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Rapid and sensitive detection of staphylococcal enterotoxin E using a time-resolved fluorescence immunochromatography assay

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Abstract

Staphylococcal food poisoning is a significant foodborne illness caused by staphylococcal enterotoxins (SEs). Immunoassays have become the primary method for rapidly detecting harmful bacteria and toxins because of their excellent sensitivity and specificity. However, these assays have limitations in that they cannot differentiate between types of SEs and do not provide rapid, on-site, quantitative testing. In this study, a time-resolved fluorescence immunochromatography assay (TRFICA) was developed specifically for detecting staphylococcal enterotoxin E (SEE), which is commonly found in dairy products. Compared with a double antibody sandwich enzyme-linked immunosorbent assay, which had a detection limit of 0.028 ng/mL, TRFICA demonstrated comparable sensitivity, enabling SEE quantification with a detection limit as low as 0.081 ng/mL in infant formula. Validation by spiking infant formula samples confirmed no cross-reactivity with analogs (recoveries ranged from 93.17% to 128.77%). Furthermore, with an 8-min reaction time and interpretation delivered by a portable TRFICA strip reader, our method demonstrates potential for use in mobile and on-site detection. This study describes a rapid, easy, and reliable method for detecting trace levels of SEE in infant formula, which could serve as an early screening tool toward preventing food poisoning in infants and children.

Keywords Staphylococcus enterotoxin E, Time-resolved fluorescence immunochromatography assay, Double antibody sandwich ELISA, Infant formula

Introduction

Staphylococcal food poisoning (SFP) has emerged as a significant foodborne illness primarily because of the presence of staphylococcal enterotoxins (SEs) produced by *Staphylococcus aureus* [1]. This includes classical SE serotypes such as staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C (SEC) (including variants SEC1, SEC2, and SEC3), staphylococcal enterotoxin D (SED), and staphylococcal enterotoxin E (SEE), collectively responsible for nearly 95% of SFP outbreaks. Notably, dairy-related cases are predominate [2–4]. For instance, in 2004, a significant outbreak in China was linked to powdered infant formula

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contaminated with SEs, affecting approximately 150 infants and young children [5]. Many of these young children were hospitalized due to distressing symptoms such as vomiting, diarrhea, and fever. Consequently, in 2005, the European Union introduced legislation to protect individuals from harmful SE residues. It specifically mandated that SEs must not be detected in five independent 25 g samples taken from milk products [6].

Among the classical SE types, SEE, in particular, has gained prominence as one of the leading causal agents of SFP worldwide. Among 38 suspected SFP incidents, SEE has been detected in cultures of *Staphylococcus aureus* recovered from 17 cases [7]. SEE has been implicated in SFP outbreaks in several countries, including the USA, UK, and France [8, 9]. Notably, SEE has been consistently detected in milk and dairy products. In 2009, for instance, six SFP outbreaks in France were traced back to SEE contamination of soft cheese made from unpasteurized milk. Even more concerning is the presence of SEE in infant formula [10, 11], which poses a significant health risk to infants. Given that infant formula is considered a non-sterile and ready-to-eat product, the potential presence of SEE in milk-derived infant formula poses a substantial health risk, especially to infants and young children. Hence, routine screening for SEE in infant formula is crucial for ensuring food safety and maintaining stringent quality standards.

Various analytical methods have been developed for the detection of SEs, such as high-performance liquid chromatography (HPLC) [12] and liquid chromatography tandem mass spectrometry [13, 14]. While these instrumental methods provide high levels of sensitivity and precision, their complexity, slow processing time, and the need for expensive equipment and skilled personnel presents challenges. These features diminish their suitability for carrying out rapid, on-site testing of large sample batches. In contrast, antibody-based immunoassays, such as the enzyme-linked immunosorbent assay (ELISA) [15–17] and the lateral flow immunochromatographic assay (LFIA) [18–20], are favored for toxin detection in food because of their simplicity, sensitivity, and accuracy. Since 2017, a standardized screening method to detect SEA, SEB, SECs, SED and SEE in foodstuffs, EN ISO 19020, has been applied internationally [21]. This standard applies to the commercial Ridascreen® Staphylococcal Enterotoxin (SET) Total kit and the Vidas® Staphylococcal Enterotoxin II (SET2) kit for detecting five classical SEs in food samples. However, there remains a scarcity of products that offer rapid, on-site, quantitative detection of SEE in infant formula.

The lanthanide-based time-resolved fluorescence immunochromatography assay (TRFICA) represents a significant advancement in the field, facilitating rapid, on-site point-of-care surveillance and diagnosis [22]. It effectively meets the demand for sensitive, rapid, specific, affordable, and user-friendly detection. Furthermore, when compared with conventional colorimetric markers and fluorescent labels, lanthanide chelates offer several advantages, including an exceptionally long fluorescence decay time and an remarkably large Stokes' shift, which are instrumental in eliminating background fluorescence. These attributes collectively contribute to the high sensitivity and accuracy of this assay type [23, 24]. Recently, thanks to the ongoing development of time-resolved fluorescence detectors, the high cost that previously hindered widespread TRFICA adoption has diminished. Consequently, TRFICA strips have undergone rapid enhancements and are now widely available, even in supermarkets [25, 26]. As a result of these advancements, the TRFICA demonstrates promising potential to become a rapid, on-site method for quantitatively detecting SEE in infant formula.

In this study, we designed a new TRFICA for the detection of SEE and compared it with the currently widely used double antibody sandwich ELISA (DAS-ELISA) method, considered the gold standard for immunoassay-based detection methods.

Results and discussion

Recombinant SEE protein expression in *E. coli*

The nucleotide sequence encoding SEE from *Staphylococcus aureus*, see (GenBank accession number: WP_000750405), was ligated into a pET28a vector and transformed into *Escherichia coli* (*E. coli*) *BL21* for expression (Fig. 1A). After purification using Ni^{2+} affinity chromatography, an optimized concentration of 120 mM imidazole was used for the elution of the expressed SEE protein. This yielded a distinct protein band in the 27–34 kDa range, consistent with SEE's theoretical size, without additional nonspecific bands (Fig. 1B) [27]. The purity of the recombinant SEE proteins was assessed via SDS-PAGE analysis, confirming a purity level exceeding 90%.

Screening and characterization of anti-SEE antibodies

Based on indirect competitive enzyme-linked immunosorbent assay (icELISA) analysis, the antisera from two of the eight immunized mice exhibited the best inhibition rates and were thus chosen for B cell-myeloma cell fusion. A total of eight hybridoma cell lines were successfully generated after three subcloning steps following the limiting dilution method. SDS-PAGE analysis confirmed that all eight purified monoclonal antibodies (mAbs) displayed characteristic 50-kDa (heavy chain) and 25-kDa (light chain) bands, with extraneous proteins from the ascites entirely eliminated (Fig. S1). The analysis of half-maximal inhibitory concentration (IC_{50}) values and titers

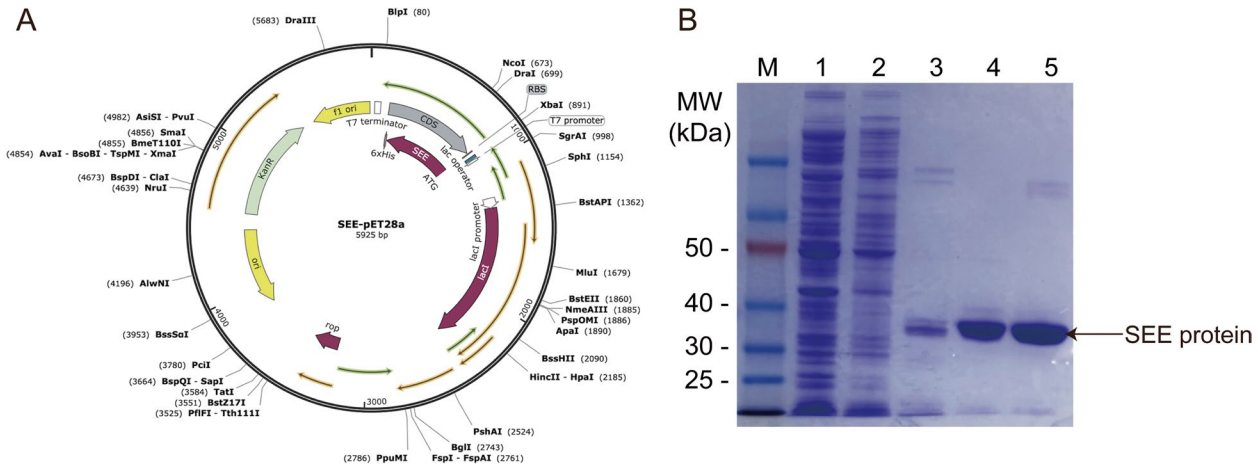

Fig. 1 Schematic illustration and construction of the recombinant SEE protein. **A** A schematic map of SEE-Pet28a. **B** SDS-PAGE analysis of the purification of recombinant SEE protein. Lane 1: Binding buffer. Lane 2–5: SEE proteins eluted by 30 mM, 80 mM, 120 mM and 500 mM imidazole, respectively. M: molecular weight marker

for the produced mAbs and polyclonal antibody (pAb) are presented in Table S1–3. The eight mAbs exhibited IC_{50} values for SEE ranging from 41 to 513 ng/mL, while one pAb showed an IC_{50} value of 125 ng/mL. Notably, mAb 9A2 exhibited the highest affinity for SEE, with an IC_{50} value of 41.152 ng/mL.

Optimization and establishment of DAS-ELISA for SEE

The pAb and eight mAbs were selected for antibody pairing. Matched antibody pairs are sets of antibodies that can recognize the same antigen, but with different epitopes or binding regions, and they are commonly used for capture and detection in sandwich ELISA or related immunoassays. Among the tested antibodies, a maximum positive/negative (P/N) value of 5.998 ± 0.127 was obtained using the mAb 1A5 and pAb (Fig. 2A). Therefore, these antibodies were selected for the development of DAS-ELISA for SEE. As shown in Fig. 2, the results of our checkerboard titration revealed that the P/N value was highest when the pAb concentration was 2.5 μg/mL and the mAb (0.5 μg/mL) dilution was 1:1000. Thus, these concentrations were selected for DAS-ELISA. Screening of various coating conditions and blocking agents found that coating at 37 °C for 2 h and blocking with 2% skimmed milk led to higher P/N values. The optimal reaction time for SEE and antibody detection was then evaluated under these conditions. Our results indicated that the P/N value gradually increased with the antigen reaction time, peaking at 60 min. Therefore, an antigen reaction time of 60 min was selected. As shown in Fig. 2G, the highest P/N value was observed at a detection antibody reaction time of 30 min. Finally, the HRP-labeled streptavidin dilution (1:1000 to 1:5000) was optimized, and we found that the P/N value was highest at a dilution of 1:3000 (Fig. 2H).

Optimization and establishment of TRFICA for SEE

To optimize the analytical performance of TRFICA, various factors were explored, including the detection antibody amount, dilutions of coating mAb, sample pad material, sample volume and detection time. The fluorescence intensity of the test lines (T lines) and the inhibition rate were used to evaluate the impact of these specific factors on TRFICA performance. Higher T values and inhibition rates indicate better sensitivity. When testing 2, 4, 8, and 16 μL of 5.67 mg/mL pAb, we showed that only 8 and 16 μL achieved a desirable and consistent signal intensity, while 16 μL showed superior signal intensity and inhibitory effect compared with 8 μL (Fig. 3A). We then investigated the most appropriate dilution for the coating mAb to enable us to minimize nonspecific adsorption and reduce false positive results [28]. The results (Fig. 3B) showed that increasing the coating mAb dilution decreased the signal intensity of T line as expected. We concluded that the T line signal intensity of the one-fold diluted mAb was too strong, which is not conducive to detecting trace amounts, and therefore a twofold dilution of mAb was employed.

A sample is applied to the TRFICA strip at the sample port, and then flows onto a sample pad. It is important to note that an even distribution and moderate transfer rate of the sample are crucial to accuracy. Different sample pad materials can influence both the release speed of the signal label and the liquid hold-up capacity, affecting sensitivity [29]. To optimize the TRFICA, three types of sample pad materials were tested: hemofiltration

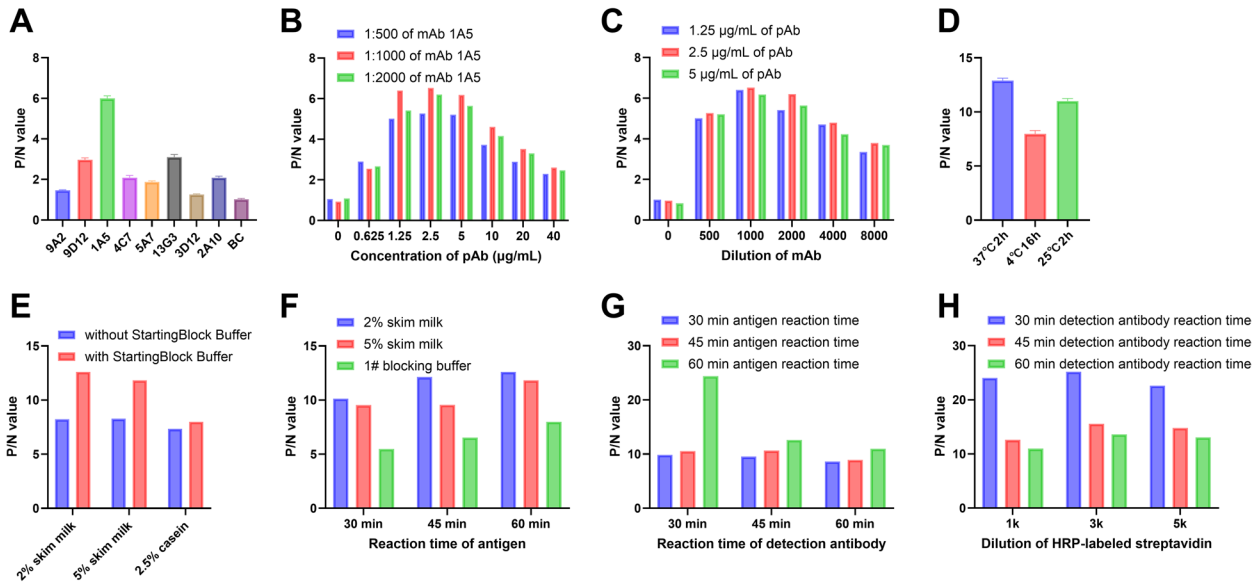

Fig. 2 Optimization of DAS-ELISA reaction conditions (illustrations in color). The maximum positive/negative (P/N) values were selected as the optimal reaction conditions. P/N > 2.1 represents a positive result. **A** Paired antibody selection. The concentrations of the reagents were as follows: rabbit pAb at 5.67 μg/mL, mAbs at 0.5 μg/mL, and SEE standards at 1 ng/mL. **B** Optimization of the concentration of pAb as capture antibody. **C** Optimization of the dilution of mAb as detection antibody. **D** Optimization of coating conditions. **E** Blocking agent selection. **F** Optimization of the reaction time of antigen. **G** Optimization of the reaction time of detection antibody. **H** HRP-labeled streptavidin dilution optimization

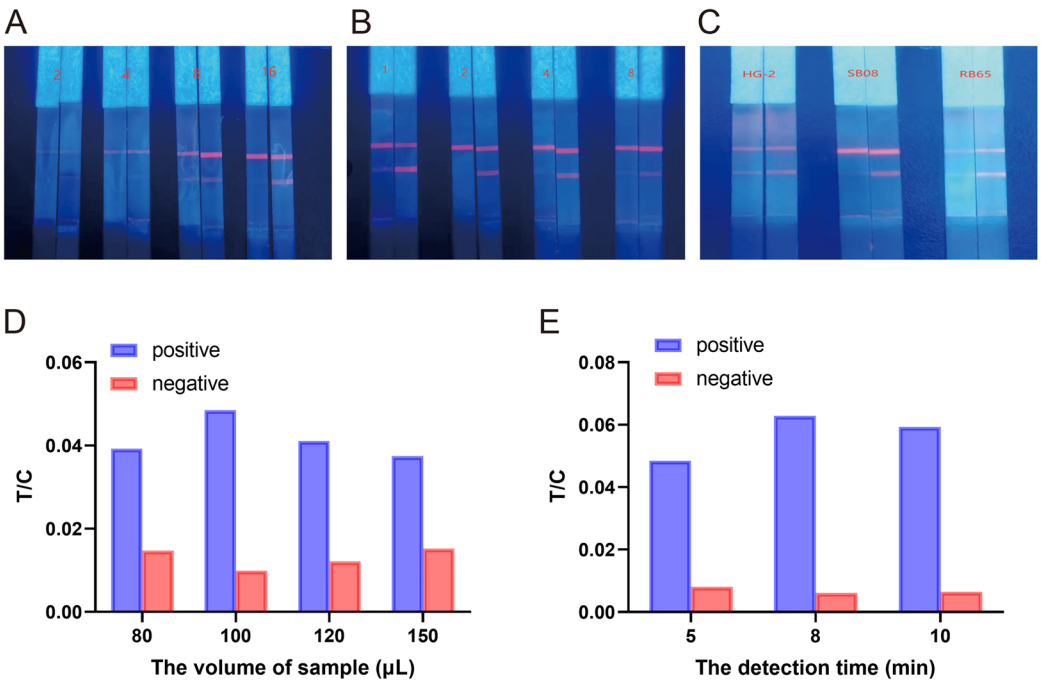

Fig. 3 Optimization of TRFICA reaction conditions (illustrations in color). Values of both positive samples (1 ng/mL SEE) and negative samples (without SEE spiking) were taken into consideration to select the optimal parameters. **A** Amount of detection antibody (2 μL, 4 μL, 8 μL, 16 μL of 5.67 mg/mL pAb). **B** Concentration of coating mAbs (1-, 2-, 4-, or eightfold dilution of mAb 1A5). The concentration of mAb 1A5 was 4.13 mg/mL. **C** Optimization of the sample pad materials (HG-2, SB08, and RB65). **D** Optimization of the sample volume (80 μL, 100 μL, 120 μL, 150 μL). **E** Optimization of the detection time (5 min, 8 min, 10 min). T/C is the ratio of the signal intensity of the test line (T line) to the control line (C line)

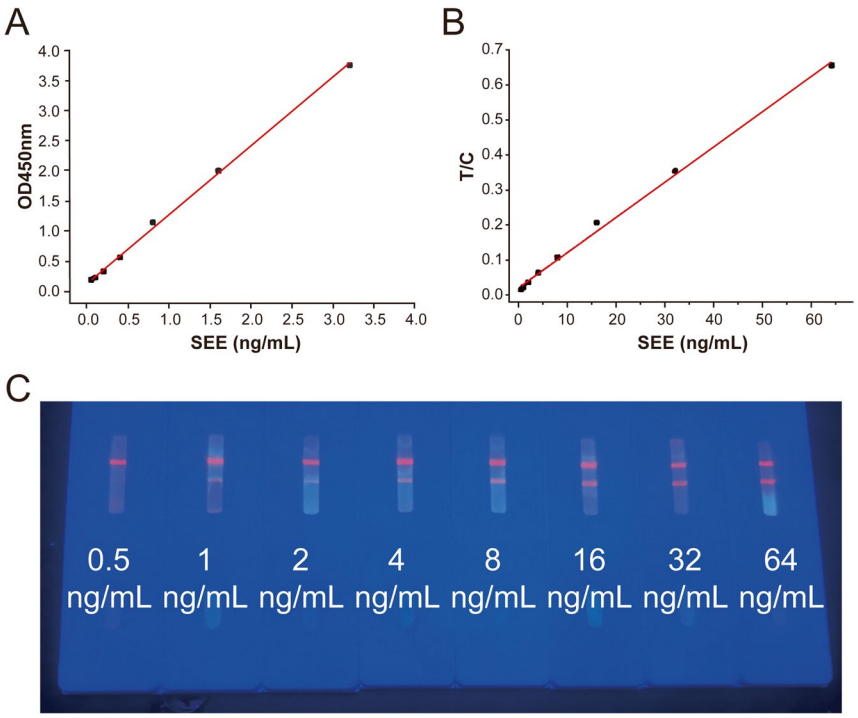

Fig. 4 Standard calibration curve of SEE by (**A**) DAS-ELISA and (**B**, **C**) TRFICA by plotting the absorbance and fluorescence intensity ratio of a series of SEE concentrations (illustrations in color). T/C is the ratio of the signal intensity of the test line (T line) to the control line (C line)

membrane (HG-2) and two glass fiber types (SB08 and RB65). The results (Fig. 3C) showed that SB08 achieved a desirable signal intensity and inhibition effect, while HG-2 and RB65 were deemed unsuitable because of poor signal strength.

The sample volume plays a crucial role in TRFICA, affecting the color development, speed, and sensitivity of the process. The signal label release rate can also affect detection sensitivity by allowing shorter or longer incubation times. This study determined that the optimal parameters for the established TRFICA were a sample volume of 100 µL (Fig. 3D) and a TRFICA reaction time of 8 min (Fig. 3E). These optimized conditions enabled our TRFICA to achieve the required fluorescence levels.

Sensitivity

To establish the standard curves for TRFICA and DAS-ELISA, a series of SEE standards were adjusted for TRFICA (0.5–64 ng/mL) and DAS-ELISA (0.05–3.2 ng/mL). The standard curve for TRFICA for SEE was linear ($y=0.01008x+0.0205$, $r^2=0.9978$) in the range of 0.5–64 ng/mL (Fig. 4B, C). The standard curve for DAS-ELISA exhibited a linear range of 0.2–3.2 ng/mL for SEE, with a correlation coefficient of 0.998 (Fig. 4A). Although the limit of detection (LOD) for the TRFICA (0.081 ng/mL) was found to be slightly higher than that of DAS-ELISA (0.028 ng/mL), the detection range was significantly wider for TRFICA (0.5–64 ng/mL) compared with DAS-ELISA (0.05–3.2 ng/mL). In a different study, comparable results were observed when using a time-resolved fluorescence immunoassay (TRFIA) to detect microcystin. The TRFIA was found to improve sensitivity and expand the detection range in comparison to ELISA and HPLC [30]. Furthermore, in various other studies that have aimed to establish TRFICAs for detecting C-peptides, procalcitonin, plasma soluble growth-stimulating gene protein 2, chlorpromazine, and fumonisin B1, consistently wide detection ranges were observed of approximately two orders of magnitude [31–35]. Our study indicates that methods using different labels could influence assay sensitivity and detection range, even when employing the same identical antibodies. Compared with DAS-ELISA, the TRFICA for SEE not only maintained similar sensitivity but also broadened the detection range, demonstrating greater adaptability.

Specificity

Additional experiments were conducted to determine the specificity of the established SEE TRFICA and DAS-ELISA by checking their cross-reactivity (CR) with the various alternative SE serotypes. As shown in Fig. 5, no CR was observed in the established DAS-ELISA systems, except for that between SEE and SED

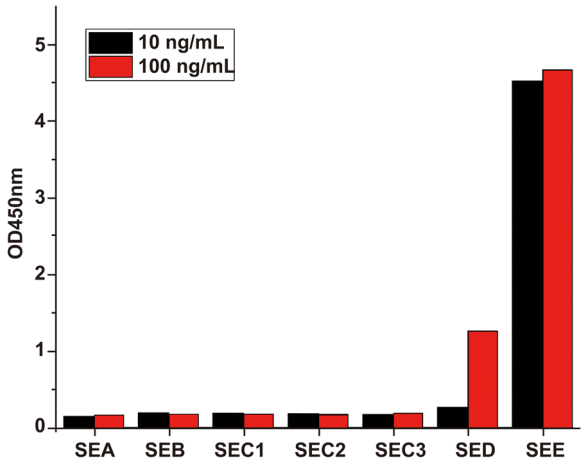

Fig. 5 The cross-reactivity of DAS-ELISA with different SE serotypes (illustrations in color). The concentrations of the cross-reacting serotypes were 10 ng/mL and 100 ng/mL

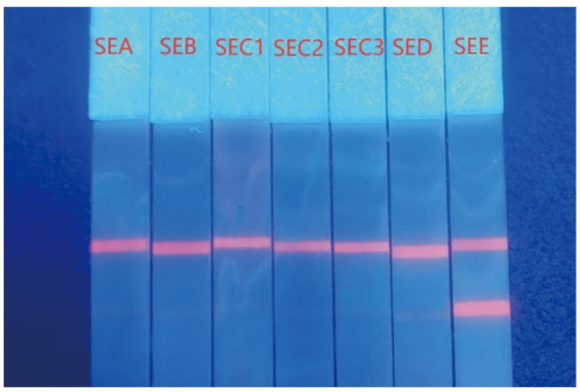

Fig. 6 The cross-reactivity of TRFICA with different SE serotypes (illustrations in color). The concentrations of the cross-reacting enterotoxins were 100 ng/mL

(only 1% CR at 100 ng/mL). The primary reason for this cross-reactivity is that SED is similarly homologous to SEE [36]. Furthermore, our established TRFICA showed no CR with other comparative antigens (Fig. 6), demonstrating better specificity for SEE detection compared with DAS-ELISA.

It is important to note that, in the development of TRFICA and DAS-ELISA, we employed opposite antibody configurations. In DAS-ELISA, we used a pAb as the capture antibody and a mAb as the detection antibody to achieve higher specificity. Conversely, in the TRFICA method, we used mAb as the capture antibody and pAb as the detection antibody, a configuration chosen based on preliminary experiments that demonstrated stronger signals with this setup. Additionally, mAbs, because of their higher specificity for antigen binding, could be used as capture antibodies to achieve high selectivity in capturing target analytes. As a detection antibody, pAb would amplify the detection signal by binding to multiple sites on the analytes [37].

This difference in antibody configuration might have significantly impacted the specificity and signal intensity of the assays. For instance, we observed some CR in DAS-ELISA, whereas no CR was detected in TRFICA. This could be attributed to higher specificity being enabled when the mAb is used as the capture antibody. This would allow for more precise recognition and capture of the target antigen, thereby reducing nonspecific binding. The use of the pAb as the detection antibody would further enhance signal intensity because of its ability to bind multiple sites on the analyte.

Accuracy and precision

The recoveries and coefficient of variation (CV) of infant formula were used to determine the accuracy and precision of both TRFICA and DAS-ELISA, as described above [38]. SEE-negative infant formula was spiked with three different brands of infant formula at three different concentrations (2, 4, 8 ng/mL for TRFICA and 0.2, 0.8, 3.2 ng/mL for DAS-ELISA). As can be seen in Table 1, the recoveries of TRFICA ranged from 93.17% to 128.77% in infant formula, with a CV lower than 12%, demonstrating the reliability of the TRFICA for detecting SEE in infant formula. When it came to DAS-ELISA, the recoveries were from 81.48% to 111.91%, with a CV lower than 6%. These results indicated that both methods are acceptable for SEE detection in terms of accuracy and precision.

Comparison of published detection methods for SEE

There have been limited reports on immunoassays specifically designed for SEE detection [15–20]. Most of the reported methods focused on the multi-residue detection of the five classical SEs simultaneously (Table 2). From a food control perspective, detecting multiple SEs has economic and social benefits. However, where a swift diagnosis of acute human poisoning or epidemiological investigations of food poisoning outbreaks are sought, grouped and undifferentiated detection may inhibit deeper epidemiological analysis. Given the importance of all five classical SEs and their association with SFP outbreaks, recent studies have focused on developing detection methods for individual enterotoxins [39, 40]. Commercial kits for specific enterotoxins are also available [41], reflecting the growing demand for targeted assays. Our study specifically focused on SEE because of its frequent association with SFP outbreaks, especially in milk and dairy products, posing a significant risk to infants. Our study has addressed the urgent need for a method to rapidly identify SEE. The development of specific methods for the sensitive

Table 1 Recoveries of SEE from infant formula by DAS-ELISA and TRFICA

Infant formula	DAS-ELISA				TRFICA			
	Spiked (ng/mL)	Found (ng/mL)	Recovery (%)	CV (%)	Spiked (ng/mL)	Found (ng/mL)	Recovery (%)	CV (%)
Yili PRO-KIDO	0.2	0.21	107.18	2.41	2	2.37	118.58	7.58
	0.8	0.79	98.66	3.50	4	4.98	124.46	5.20
	3.2	2.99	93.37	5.63	8	9.76	122.00	4.98
Junlebao LePlatinum K2	0.2	0.20	100.90	3.27	2	2.46	123.01	11.61
	0.8	0.90	111.91	2.67	4	4.87	121.81	10.57
	3.2	2.96	92.35	1.65	8	10.30	128.77	6.88
Friso Prestige	0.2	0.16	81.48	4.60	2	1.86	93.17	5.22
	0.8	0.81	101.66	3.60	4	5.01	125.35	9.05
	3.2	3.17	98.98	3.01	8	10.17	127.08	7.46

Table 2 Comparison of published methods for the detection of SEE

Reference	Method	Target analyte	Linear range of SEE (ng/mL)	LOD of SEE (ng/mL)	Required time
Vidas® SET2 kit [39]	Enzyme-linked fluorescence test (ELFA)	SEA, SEB, SEC, SED, SEE	-	0.025	80 min
Ridascreen® SET Total kit [39]	DAS-ELISA	SEA, SEB, SEC, SED, SEE	-	0.25 (liquid sample), 0.375 (solid sample)	3 h
[20]	Immunochromatographic (ICT)	SEA, SEB, SEC, SED, SEE	10–250	5	15 min
[40]	DAS-ELISA	SEA, SEB, SEC, SED, SEE	0.086–2.5	0.25	2–3 h
[41]	DAS-ELISA	SEA, SEB, SEC, SED, SEE	0.05–100	0.4	> 2 h
This study	DAS-ELISA	SEE	0.2–3.2	0.028	2–3 h
This study	TRFICA	SEE	0.5–64	0.081	8 min

and individual detection of SEE using TRFICA and DAS-ELISA will enable future investigations into the unique role of SEE in SFP outbreaks.

Sensitive methods are crucial for SEE detection. One of the most challenging aspects of immunoassays is finding the right raw materials, i.e., antibodies, as they directly impact sensitivity, specificity, and assay stability. In our study, both TRFICA and DAS-ELISA demonstrated detection limits for SEE superior to those reported in the literature [20, 41–43] and were comparable to the commercial Vidas® SET2 kit. The enhanced performance of TRFICA and DAS-ELISA can be attributed to the development of novel monoclonal and polyclonal antibodies with high affinity for SEE, which contributed significantly to the increased sensitivity of these assays.

It is important to note that antibody-based immunoassays can vary in procedure time and sensitivity because of differences in assay design and the technology used for signal detection. For instance, traditional LFIAs are favored for their ease of use, cost-effectiveness, and rapid format. However, they often suffer from lower sensitivity and specificity compared with ELISA methods [44]. In this context, our study introduced the TRFICA method, which combines the advantages of fluorescence analysis and LFIAs. This novel approach achieved sensitivity levels comparable to ELISA and outperformed traditional LFIAs [20]. Additionally, TRFICA offered the advantage of a significantly shorter reaction time, only eight minutes, making it a simpler and more flexible option for the simultaneous testing of single or multiple samples. This groundbreaking detection methodology holds great potential for SEE detection in various applications, and particularly for the efficient screening of infant formula.

Conclusion

Overall, our developed TRFICA method, which combines fluorescence analysis and lateral flow immunochromatography, offers a valuable solution for on-site detection of SEE in infant formula. It has achieved sensitivity

levels comparable to ELISA, while providing new levels of simplicity, flexibility, and shorter reaction times. To the best of our knowledge, this study represents the first report of TRFICA specifically for SEE analysis using antibodies. Our findings emphasize the significance of careful antibody selection, assay design, and technological approaches in developing sensitive and specific immunoassays for detecting target analytes, especially in the realm of food safety and public health.

Materials and methods

Materials

Highly purified standard toxins, including SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE (purity more than 95%), were obtained from Toxin Technology (Sarasota, FL, USA). Three Chinese infant formulas, namely PROKIDO, LePlatinum K2, and Friso Prestige, were purchased from Yili Group, Junlebao Dairy Company (Shijiazhuang, China), and FrieslandCampina (Amersfoort, the Netherlands). White, opaque, polystyrene 96-well microplates were obtained from Costar Inc. (Milpitas, CA, USA). Goat anti-mouse IgG and TMB were obtained from Jackson ImmunoResearch (West Grove, PA, USA). The time-resolved fluorescence microspheres were commercially obtained from Bangs Laboratories (Fishers, IN, USA). Sample pads (HG-2, RB65, SB08) were purchased from Liangxin Technology Co., Ltd. (Shanghai, China). The HRP conjugation labeling kit was obtained from Abcam (Toronto, Canada). Fetal calf serum and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco BRL (Carlsbad, CA, USA). Polyethylene glycol 1500 (PEG 1500), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), and HRP were purchased from Sigma-Aldrich (St. Louis, MO, USA). The portable TRFICA reader was bought from Nanjing Microdetection Biotech Co., Ltd. (Nanjing, China).

Expression and purification of a recombinant SEE protein

SEE protein expression constructs for bacterial expression were generated by gene synthesis (Genscript). Briefly, gene sequence for SEE was obtained from GenBank (accession no. WP_000750405). The SEE sequences were synthesized and cloned into pET-28a (+) expression vectors. The resulting recombinant plasmid was transformed into the *E. coli BL21* and selected by screening colonies on Luria–Bertani (LB) media containing antibiotics. Bacteria were grown in LB medium at 37 °C with 0.525 g (150 rpm) shaking on a shaking incubator with a rotational radius of 25 mm until they reached the logarithmic phase. Recombinant fusion protein expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Subsequently, the *E. coli* cells were collected and disrupted by sonication. After centrifugation, the expressed SEE protein was purified by nickel affinity chromatography. This was followed by elution with wash buffer (phosphate buffer) containing different concentrations of imidazole (30, 80, 120, and 500 mmol/L) to obtain the pure SEE protein. The purity of the collected samples was verified by SDS-PAGE followed by staining with Coomassie brilliant blue R250. Protein concentration was determined via BCA assay (Thermo Scientific, Waltham, MA, USA).

Recombinant SEE protein immunization of animals

Eight 6-week-old female BALB/c mice were immunized five times every three weeks, following our previously described procedure [45]. This experiment was approved by China Agricultural University and complied with the Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines. In brief, the initial injection consisted of 200 μg of expressed SEE protein emulsified with CFA, succeeded by four subcutaneous injections of 100 μg of expressed SEE protein with IFA. From the second immunization onwards, antisera were obtained from the mice's orbital venous plexus on the tenth day post-immunization, and their affinity was evaluated using icELISA.

Following our previously described procedure [46], a female New Zealand white rabbit was immunized four times to produce pAbs. The rabbit was immunized subcutaneously with 1 mg of expressed SEE protein emulsified with CFA for the first immunization. For the booster immunizations, an equal quantity of the immunogen emulsified with IFA was injected at 2-week intervals. Rabbits were bled through an ear vein 10 days after each booster injection, and the antisera affinity was monitored by icELISA.

The affinity of antisera was determined using icELISA and expressed as inhibition ratio values as follows. The microplates were coated with 100 μL of expressed SEE protein at 37 °C for 2 h. The coating solution in the plates was then discarded. Blocking buffer was then added to the plates (150 μL/well), which were then placed in an incubator for 1 h at 37 °C. The SEE standards (50 μL) were added to the wells, along with 50 μL of antisera, which was serially diluted. After an incubation of 30 min at 37 °C, the plates were washed by PBST three times. Goat-anti-mouse IgG-HRP (1:3000, 100 μL/well) was then added, and incubated for 30 min at 37 °C. After four washes, 100 μL of TMB substrate was added to each well and incubated for 15 min at 25 °C. Then, 2 M H_2SO_4 (50 μL/well) was used to stop the enzymatic reaction, and the OD value at 450 nm was measured. The inhibition ratio was calculated using the following equation: Inhibition ratio (%) = $(B_0 - B) / B_0 \times 100\%$, where B_0 and B are the

OD values in the absence of SEE standards and in the presence of SEE standards, respectively.

Preparation and identification of mAbs against SEE standards

Our group previously described the fusion experimental procedures [45]. Three days prior to cell fusion, the mice expressing the highest affinity antisera were intraperitoneally injected with 300 μg of expressed SEE protein diluted in PBS. The spleens were obtained and dissociated from the euthanized mice under sterile conditions. The splenocytes were fused with SP2/0 myeloma cells using PEG 1500 after the red blood cells were lysed. The resulting hybridoma cells were propagated in a HAT selection medium, and plated in 96-well micro-culture plates. Hybridoma cells that secrete antibodies specific to SEE standards were screened for by non-competitive ELISA and icELISA. Clones with high IC_{50} values were subcloned three times using the limiting dilution method before ascites production. The mAbs were then purified from ascites through protein A affinity chromatography columns and conjugated to biotin as a secondary antibody for subsequent experiments.

Rabbit pAb preparation

Two weeks after the final immunization injection, the rabbit exhibiting the highest affinity for SEE was selected and injected intravenously with 1 mg of expressed SEE protein diluted in PBS. Antisera samples were collected 10 days later, and the antibodies were purified from the serum using protein A affinity chromatography columns. The resulting pAbs were then stored at − 80 °C.

Development and optimization of a double antibody sandwich ELISA

To establish the DAS-ELISA, pAbs were used as capture reagents, and eight mAbs were used as detection reagents. Selection of the best pairing of mAbs to our pAbs was based on the P/N value. Appropriate coating concentrations, temperature conditions, blocking agents, mAb dilution, and incubation time were determined as follows. 96-well plates were coated with capture antibody in various concentrations using two-step dilutions from 40 to 0.625 μg/mL (100 μL/well). The plates were then incubated for 2 h at 37 °C, 2 h at 25 °C, or 16 h at 4 °C. The microplates were then washed three times with PBST and subsequently blocked with either 2% non-fat milk-PBS, 5% non-fat milk-PBS, or 2.5% casein at 37 °C for 2 h. Aqueous SEE standards were diluted in PBS, and incubated for 30 min, 45 min, and 60 min at 37 °C. Biotinylated mAbs were analyzed as detection reagents at 1:500, 1:1000, 1:2000, 1:4000, and 1:8000 dilutions in PBS for 30 min, 45 min, and 60 min at 37 °C. In each plate, the reference negative control was included, and the experiments were repeated three times. After three washes, HRP-labeled streptavidin was added to each well at 1:1000, 1:3000, and 1:5000 dilutions for 30 min, 45 min, and 60 min at 37 °C. Following three washes with PBST, the TMB ELISA substrate was added and incubated in the dark at room temperature (25 °C) for 15 min. The absorbance was measured at 450 nm using a microplate reader after the reaction was stopped by the addition of 50 μL of 2 M H_2SO_4. Optimal conditions were determined based on the highest P/N value.

Development and optimization of time-resolved fluorescence immunoassay

Time-resolved fluorescence probes were prepared by conjugating rabbit serum pAbs to europium microbeads. The microbeads have a carboxylic acid that can bind to antibodies by 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS) activation. For conjugation, the beads were suspended in 104 μL of activation buffer and then treated by sonication for 2 min. 20 μL of EDC (1 mM) and 20 μL of Sulfo-NHS (1 mM) were added, the solution shaken for 15 min at 37 °C, and then centrifuged at 20,000 g for 15 min at 4 °C. After the supernatant was removed, the beads were washed with 200 μL of coupling buffer containing 50 mM sodium borate and 200 mM boric acid, and collected by centrifugation (15 min, 20,000 g, 4 °C). Then, different concentrations of pAbs (2, 4, 8, or 16 μL of 5.67 mg/mL solution in coupling buffer) were added, and the mixture was shaken for 2 h at 37 °C, and then centrifugated at 15,000 g for 15 min. After the supernatant was removed, the beads were resuspended in 100 μL of blocking buffer and further shaken for 2 h at 37 °C. Following two wash steps, the final solution was stored at 4 °C until use.

We also determined the appropriate dilutions of coating mAbs, sample pad material, sample volume, and detection time. On a nitrocellulose membrane, the T line was coated with different dilutions of mAbs (1-, 2-, 4-, or eightfold dilution of mAb 1A5). The concentration of mAb 1A5 was 4.13 mg/mL. The C line was coated with goat anti-mouse IgG, and then dried at 37 °C for 2 h. Three types of sample pads were also investigated, including hemofiltration membrane (HG-2) and glass fibers (RB65 and SB08) as previously described [47]. To determine the optimum sample volume and detection time, different sample volumes (80, 100, 120, or 150 μL) were added into the sample well. After 5, 8, or 10 min of incubation, the signal intensity of the TRFICA was read by the strip reader, and the T/C signal ratio was used for quantification. In the optimization process described above, T/C values were determined in relation to both

the absence (negative sample) and presence (positive sample) of SEE. The concentration of SEE in the spiked samples for TRFICA was 1 ng/mL. The ideal detection time was determined as the time that T/C value remained unchanged and was acquired by the following procedure: after the sample was added to the test strip, the test strip was inserted into the immunofluorescence analyzer. The signal intensities were recorded after 5, 8, and 10 min and the point where the T/C value reached equilibrium was selected as the detection time point.

Sensitivity

To establish a standard curve, standardized SEE solution was spiked in the assay buffer at 0.5, 1, 2, 4, 8, 16, 32, and 64 ng/mL for TRFICA, and 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 ng/mL for DAS-ELISA and measurements taken in triplicate. The standard curve was graphed using OriginPro 8.0 software. To determine the LOD for the TRFICA and DAS-ELISA developed here, 20 SEE-negative samples were also tested. The LOD for interpretation of positive and negative results was based on the mean signal value obtained from the values of 20 negative samples ($n=20$) plus three times the standard deviations [48].

Specificity

To evaluate the specificity of the TRFICA and DAS-ELISA, classical SE serotypes, namely SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE, were used to evaluate the assays' CR. The concentrations of the SEs were adjusted to 10 and 100 ng/mL, which was deemed sufficient for determining specificity. The TRFICA and DAS-ELISA systems were then trialed, and the CR value obtained using the following formula:

$$CR = IC_{50(SEE)}/IC_{50(\text{analog})} \times 100\% \quad (1)$$

Accuracy and precision

Recoveries and CVs were calculated to evaluate the accuracy and precision of both methods. Three Chinese infant formula brands, namely Yili PRO-KIDO, Junlebao LePlatinum K2 and Friso Prestige were analyzed. 25 g of infant formula was added to 100 mL 0.25 M Tris buffer (pH = 8.0), and the top lipid layer was discarded following centrifugation at 15,000 g, 4 °C for 10 min. The sample was then diluted by a factor of 20 with deionized H_2O. The samples were spiked with SEE standards at different concentrations (2, 4, 8 ng/mL for TRFICA and 0.2, 0.8, 3.2 ng/mL for DAS-ELISA) and each concentration was tested in triplicate ($n=3$). The formula used to calculate recovery was:

$$\text{Recovery}(\%) = (\text{detected level}/\text{spiked level}) \times 100\% \quad (2)$$

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44280-024-00063-x.

Supplementary Material 1.

Authors' contributions

L.N. contributed to the development of the TRFICA and DAS-ELISA, and was a major contributor to writing the manuscript; J.X. completed the preparation of the mAbs and pAb; Q.L. performed the expression and purification of a recombinant SEE protein; G.M.M and X.Y. analyzed the data; K.W. provided discussion and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Animal care and all animal procedures were carried out in compliance with the Guidelines for the Care and Use of Laboratory Animals of China Agricultural University. The study was approved by the China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Committee. The license number is AW61013202-2–2.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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