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Carcinoscorpius and Tachypleus lysates assay for detecting endotoxin in milk and groundwater: Toward reducing reliance on Limulus amebocyte lysate

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ABSTRACT

As one of the emerging pollutants, the presence of endotoxins became an increasingly urgent issue due to their impact on human health when found in drinking water and groundwater. A simple and rapid assay for assessing bacterial biomass in these water samples was essential, particularly in situations where access to laboratory facilities was limited. The bacterial endotoxin test (BET) using the *Limulus* amebocyte lysate (LAL) test was established as an effective method. However, the application of BET employing *Carcinoscorpius* or *Tachypleus* Amebocyte Lysate (CAL/TAL), derived from *Carcinoscorpius rotundicauda* and *Tachypleus gigas,* remained rarely performed. This study aimed to explore the CAL and TAL potential for detecting bacterial endotoxins in milk and groundwater. In this assay, 94 blood samples of horseshoe crabs collected from the Banyuasin Waters of South Sumatra (79 samples of *C. rotundicauda* and 15 samples of *T. gigas*) were used. The assay was carried out using the gel-clot method. The results revealed that the CAL and TAL were able to detect small concentrations of endotoxin (up to a concentration of 0.0156 EU/mL) in raw milk, pasteurized milk, well water, and L1 HPV 52 protein based on the gel-clot test. Both CAL and TAL could be a potential substitute for the LAL assay, especially for detecting bacterial biomass in milk and groundwater. Furthermore, these findings were essential as initial scientific information for developing the CAL/TAL tests toward the development of recombinant Factor C (rFC).

1. Introduction

The horseshoe crab blood plays a vital role in the biomedical and pharmaceutical industries due to their simple yet remarkable immune system (Krisfalusi-Gannon et al., 2018; Kumar et al., 2015). One of the most important features of their blood, or hemolymph, is its ability to coagulate in response to bacterial presence, rendering the bacteria harmless. This coagulation mechanism is crucial for testing vaccines, injectable medicines, and sterilizing medical equipment (Kumar et al., 2015). The hemolymph contains amebocytes, specialized cells that carry proteins essential for blood clotting and trapping foreign bacteria (Kumar et al., 2016). Amebocyte lysate, a lyophilized product derived from the amebocytes of horseshoe crabs, has become indispensable for endotoxin detection in the applications of biomedical (Hashmi and Thakur, 2019; John, B et al., 2020; Putra et al., 2019), drinking water (Abdulraheem et al., 2012; Flórez et al., 2023; Suzuki et al., 2016), and water sources (Can et al., 2013; Sattar et al., 2022; Zhang et al., 2019).

The lysate from *L. polyphemus* is known as *Limulus* Amebocyte Lysate (LAL), while lysates from *T. tridentatus* and *T. gigas* are referred to as *Tachypleus* Amebocyte Lysate (TAL), and *Carcinoscorpius* Amebocyte Lysate (CAL) as the term for lysate from *C. rotundicauda* (Hashmi and Thakur, 2019; John et al., 2012; Krisfalusi-Gannon et al., 2018; Vestbo

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et al., 2018). Both LAL and TAL have been widely applied to detect bacterial endotoxin (Gauvry, 2015), and CAL has indicated potential for similar applications (Rao and Bhagirathi, 1989). Endotoxins, which are lipopolysaccharide (LPS) toxins found in the cell walls of Gram-negative bacteria, can be detected and quantified using the bacterial endotoxin test (BET) through amebocyte lysate (Hashmi and Thakur, 2019). The BET is essential for quality control and safety in pharmaceutical manufacturing (Gorman, 2020).

LAL has been produced by global pharmaceutical manufacturers for over 45 years to detect endotoxin pyrogens (Dubczak et al., 2021), while TAL is primarily produced in Japan and China (Dolejš and Vaňousová, 2015). On the other hand, the distribution of Asian horseshoe crabs, including *C. rotundicauda* and *T. gigas*, has been documented in several regions of Indonesia, including Sulawesi, Kalimantan, Sumatra, and Java (Meilana and Fang, 2020). In South Sumatra, specifically, these species have been found in the Banyuasin Waters (Fauziyah Mustopa et al., 2021, 2023; Fauziyah, 2019a, 2019b). However, there has been limited research exploring the potential of TAL from these waters for endotoxin testing, especially in water sources and drinking water. Even though, water is an essential component of human life, serving purposes such as drinking, washing, bathing, and use in injectable solutions (Sattar et al., 2022). While access to clean drinking water is standard in developed countries, many individuals in developing countries face challenges in securing safe water.

Three primary techniques are used for the quantitative BET, namely the turbidimetric technique, the gel clot technique, and the chromogenic technique (Hashmi and Thakur, 2019). In this study, the gel clot technique was employed to detect and quantify endotoxins from Gram-negative bacteria using TAL and CAL lysates extracted from horseshoe crabs collected in the Banyuasin Waters of South Sumatra, Indonesia. This technique provides a simple, effective, and rapid method for detecting bacterial endotoxins through a clotting response. Furthermore, the successful use of amebocyte lysate from these crabs may present an alternative source of lysate for BET, which is critical given the limited availability of LAL (John et al., 2012).

Therefore, this study is an important initial step in assessing the potential of CAL and TAL for future development in recombinant lysate production and BET applications. This study aimed to explore the potential of amebocyte lysate extracted from *C. rotundicauda* and *T. gigas* for detecting bacterial endotoxins in milk and groundwaters.

2. Materials and methods

2.1. Material

In this study, the amebocyte lysate (TAL/CAL) was obtained by bleeding two horseshoe crabs (*T. gigas* and *C. rotundicauda*) collected from Banyuasin Waters, South Sumatra, Indonesia (Fig. 1). The bleeding method reported by Romadhon et al. (2018) was adopted. The identified species were bleeding to obtain 4–10 mL of blood and collected into 3 mL EDTA tubes. All horseshoe crabs were immediately released back into their habitat after the bleeding process. The blood samples were transferred to the laboratory in a cool box and then stored in a freezer at −20 °C for further analyses. In this case, 94 blood samples of horseshoe crabs (79 samples of *C. rotundicauda* and 15 samples of *T. gigas*) were used to detect bacterial endotoxins.

2.2. Methods

In this assay, the BET was used to detect/quantify endotoxin from the gram-negative bacteria using CAL and TAL, following the gel-clot method. This method was originally described by Levin and Bang

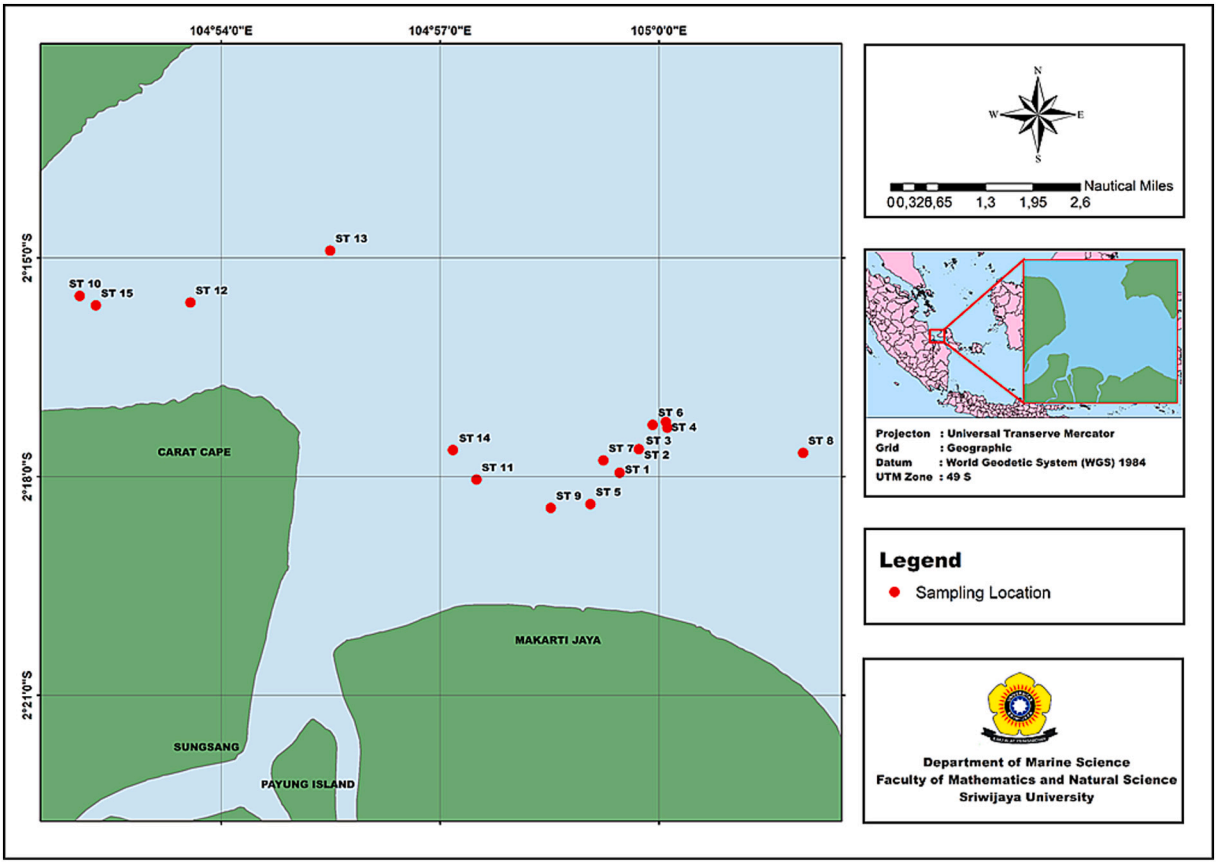

Fig. 1. Sampling locations of horseshoe crabs in the Banyuasin Waters, South Sumatra, Indonesia. Red dots represent specific sampling sites distributed along coastal and estuarine areas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(1964a,1964b). The assay procedure was adapted from Putra et al. (2019), as illustrated in Fig. 2.

A 10 EU/mL Control standard endotoxin (CSE) was reconstituted in 1 mL of BET water and homogenized for 15 min. The CSE solution was then serially diluted to obtain a concentration of 1 EU/mL, 0.5 EU/mL, 0.25 EU/mL, 0.125 EU/mL, 0.0625 EU/mL, 0.0312 EU/mL, and 0.0156 EU/mL. These dilutions were used for the confirmation test and the spiking experiment in milk and groundwater samples.

Three types of samples were utilized in this study, including raw milk, pasteurized milk, and groundwater. All samples were stored at 4 °C before analysis. Before testing, milk samples were gently homogenized by inversion to ensure an even distribution of endotoxins. Groundwater samples were filtered using a 0.22 μm pyrogen-free membrane filter to remove particulate contaminants.

The applicability of CAL and TAL for detecting endotoxins in milk and groundwater was evaluated using two experimental approaches. First, in the spiking assay, each sample (raw milk, pasteurized milk, or groundwater) was spiked with CSE at predefined concentrations (1 EU/mL, 0.5 EU/mL, 0.25 EU/mL, 0.125 EU/mL, 0.0625 EU/mL, 0.0312 EU/mL, and 0.0156 EU/mL) to assess assay sensitivity. After CSE addition, the samples were vortexed for 15 s to ensure homogenization before being subjected to the BET assay using CAL or TAL. Second, in direct testing, raw milk, pasteurized milk, and groundwater samples were tested without CSE addition to identify naturally occurring endotoxins. The samples were analyzed directly using the BET gel-clot method. For both experimental approaches, the BET gel-clot assay was performed as follows:

(1) 100 μL of each prepared sample (either CSE-spiked or untreated) was mixed with 100 μL of CAL or TAL reagent in a 1.5-mL pyrogen-free Eppendorf tube.
(2) A negative control (BET water without CSE) and a positive control (known endotoxin concentration) were included.
(3) The mixtures were gently homogenized and incubated at 37 °C for one hour without disturbance.
(4) After incubation, clot formation was assessed based on the established grading criteria (Fig. 3).

The clot formation was assessed following the grading criteria established in the previous studies (Bishop and White, 1986; Tinker-Kulberg et al., 2020), namely no noticeable increase in viscosity/opacity (−); weak gel with slight to moderate opacity, and starch-like flocculants adhesion to the tube sides when obliqued (±); soft gel with moderate to considerable opacity, and tube gluing when rotated 90° (+); and firm gel with considerable opacity, remaining stable after being rotated 180° (++).

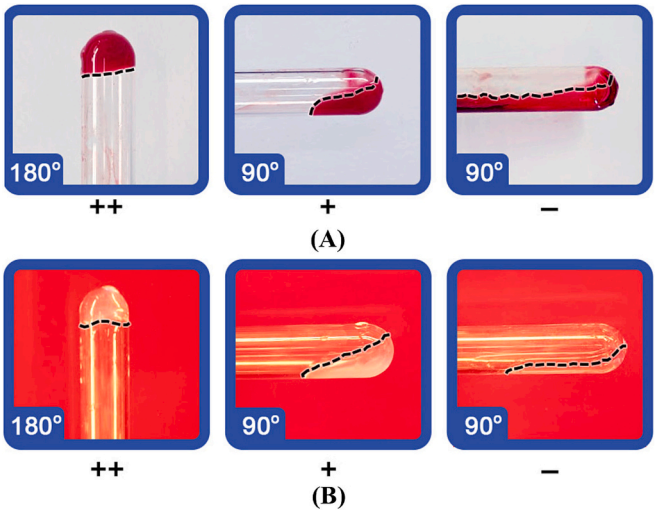

Fig. 3. Visual clot grading of LAL in the presence of bacteria or LPS in (A) human blood or (B) a standard control (endotoxin-free water) (Bishop and White, 1986; Tinker-Kulberg et al., 2020). The clot grade consists of no noticeable increase in viscosity/opacity (−); weak gel with slight to moderate opacity, and starch-like flocculants adhesion to the tube sides when obliqued (±); soft gel with moderate to considerable opacity, and tube gluing when rotated 90° (+); and firm gel with considerable opacity and stayed stable after rotated 180° (++).

3. Results and discussion

Among the 94 blood samples of horseshoe crabs, 30 % (28 samples) successfully detected endotoxin at all four tested concentrations of control standard endotoxin (CSE), while 25 % (24 samples) detected endotoxin at three concentrations, and 15 % (14 samples) at two concentrations. Only 13 % (12 samples) detected endotoxin at one concentration. In total, 83 % of the samples showed endotoxin detection capabilities across different CSE concentrations. The remaining 17 % (16 samples) were unable to detect endotoxin at concentrations ranging from 0.125 to 1 EU/mL, as no gelation was observed (Table 1).

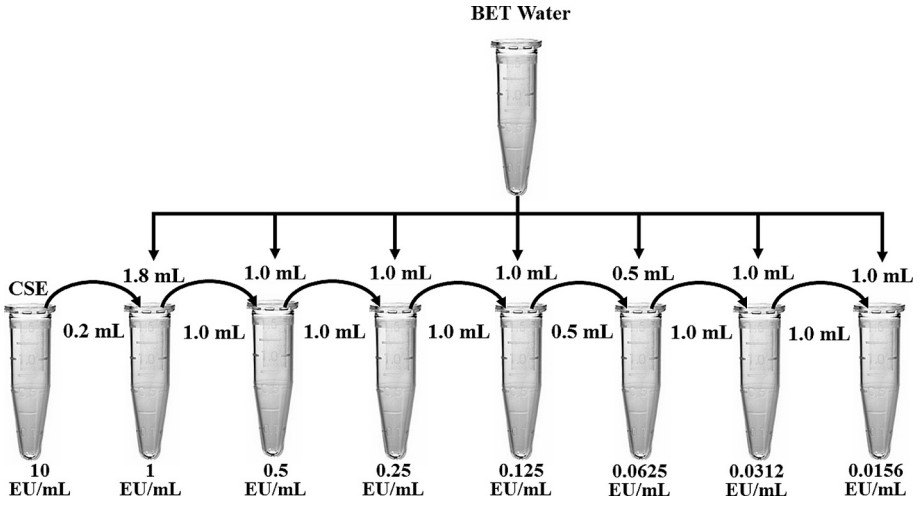

Fig. 2. Serial dilution scheme for bacterial endotoxin concentration in the BET verification test. The dilution process starts with Control Standard Endotoxin (CSE) at 10 EU/mL, followed by stepwise dilution using BET water to achieve final concentrations ranging from 1.0 to 0.0156 EU/mL. Arrows indicate the transfer volumes between dilution steps. The dilution scheme was adapted from Putra et al. (2019).

Table 1
Summary of CAL and TAL assay results for endotoxin detection.

The BET results	CAL ($n = 80$)			TAL ($n = 14$)			Overall Blood Samples ($n = 94$)	
	♂	♀	Σ	♂	♀	Σ	Total	%
Capable of detecting four CSE concentration	9	13	22	5	1	6	28	30
Capable of detecting three CSE concentration	8	15	23	0	1	1	24	25
Capable of detecting two CSE concentration	2	8	10	4	0	4	14	15
Only capable of detecting one CSE concentration	6	5	11	0	1	1	12	13
Not able to detect any CSE concentration	3	10	13	2	1	2	16	17

CSE concentrations tested: 1.0, 0.5, 0.25, and 0.125 EU/mL. CAL = *Carcinoscorpius* amebocyte lysate; TAL = *Tachypleus* amebocyte lysate.

Of the 28 blood samples detecting endotoxin at all four CSE concentrations (Table 2), 22 were CAL samples (♂ = 9; ♀ = 13), while 6 were TAL samples (♂ = 5; ♀ = 1). Similarly, among the 24 samples that detected endotoxin at three concentrations, 23 were CAL samples (♂ = 8; ♀ = 15), and only 1 was a TAL sample (♂ = 0; ♀ = 1). For those detecting endotoxin at two concentrations, 10 were CAL samples (♂ = 2; ♀ = 8), and 4 were TAL samples (♂ = 4; ♀ = 0). Finally, of the 16 samples that failed to detect endotoxin, 13 were CAL samples (♂ = 3; ♀ = 10), and 3 were TAL samples (♂ = 2; ♀ = 1). Fig. 4 provides a visual representation of lysate gelation in CAL samples in response to different CSE concentrations, highlighting the differences between positive and negative endotoxin detection.

In total, 84 % of the CAL samples (66 CAL samples; ♂ = 25; ♀ = 41) and 80 % (12 TAL samples; ♂ = 9; ♀ = 3) were able to detect endotoxin. All positive samples exhibited a coagulation response lasting over 3 h, consistent with prior findings by Putra et al. (2019), which demonstrated TAL's ability to detect endotoxins at concentrations as low as 0.25 and 0.5 EU/mL.

This study confirms the capability of both CAL and TAL lysates to detect endotoxins via the gel clot method. These findings align with Huovinen et al. (1990), who reported that the source species does not significantly affect the performance of LAL or TAL reagents in the gel clot assay. Furthermore, TAL derived from *T. tridentatus* was shown to be as effective as LAL derived from L. *polyphemus*, and CAL has been proposed as a viable alternative to LAL in endotoxin testing (Rao and Bhagirathi, 1989).

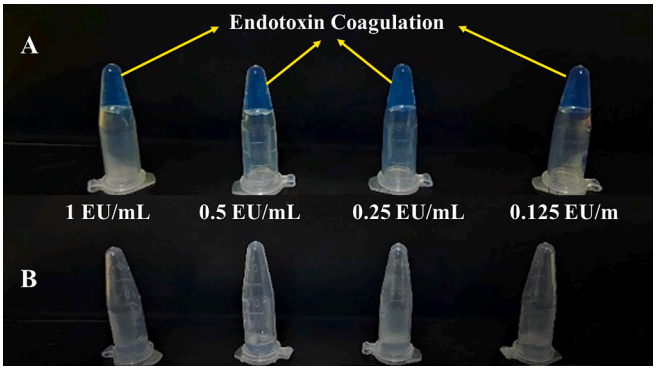

Fig. 4. Visual examples of lysate gelation in response to control standard endotoxin (CSE) concentrations: (A) Positive gel formation at varying CSE concentrations (1.0–0.125 EU/mL), indicating endotoxin presence; (B) No gel formation in the absence of endotoxin, indicating a negative result.

Among the 28 samples that successfully detected endotoxin across four CSE concentrations (0.125–1 EU/mL), 23 samples (17 CAL, 6 TAL) were further tested at lower concentrations (0.0625, 0.0312, and 0.0156 EU/mL), as shown in Table 3. At these lower concentrations, 43.5 % of the samples ($N_{CAL} = 7$; $N_{TAL} = 3$) detected endotoxin at all tested levels (0.0156–0.0625 EU/mL). A smaller fraction, 4.3 % ($N_{CAL} = 1$), detected endotoxin at two concentrations (0.0156–0.0312 EU/mL), while 13 % ($N_{CAL} = 1$; $N_{TAL} = 2$) detected endotoxin only at 0.0625 EU/mL. However, 39.1 % ($N_{CAL} = 8$; $N_{TAL} = 1$) were unable to detect endotoxin at these lower concentrations. This outcome is consistent with findings

Table 3
The assay results of horseshoe crabs' blood for detecting endotoxin concentrations up to 0.0156 EU/mL. Clot ratings followed the previous study (Bishop and White, 1986; Tinker-Kulberg et al., 2020).

CSE Concentration (EU/ml) and clots rating			Horseshoe Crab				Total	Percentage (%)
			CR		TG			
0.0625	0.0312	0.0156	♂	♀	♂	♀		
++	++	++	5	2	3	0	10	43.5
++	++	–	1	0	0	0	1	4.3
++	–	–	0	1	1	1	3	13.0
–	–	–	2	6	1	0	9	39.1
	Total (N)		8	9	5	1	23	100

CR is *Carcinoscorpius rotundicauda,* TG is *Tachypleus gigas,* ++ is firm gels, + is soft gels, ± is weak clots - is no gelation, ♂ is male, and ♀ is female.

Table 2
The test results of horseshoe crabs' blood for detecting endotoxins according to CSE concentration, species, and sex. Clot ratings followed the previous study (Bishop and White, 1986; Tinker-Kulberg et al., 2020).

The number of CSE concentration	CSE Concentration and clots rating (EU/ml)				Horseshoe Crab				Total	Overall
					CR		TG			
	1	0,5	0,25	0,125	♂	♀	♂	♀		
4	++	++	++	++	9	13	5	1	28	28
3	++	++	++	–	3	3	0	1	7	24
	++	++	–	++	2	2	0	0	4	
	++	–	++	++	1	3	0	0	4	
	–	++	++	++	2	7	0	0	9	
	++	++	–	–	1	3	2	0	6	
	–	–	++	++	0	2	0	0	2	
2	–	++	++	–	1	2	1	0	4	14
	++	–	++	–	0	1	0	0	1	
	++	–	–	++	0	0	1	0	1	
	++	–	–	–	1	2	0	0	3	
1	–	++	–	–	3	2	0	1	6	12
	–	–	–	++	2	1	0	0	3	
0	–	–	–	–	3	10	2	1	16	16

CR is *Carcinoscorpius rotundicauda,* TG is *Tachypleus gigas,* ++ is firm gels, + is soft gels, ± is weak clots - is no gelation, ♂ is male, and ♀ is female.

from Santosa et al. (2020), where the gel clot LAL assay demonstrated sensitivity to endotoxin concentrations as low as 0.01 EU/mL.

Endotoxin tests were applied to various liquid samples, including raw milk, pasteurized milk, well water, and L1 HPV 52 protein, using blood samples that could detect endotoxins at 0.0156 EU/mL (Table 4). Notably, no endotoxins were detected in pasteurized milk or L1 HPV 52 protein, while all samples from *C. rotundicauda* and *T. gigas* successfully detected endotoxins in well water. Interestingly, 20 % ($n = 2$) of the blood samples detected endotoxin in raw milk. These results align with previous research by John et al. (2012), which demonstrated TAL's application in detecting endotoxins in various biological liquids. Furthermore, the simplicity of the gel-clot method allows for potential on-site application by directly adding a few drops of water into pre-filled tubes containing the amebocyte reagent. This approach enables preliminary endotoxin detection at the point of collection, facilitating immediate assessment while researchers still have access to additional relevant samples. The detection of endotoxins in raw milk is particularly relevant for distinguishing between Gram-negative and Gram-positive mastitis, which is crucial for clinical management (Flórez et al., 2023). A rapid and robust endotoxin quantification method could contribute to the early diagnosis of coliform mastitis in dairy cows, thereby improving treatment strategies. Previous studies have validated the use of kinetic turbidimetric assays based on LAL for endotoxin quantification in milk, demonstrating its effectiveness in minimizing interference from milk components (Flórez et al., 2023). Our findings further support the potential of CAL and TAL as alternative tools for endotoxin detection in raw milk, which may provide a more sustainable and regionally accessible solution. Given these promising results, it is essential to consider the broader implications of relying on amebocyte lysates for endotoxin testing, particularly in the context of sustainability and conservation.

The global reliance on TAL for endotoxin detection underscores the important role of horseshoe crabs in the endotoxin testing industry, particularly in emerging markets (Gauvry, 2015). However, the species used to produce TAL are facing serious population declines due to overharvesting and habitat loss (Gauvry, 2015; John et al., 2021; Laurie et al., 2019). This reliance on a dwindling resource highlights the urgent need for alternative endotoxin detection methods and sustainable practices in the industry (Gauvry, 2015). In response to these challenges, this study demonstrates the potential of CAL and TAL as viable alternatives to LAL, particularly in Southeast Asia, where these species are more abundant.

Overall, our findings confirm that CAL and TAL can detect endotoxins at concentrations as low as 0.0156 EU/mL using the gel-clot method. Unlike conventional LAL assays that rely *on* L. *polyphemus*, CAL and TAL offer a sustainable regional alternative that reduces dependence on the declining populations of American horseshoe crabs. The ability of CAL and TAL to detect endotoxins in milk and groundwater further expands their potential applications beyond conventional bacterial endotoxin testing. The sensitivity of CAL and TAL in detecting endotoxins at lower concentrations is comparable to or even surpasses the detection limits reported for LAL assays, which typically range between 0.03 and 0.06 EU/mL (Dehghan et al., 2023).

These findings provide critical foundational knowledge for advancing recombinant Factor C (rFC)-based endotoxin detection, which offers a sustainable, animal-free alternative. The U.S. Patent US6645724B1 employs fluorometric and chromatographic techniques for endotoxin quantification, while the European Patent EP1409984B1 utilizes an rFC-based assay to replace traditional horseshoe crab lysates. In contrast, our study focuses on the direct use of CAL and TAL lysates through a gel-clot assay, offering a simpler and cost-effective alternative for endotoxin detection. Unlike these patents, which primarily target pharmaceutical and clinical applications, our study broadens the scope to include environmental monitoring (groundwater) and food safety assessment (raw and pasteurized milk). By demonstrating the efficacy of CAL and TAL in diverse sample types, this study highlights the potential of these lysates for real-world applications beyond biomedical settings. This approach reinforces the significance of our findings in reducing reliance on traditional LAL assays while promoting more sustainable and ethical endotoxin testing methodologies.

This study introduces a novel and state-of-the-art approach to endotoxin detection by utilizing CAL and TAL as alternatives to LAL. Unlike conventional methods that are exclusively LAL-based, our research demonstrates that CAL and TAL exhibit comparable sensitivity in detecting endotoxins, with a detection threshold as low as 0.0156 EU/mL. The methodology presented here builds upon and refines existing techniques by validating the efficacy of CAL and TAL lysates through gel-clot assays applied to various biological (milk) and environmental (groundwater) samples. Additionally, this study introduces a field-adaptable approach, where a few drops of a sample can be directly tested in pre-filled amebocyte reagent tubes, allowing for real-time endotoxin assessment during sample collection. Unlike traditional LAL-based assays that require controlled laboratory conditions, this CAL/TAL-based method allows for more flexible and immediate detection of endotoxins, particularly in field settings. In contrast to previously reported studies, which have primarily focused on LAL-based assays, this research highlights a significant advancement by expanding the application of CAL and TAL beyond conventional bacterial endotoxin testing.

These findings are significant as foundational scientific information for further development of CAL and TAL-based assays, particularly in the context of rFC development. The potential use of rFC could help reduce the need for harvesting horseshoe crabs for lysate production, promoting more ethical practices in the biomedical industry while offering a more accurate, sensitive, and reliable method for endotoxin detection. By bridging the gap between natural lysate-based assays and recombinant alternatives, this study plays a crucial role in shaping the future of endotoxin detection technologies.

Table 4
The BET application toward several drinking waters and L1 HPV 52 protein using CAL/TAL samples which have been proven capable of detecting endotoxin up to the CSE concentration of 0.0156 EU/mL. Clot ratings followed the previous study (Bishop and White, 1986; Tinker-Kulberg et al., 2020).

Station	Blood code	Species	Sex	Raw Milk	Pasteurized Milk	Well water	L1 HPV 52 protein
3	8C	TG	♂	−	−	++	−
4	26E	CR	♀	−	−	++	−
6	12D	CR	♂	−	−	++	−
9	37G	CR	♂	−	−	++	−
10	33G	TG	♂	++	−	++	−
11	38G	CR	♂	−	−	++	−
	39F	TG	♂	−	−	++	−
12	14	CR	♂	−	−	++	−
13	25	CR	♂	++	−	++	−
15	39	CR	♀	−	−	++	−

CR is *Carcinoscorpius rotundicauda,* TG is *Tachypleus gigas,* ++ is firm gels, + is soft gels, ± is weak clots - is no gelation, ♂ is male, and ♀ is female. Pasteurized milk was obtained from commercially available products.

4. Conclusion

This study demonstrated that CAL and TAL assays effectively detected endotoxin in milk and groundwater, achieving a sensitivity of up to 0.0156 EU/mL) using the gel-clot test. These findings highlight the potential of CAL and TAL as viable alternatives to LAL assays, supporting efforts to reduce reliance on *Limulus* species for bacterial endotoxin testing. Compared to previously published methods, including those described in U.S. Patent US6645724B1 and European Patent EP1409984B1, this study presents a distinct advantage by utilizing alternative horseshoe crab species, thereby expanding the resource base for endotoxin detection. Moreover, this research provides valuable insights for advancing the development of rFC-based assays, which offer a sustainable, animal-free alternative with consistent performance and minimal ecological impact, aligning with global efforts to promote ethical and environmentally responsible biomedical testing.

Ethics statements

This study was conducted following ethical guidelines and regulations. Ethical approval was obtained from the Medical and Health Research Ethics Committee, Sriwijaya University with approval number: 126–2021.

CRediT authorship contribution statement

Fauziyah: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Apon Zaenal Mustopa:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis. **Fatimah:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation. **Nabila Aprianti:** Writing – review & editing, Visualization, Validation, Data curation. **Rahmi Damarani:** Writing – original draft, Visualization, Software, Resources, Methodology, Formal analysis. **Amanda Astri Pratiwi Febrianti:** Writing – review & editing, Validation, Supervision, Resources, Investigation, Data curation. **Dina Permata Wijaya:** Writing – review & editing, Visualization, Validation, Resources, Methodology. **Fitri Agustriani:** Writing – review & editing, Validation, Software, Resources, Investigation, Data curation. **Rozirwan:** Writing – review & editing, Visualization, Validation, Methodology, Investigation.

Declaration of generative AI and AI-assisted technologies in the writing process

Generative Artificial Intelligence (AI) or AI-assisted technologies were not used in the writing or generation of any of the sections of this manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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