Nonetheless, the overall

Table 1. Results of the Inhibition and Potentiation test for samples of antibothropic (SAB), anti-rabies (SAR), and tetanus antitoxin sera (SAT).

Sample dilution	Replicate number (n)	Sample without a spike		Positive Product Control (5 EU/mL)	
		Mean (EU/mL)	CV (%)	Recovery ± SD (%)	CV (%)
SAB no dilution	5	< 0.050	0.0	26.7 ± 1.9	7.1
SAB 1:10	5	< 0.050	0.0	69.7 ± 3.3	4.7
SAB 1:100	5	< 0.050	0.0	99.2 ± 11.1	11.2
SAR no dilution	5	< 0.050	0.0	26.5 ± 1.6	6.0
SAR 1:10	5	< 0.050	0.0	134.5 ± 9.4	7.0
SAR 1:100	5	< 0.050	0.0	108.2 ± 7.2	6.7
SAT no dilution	5	< 0.050	0.0	0.5 ± 0.7	140.0
SAT 1:10	5	< 0.050	0.0	56.7 ± 3.4	6.0
SAT 1:100	5	< 0.050	0.0	103.3 ± 12.1	11.7

Source: Prepared by the authors, 2025.
SD: standard deviation; CV: coefficient of variation.

performance at 1:100 was considered to be more consistent and reliable. For this reason, 1:100 was defined as the working dilution to be used during the performance evaluation method.

To assess the potential interference related to the matrix (0.35% phenol), the phenol solution was diluted in pyrogenic-free water to a final concentration of 0.0035%. Ten replicates of this solution were tested to evaluate endotoxin recovery levels from the overload performed in wells identified as positive product control (PPC). All ten replicates showed results below 0.05 EU/mL and average endotoxin recovery within the test validity criterion (50%–200%), allowing us to conclude that the phenol solution used as a vehicle in the manufacture of the hyperimmune sera does not interfere with the recovery of added endotoxin. Kang et al.[32] highlighted that the rFC assay provides specificity and sensitivity comparable to the traditional LAL method when applied to biopharmaceuticals. While some samples showed higher levels of interference when tested with the rFC assay, these challenges were effectively managed through appropriate dilution strategies.

It is important to highlight that phenol is the pharmacotechnical adjuvant present in the formulation. Therefore, no additional matrix components are known to be present that would be expected to interfere with the assay. Based on the available information and the results obtained, no interference was observed under the tested conditions, and the phenol concentration used appears to be compatible with reliable test performance.

For the six concentrations tested within the range from 0.05 to 10 EU/mL, the CV for precision remained below 25%, meeting the requirement[33] for the three evaluated sera (Table 2). Recent advancements in the rFC assay have allowed for more accurate detection of low endotoxin concentrations (0.05 EU/mL), with precision values well below the 25% threshold, as demonstrated in the study by Kang et al[32].

The results obtained by operators A and B for samples spiked with 0.05 and 0.5 EU/mL were analyzed to assess intermediate precision using the Student's *t*-test for two samples. This test was performed to determine whether differences between the analysts could account for the variability in the measurements (Table 3). The interaction between the day and the operator was not statistically significant at the 5% significance level, as *p*-values exceeded 0.05 for each endotoxin concentration tested (0.05 and 0.5 EU/mL) across the three sera.

Table 2. Precision and accuracy results from 0.05 to 10 EU/mL.

Spike (EU/mL)	Replicate number (n)	Sample spiked		Positive Product Control (5 EU/mL)	
		Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)
Anti-bothropic hyperimmune serum (SAB)					
0.05	10	126.20 ± 14.6	11.6	108.2 ± 14.8	13.7
0.50	10	118.90 ± 12.5	10.5	117.4 ± 11.3	9.6
1.00	5	89.26 ± 11.7	13.1	119.0 ± 14.8	12.4
2.00	5	112.87 ± 8.6	7.7	93.9 ± 5.9	6.3
5.00	5	79.90 ± 5.0	6.3	110.5 ± 14.1	12.8
10.00	5	71.40 ± 15.1	21.1	44.8 ± 15.9	35.5
Anti-rabies hyperimmune serum (SAR)					
0.05	10	120.60 ± 15.6	12.9	115.1 ± 10.2	8.9
0.50	10	115.06 ± 9.1	7.9	115.8 ± 9.7	8.4
1.00	5	116.60 ± 4.0	3.4	111.6 ± 12.0	10.8
2.00	5	89.78 ± 11.2	12.5	116.3 ± 14.3	12.3
5.00	5	96.24 ± 9.8	10.2	112.2 ± 12.0	10.7
10.00	5	72.85 ± 18.8	25.8	108.5 ± 62.0	57.1
Tetanus antitoxin hyperimmune serum (SAT)					
0.05	10	124.80 ± 16.2	13.0	108.1 ± 9.4	8.7
0.50	10	99.06 ± 15.2	15.3	113.6 ± 11.7	10.3
1.00	5	102.10 ± 5.6	5.5	107.6 ± 10.0	9.3
2.00	5	116.31 ± 5.2	4.5	110.1 ± 13.6	12.4
5.00	5	93.60 ± 8.4	9.0	114.2 ± 13.6	11.9
10.00	5	66.44 ± 8.7	13.1	67.4 ± 30.5	45.3

Source: Prepared by the authors, 2025.
SD: standard deviation; CV: coefficient of variation.

The CV values for the intermediate precision (Table 3), for 0.05 EU/mL (n = 20) and 0.5 EU/mL (n = 20), showed compliance with the pharmacopoeial acceptance criterion[33] for the sera evaluated.

As highlighted by Marius et al.[19] and Kang et al.[32], the rFC assay showed promising results for intermediate precision, particularly in multi-operator settings, reinforcing the robustness of this method. The CV values for repeatability and intermediate precision consistently remained below 15%, indicating high method reliability even across different laboratories and analysts[33].

The endotoxin recovery values for the accuracy results for concentration levels between 0.05 and 10 EU/mL are presented in Table 2. The mean recovery percentages calculated for samples with concentrations between 0.05 and 5 EU/mL ranged from 79.9 to 126.2% for SAB, from 89.8 to 120.6% for SAR, and from 93.6 to 124.8% for SAT, demonstrating compliance with the compendial requirements[31] (50 to 200%). Recent studies[8,19,32] have reinforced the reliability of the rFC assay in terms of accuracy, particularly at low endotoxin levels, where traditional LAL assays may struggle with interference.

The lowest endotoxin concentration evaluated (0.05 EU/mL) presented the highest mean recovery values for the three sera. However, for the concentration of 10 EU/mL, despite meeting the requirement for the recovery value of the overloaded sample for SAB and SAT, the mean recovery value obtained for the CPP did not meet the assay validity criterion[31] in any of the three sera evaluated. For the CPP related to the SAB, SAR, and SAT sample tests, high data variability was evidenced about the average, a fact assessed through the standard deviation and CV data (Table 2).

Bolden and Smith[8] reported that the rFC assay was as accurate as the LAL assay when using a reference standard endotoxin to evaluate bacterial endotoxins in pharmaceutical products. Expanding on this perspective, Marius et al.[19] conducted an in-depth analysis comparing the performance of the rFC assay with the LAL method using biological samples of varying compositions. Their findings showed that the rFC assay demonstrated performance equivalent to or even superior to that of the LAL method in terms of specificity, precision, and robustness, especially when applied to complex biological matrices.

Table 3. Intermediate Precision results for 0.05 and 0.5 EU/mL.

Sample	Day / Operator	Spike (EU/mL)	Replicate number (n)	Sample spiked		Positive Product Control (5 EU/mL)	
				Mean ± SD (EU/mL)	CV (%)	Mean ± SD (%)	CV (%)
SAB	A	0.05	10	0.063 ± 0.007	11.6	108.2 ± 14.8	13.7
	B	0.05	10	0.062 ± 0.011	17.7	97.0 ± 15.6	16.1
	A	0.50	10	0.595 ± 0.062	10.5	117.4 ± 11.3	9.6
	B	0.50	10	0.616 ± 0.062	10.1	115.8 ± 18.8	16.2
SAR	A	0.05	10	0.060 ± 0.008	12.9	115.1 ± 10.2	8.9
	B	0.05	10	0.064 ± 0.015	23.4	111.5 ± 10.6	9.5
	A	0.50	10	0.575 ± 0.046	7.9	115.8 ± 9.7	8.4
	B	0.50	10	0.541 ± 0.033	6.1	118.1 ± 8.8	7.5
SAT	A	0.05	10	0.062 ± 0.008	13.0	108.1 ± 9.4	8.7
	B	0.05	10	0.059 ± 0.006	10.2	114.6 ± 20.1	17.5
	A	0.50	10	0.495 ± 0.076	15.3	113.6 ± 11.7	10.3
	B	0.50	10	0.539 ± 0.113	21.0	111.0 ± 17.0	15.3

Sample	Spike (EU/mL)	p-value (Student's t-test; CI = 95%)
SAB	0.05	0.804
	0.5	0.608
SAR	0.05	0.294
	0.5	0.074
SAT	0.05	0.274
	0.5	0.328

Source: Prepared by the authors, 2025.
SAB: antibothropic serum; SAR: anti-rabies serum; SAT: tetanus antitoxin serum; SD: standard deviation; CV: coefficient of variation; CI: confidence interval.

Table 4. Results of the detection and quantification limit determination.

Sample	dRFU Mean	SD	Detection limit	Quantification limit
Blank	50.7	11.1	-	-
SAB + 0.05 EU/mL	348.2	49.6	0.05 EU/mL	0.05 EU/mL
SAR + 0.05 EU/mL	218.1	48.1	0.05 EU/mL	0.05 EU/mL
SAT + 0.05 EU/mL	293.5	47.1	0.05 EU/mL	0.05 EU/mL

Source: Prepared by the authors, 2025.
dRFU: relative fluorescence unit (RFU) difference between the first and last assay read; SD: standard deviation; Limit of detection: dRFU Mean(0,05 EU/mL) dRFU Mean(Blank) ≥ 3.3 x SD (Blank); Limit of quantification: dRFU Mean(0,05 EU/mL) dRFU Mean(Blank) ≥ 1.6 x (SD(Blank) + SD(0.05 EU/mL)); SAB: antibothropic serum; SAR: anti-rabies serum; SAT: tetanus antitoxin serum; SD: standard deviation; CV: coefficient of variation.

The data presented in Table 4, along with the equations described in its accompanying note, were used to determine the detection and quantification limits of the method at the lowest endotoxin concentration tested (0.05 EU/mL) for each serum sample.

Since the difference between the mean dRFU values for samples with an addition of 0.05 EU/mL (SAB, SAR, and SAT) and the blank was greater than 3.3 times the value of the standard deviation of the blank, it can be stated that the concentration of 0.05 EU/mL represents the detection limit of the method. Since the difference between the mean dRFU values for samples with the addition of 0.05 EU/mL (SAB, SAR, and SAT) and the blank was greater than 1.6 times the value of the sum of the standard deviation of the blank with the standard deviation of samples with the addition of 0.05 EU/mL, it can be stated that this concentration also represents the quantification limit of the method. The concentration level of 0.05 EU/mL, in addition to presenting positive detection in all replicates analyzed, met the acceptance criteria for the parameters precision, accuracy, and linearity for the three sera evaluated (Table 2; Figure). For this reason, the detection and quantification limit of the method was set to 0.05 EU/mL.

The regression analysis performed to evaluate the linearity parameter confirmed a strong linear relationship between the nominal endotoxin concentrations and the measured concentrations in the SAB, SAR, and SAT samples, with R^2 values of 0.988, 0.994, and 0.993, respectively, across the range from 0.05 to 5 EU/mL (Figure). The residual plots from the regression analysis were further evaluated for the normality, homoscedasticity, and independence of the residuals. This analysis is consistent with the findings of Kang et al.[32], who demonstrated high linearity and minimal residual errors in rFC assays across endotoxin concentrations.

The study demonstrated that the rFC assay achieved correlation coefficients above 0.98 and recovery values within the range of 97.4 to 121%, fully meeting the international acceptance criteria. Although the rFC assay showed increased sensitivity to interferences from aluminum-based adjuvants in some vaccines, these were mitigated through dilution adjustments, reinforcing its broad applicability in complex biological matrices.

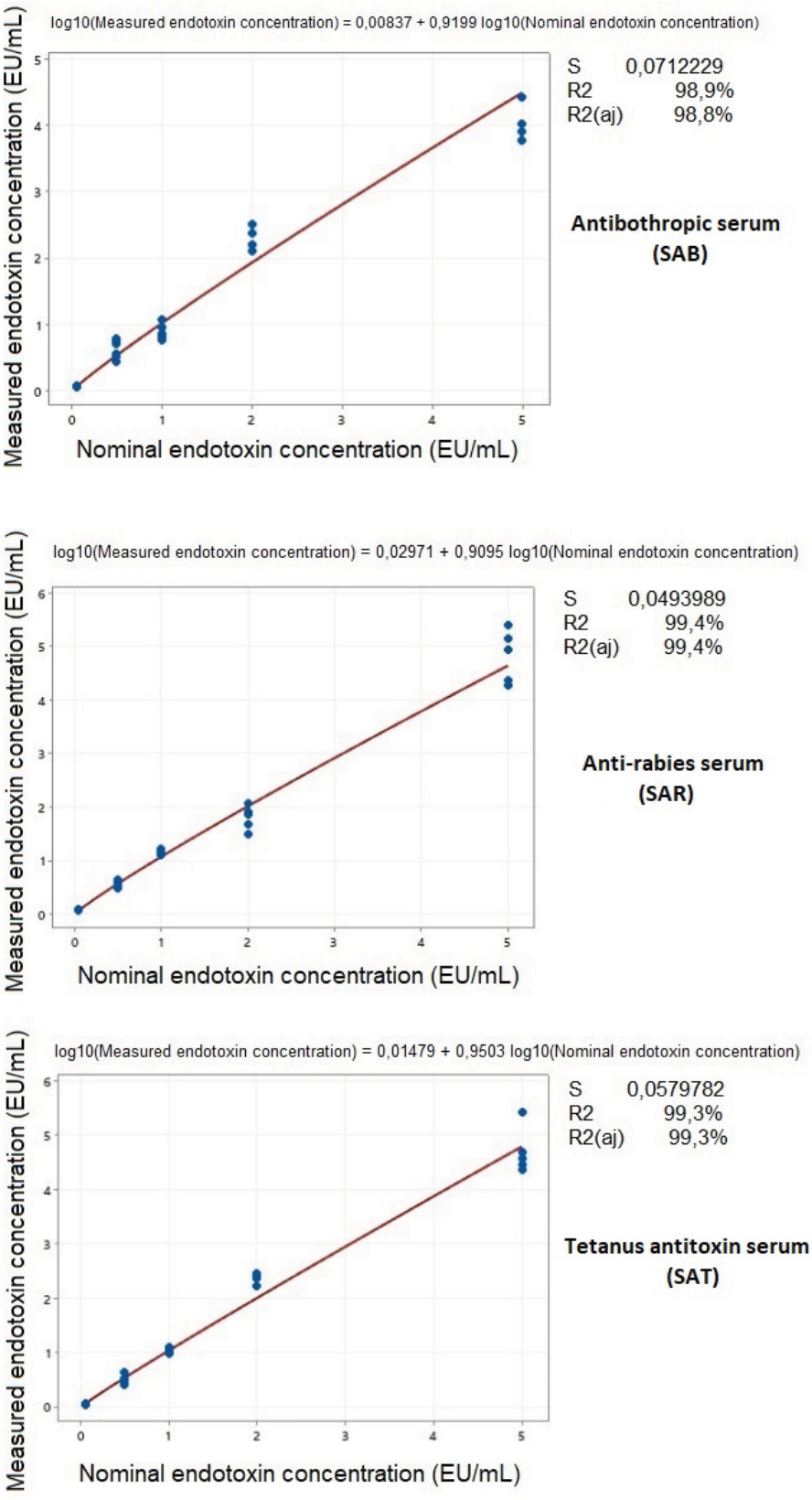
The normality of the standardized residuals was assessed using normal probability plots, which indicated no significant deviation from normality (p-value > 0.05) for the concentration range from 0.05 to 5 EU/mL at a 95% confidence interval. Residual versus fitted value plots demonstrated a random distribution, supporting the assumption of homoscedasticity. This was confirmed by the Levene test, which yielded p-values greater than 0.05 for all concentration ranges within 0.05 to 5 EU/mL, indicating equal variances. Finally, the independence of the residuals was verified using the residual versus order plots generated in the Minitab® software, confirming no systematic patterns.

Grallert et al.[18] investigated the behavior of different LPS species across a wide concentration range using EndoLISA® and the LAL reagent. Their findings revealed a linear correlation (R^2 = 0.91) between the endotoxin measurements obtained with both methods throughout the tested range. Additionally, the study assessed the impact of various substances commonly used in the production of biological products on endotoxin recovery using EndoLISA®. Compared with the LAL assay, EndoLISA® demonstrated reduced interference from high concentrations of salts, chaotropic agents, organic solvents, and detergents.

Minimizing invalid results is a critical factor for implementing analytical methods for endotoxin detection. The percentage of invalid results was evaluated within the concentration range from 0.05 to 5 EU/mL. Assays were considered invalid if the recovery values of the PPC were below 50% or above 200%[31] or if the CV between duplicate samples and/or PPC exceeded 25%[33].

In this study, across all tests conducted with the three sera, 7.7% (n = 20) of the results were invalid. Of these, 25.0% (n = 5) were due to the PPC recovery values, and 75.0% (n = 15) were due to the sample CV and/or PPC CV values. For SAB, 9.0% (n = 8) of the results were invalid, with 25.0% (n = 2) related to PPC recovery and 75.0% (n = 6) related to CV values. For SAT, 8.0% (n = 7) of the results were invalid, with 28.6% (n = 2) due to PPC recovery and 71.4% (n = 5) due to CV values. For SAR, 6.0% (n = 5) of the results were invalid, with 20.0% (n = 1) related to PPC recovery and 80.0% (n = 4) related to CV values.

Grallert et al.[18] also demonstrated that when comparing the results from the LAL and rFC methods, the rFC method produced fewer invalid results over a broader working range (0.05 to 500 EU/mL) and was less affected by complex samples or inhibitory constituents. However, in terms of batch-to-batch variability, the rFC test kits exhibited greater variability compared with the previously reported results for the LAL assay.



Source: Prepared by the authors, 2025.

Figure. Linear regression analysis from 0.05 to 5 EU/mL - measured versus nominal endotoxin concentration.

CONCLUSIONS

The data presented support the conclusion that the alternative method using the EndoLISA® kit could be considered for quantifying bacterial endotoxins during the quality assessment of hyperimmune sera, including Anti-Rabies, Pentavalent Antibothropic, and Tetanus antitoxin, within the range from 0.05 to 5 EU/mL (1:100 dilution). The method demonstrated the ability to detect and quantify endotoxins at concentrations as low as 0.05 EU/mL with accuracy, precision, and linearity.

However, the EndoLISA® kit is not recommended for evaluating hyperimmune sera where endotoxin concentrations are expected to exceed 5 EU/mL at a dilution of 1:100. For samples spiked with 10 EU/mL, the method failed to meet the required criteria for accuracy, precision, and linearity.

Considering the critical role of hyperimmune sera in Brazil's public health and epidemiological initiatives, in addition to the current requirement of the rabbit pyrogen test for biological safety assessment in pharmacopoeial monographs, alternative methodologies, such as the rFC method evaluated in this study, emerge as promising options.

Although they do not replace tests performed using in vivo models, these alternatives ensure reliable quantification of bacterial endotoxins and show significant potential to contribute to the reduction of animal use in in-process quality control testing of hyperimmune sera.

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Acknowledgement

To the São Paulo Research Foundation (Fapesp) and the National Council for Scientific and Technological Development (CNPq) for the financial support granted through processes nº 2018/19237-4 and 42.0441/2018-2, respectively.

Carla Lilian de Agostini Utescher and Wagner Quintilio received a productivity fellowship from Fundação Butantan.

Authors' contributions

Hilinski EG, Ghisleni DDM, Utescher CLA, Quintilio W, Pinto TJA - Conception, planning (study design), acquisition, analysis, data interpretation and writing of the work. All the authors approved the final version of the paper.

Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.