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Original article

DEVELOPMENT OF IMMUNOCHROMATOGRAPHIC LATERAL FLOW TEST FOR RAPID DETECTION OF *CLOSTRIDIUM PERFRINGENS* Α, Β AND Ε TOXINS IN CLINICAL SAMPLES

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Summary

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In the present work a lateral flow immunochromatographic test (LFT) for rapid detection of *Clostridium perfringens* toxins types, alpha (α), beta (β) and epsilon (ε) in clinical samples was developed. *C. perfringens* toxins were prepared, purified and inactivated with 0.2% formalin. Polyclonal antibodies specific to *C. perfringens* toxins types α, β and ε toxoids were prepared in rabbits and guinea pigs. The toxoid specific polyclonal antibodies prepared in rabbits were labelled with gold chloride nanoparticles. The prepared toxin specific rabbit and guinea pigs antibodies and goat anti-rabbit antibodies were utilised in development of a lateral flow immunochromatographic test and the latter – evaluated for detection of *C. perfringens* α, β and ε toxins in clinical samples. The sensitivity and specificity and accuracy of the developed LFT were determined by comparison with a commercially available ELISA used for detection of these toxins. The prepared LFT was capable to detect *C. perfringens* α, β and ε toxins in quantities of 2 μg/ml, 250 ng/ml and 60 ng/ml, respectively. One hundred poultry suspected faecal samples was examined both with the prepared LFT and commercial ELISA to test the validity of developed LFT. The sensitivity, specificity and accuracy of the LFT for detection of *C. perfringens* toxins were 81%, 95.2% and 90%, respectively, for α toxin, 76.6%, 98.5% and 72%, respectively, for β toxin and 66.6%, 98.8% and 95%, respectively, for ε toxin.

Key words: *C. perfringens* α, β and ε toxins*,* lateral flow test (LFT)

INTRODUCTION

Clostridium perfringens is a Gram-positive anaerobic spore-forming bacterium that causes life-threatening diseases such as gas gangrene and mild enterotoxaemia in humans and animals, although it colonises humans and animals as a part of normal intestinal flora (Havelaar *et al.,* 2015). *Clostridium perfringens,* which is considered as one of the largest toxin producing bacteria, is classified into five

types designated A, B, C, D and E according to their ability to produce the four major lethal toxins, namely, the alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) which cause a variety of diseases. The activities of major *C. perfringens* lethal toxins are the basis of the pathogenesis of classical enterotoxaemia attributed to this organism. Recently, it has been recognised that *C. perfringens* produces other toxins of probable importance in animal disease, such as enterotoxin and a cytotoxic beta-2 toxin (Radostitis *et al.,* 2007*)*.

In domestic farm animals, *C. perfringens* infection is associated with several types of enteritis, such as necrotising enteritis in young animals (piglets, foals, calves), and enteritis or colitis in adult goats, dogs and horses (Uzal *et al.,* 2010). Also, *C. perfringens* causes major losses in poultry by inducing necrotic enteritis in broilers (Timbermont *et al.,* 2011) at a global scale.

Clostridium perfringens alpha toxin (CPA) is associated with yellow lamb disease, gas gangrene in humans and malignant oedema in domestic animals including sheep, goats, cattle and horse (Uzal *et al.,* 2010). Its role is still frequently blamed for enteritis, and/or enterotoxaemia in cattle (Manteca *et al.,* 2002; Songer & Miskimins, 2005), horses (Bacciarini, 2003; Water *et al.,* 2005), goats (Songer, 1998) and pigs (Saenz *et al.,* 2007).

Beta toxin (CPB) is a major lethal toxin produced by both type B and C strains of *C. perfringens.* It underlines several animal diseases that are often accompanied by sudden death or acute neurological signs. In spite of the importance of beta toxin in veterinary medicine, the biological activity of this protein is poorly defined (Gkiourtzidis *et al.,* 2001). Beta toxin causes often fatal haemorrhagic dysentery in sheep, struck of sheep, enterotoxaemia of lambs, calves and piglets and necrotic enteritis of man and fowls (Cavalcanti *et al.,* 2004).

Clostridium perfringens epsilon toxin (ETX) is secreted by type B and D strains (McClane *et al.,* 2004). It is produced as a prototoxin that is activated by proteolytic enzymes produced by the same organism (Willis, 1969). The epsilon toxin is the third most potent clostridial toxin, after botulinum and tetanus toxins. A characteristic feature of epsilon toxin is its potent neurotoxicity, which is not observed for other structurally well-defined pore-forming toxins (Miyata *et al.,* 2001). This toxin causes blood pressure elevation, increased contractility of smooth muscle, vascular permeability increase, as well as brain and lung oedema in multiple animal species, while in goats ETX also causes colitis (Uzal *et al.,* 2004) and is the commonest cause of clostridial enterotoxaemia in sheep and goats (Uzal & Kelly, 1997).

The conventional diagnosis of *C. perfringens* infections and intoxication depends upon detection of the organism using bacteriological tools, application of PCR for the identification of the toxin and the producing bacteria (Miyamoto, 2012), the use of cytotoxicity assays and immunoassays such as latex agglutination, immunodiffusion and ELISA. All of these techniques, however, have diagnostic limitations (Rumah *et al*., 2013). Despite the fact that toxin detection is of paramount importance for diagnosis, the cytotoxicity assays are too complex and the conventional toxin immunoassays like ELISA are cumbersome requiring specialised equipped lab and well-trained personnel.

The lateral flow immunochromatographic technique (LFT) is a simple strip

or device assay, which gains more and more popularity as a rapid diagnostic method that, can be used for direct diagnosis at the production line or in the field. This technique is among the most widely used techniques for detection of microbial analytes in clinical specimens. The LFT has many advantages compared to some laboratory tests in terms of ease of use, rapidity, portability, reliability and cost. Because of these advantages, LFT have been widely applied as rapid tests for food contaminants including bacteria (Bruno, 2014), viruses (Hagstrom *et al.,* 2015), pesticides (Wang, 2014) and toxins (Ching *et al.,* 2015).

The LFT utilises antigen-antibody interactions in a manner, which provides a rapid detection of the analyte in question. With a very user-friendly format including short testing times and long-term stability within a broad range of climates, these tests are anticipated for onsite testing by untrained personnel. Although easy to use, the development of these tests is very crucial and has to be followed very diligently and precisely.

The aim of the current work was to develop an immunochromatographic lateral flow test for rapid detection of *C. perfringens* α, β and ε toxins in clinical samples. The test is designed to identify any of these toxins which are excreted from the *C. perfringens* pathogens in less than 5 minutes and in one step.

MATERIALS AND METHODS

Bacterial strain and toxins

C. perfringens bacterium types A, B and D were kindly obtained from the Anaerobic Vaccine Department in Veterinary Serum and Vaccine Research Institute, Abbasia, Egypt. The α, β and ε toxins were prepared from these strains according to the manual of the department.

Molecular identification of C. perfringens strains and their toxin genes

The total bacterial DNA of the three *C. perfringens* A, B and D strains were extracted using easy pure bacteria Genomic kit (Transgen, EE161-01, China) using the primers listed in Table 1. Multiplex PCR was carried out according to Park *et al.* (2015).

Table 1. Multiplex PCR primers sequences for detection of toxins genes of *C. perfringens*

Toxin gene	Primer	Sequence (5–3)	Product
C. perfringens type A			
cpa (a-toxin)	CP alpha F	GCTAATGTTACTGCCGTTGA	324 bp
	CP alpha R	CCTCTGATACATCGTGTAAG	
C. perfringens type B			
cpa (b-toxin)	CP beta F	AAATATGATCCTAACCAAMaAA	548 bp
	CP beta R	CCAAATACTYbTAATYGATGC	
C. perfringens type D			
cpa (ε-toxin)	CP epsilon F	TGGGAACTTCGATACAAGCA	376 bp
	CP epsilon R	AACTGCACTATAATTTCCTTTTCC	

F: forward primer; R: reverse primer.

Preparation of polyclonal antibodies

Preparation of polyclonal antibodies (PAb) against *C. perfringens* α, β and ε toxins in rabbits was done according to Siqueira *et al.* (2018). Each of these toxins was prepared, separated, purified, adjusted in to 25 mg/mL, inactivated using 0.2% formalin and converted into toxoids. Briefly, nine Boskat rabbits were used for immunisation with the prepared *C. perfringens* toxoids (3 rabbits for each toxoid type). The rabbits were randomly divided into the three groups and were injected subcutaneously as followed. Each rabbit was injected with 25 mg of *C. perfringens* toxoid emulsified in complete Freund's adjuvant (day one), and with 50 mg and 100 mg of immunogen emulsified in incomplete Freund's adjuvant at days 15 and 30, respectively. Blood samples were collected from the immunised rabbits at day 45, serum was separated and the toxin-specific polyclonal antibodies were purified.

Preparation of PAb against *C. perfringens* α, β and ε toxins in guinea pigs used a method for immunisation that was similar to that used for immunisation of rabbits. Five guinea pigs were used for each *C. perfringens* toxoid.

Purification of IgG from Pab

Purification of IgG from rabbit and guinea pigs polyclonal antibodies was done using caprylic acid according to Elke *et al*. (2008). Twenty five mL of each serum was centrifuged at 10000×g for 20–30 min and the pellet was discarded. The serum was mixed with 50 mL of 0.06M sodium acetate buffer pH 4.6 in a beaker and placed on a magnetic stirrer. Caprylic acid (2.02 mL) was added slowly dropwise while stirring at room temperature for 30 min and then centrifuged at 10000×g for 20 min. The supernatant was retained and the pellet was discarded. The supernatant was dialysed against PBS buffer at 4 ^{o}C overnight with three buffer changes. Finally the concentration of purified IgG was measured spectrophotometrically.

Preparation of colloidal gold nanoparticles

Preparation of colloidal gold (CG) nanoparticles was done according to Herizch *et al*., (2014). CG nanoparticles were adjusted at 40 nm diameter size. Fifty mL of purified water containing 0.01% (w/v) sodium citrate was boiled with vigorously stirred. One mL of 1% $HAuCl_4$ was added rapidly. When the colour of solution changed to red (about 2 min) the solution was boiled for another 10 min. Finally, 0.02% (w/v) of sodium azide was added. After cooling the diameter of the prepared nanoparticles was checked by scanning within the range 400-600 nm using spectrophotometer.

Conjugation of the purified C. perfringens toxoid-specific IgG with colloidal gold

Conjugation of the purified *C. perfringens* toxoid-specific rabbit IgG with colloidal gold (CG) was performed according to Kong *et al*. (2017). First, the CG was adjusted to pH 8.5 using 0.02M K_2CO_3. With gentle stirring, 0.5 mL of purified rabbit IgG (1 mg/mL) was added to 50 mL of adjusted CG then gently mixed for 10 min. PEG (20000 1% w/v final concentration) was added for blocking with gentle stirring for another 10 min followed by centrifugation at 10000×g for 30 min. The conjugated CG was suspended in 1 mL conjugated CG diluted buffer (20 mM Tris containing 3% w/v sucrose, 1% w/v BSA and 0.02 % w/v sodium azide, and stored at 4˚C.

LFT development

The preparation of the LFT according to Guo *et al.* (2015) involved preparation of:

- Sample pad

It was made from glass fiber, pre-treated with sample pad solution (pH 8.5) composed of purified water containing 3.81% (w/v) Borax, 1% (w/v) polyvinyl pyrrolidone (PVP), 2% (w/v) Triton X 100, 0.1% (w/v) casein sodium salt, 0.5% (w/v) sodium chloride, 0.15% (w/v) SDS and 0.02% (w/v) sodium azide then dried at 37 °C.

- Conjugation pad

Glass fiber conjugation pad was pre-treated with conjugation treatment solution pH 7.4 – 20mM PBS containing 2% (w/v) BSA, 2.5% (w/v) sucrose, 0.3% (w/v) PVP, 1% (w/v) Triton X 100 and 0.02% (w/v) sodium azide, then dried at 37 °C and kept in dry condition. Finally the conjugation pad was saturated with CG-conjugated toxin-specific rabbit IgG and dried at 37 °C for 1 h and kept in dry condition.

- Nitrocellulose membrane

The dispenser (Iso flow) was used to dispense two lines on the nitrocellulose (NC) membrane (25 mm × 300 mm). The purified toxin-specific guinea pig IgG (1 mg/mL) was dispensed around the bottom of the test line (1 μL/1 cm line) whiles the goat anti rabbit antibodies 0.5 mg/mL (MILIPORE Cat. No. AP132) were dispensed at the upper position as the control line (1 μL/1 cm line). The distance between two lines was 5 mm. The loaded NC membrane was dried at 37 °C for 2 h and kept in dry condition.

The treated sample pad, treated conjugation pad, loaded NC membrane and absorption pad were stick down in the PVC card. After that, the collected PVC card was cut into 3.9 mm width test-strips by using an automated cutter machine.

Sensitivity, specificity and validity of the developed LFT

Sensitivity of the LFT: C. perfringens α, β and ε standard toxins containing the MLD were two fold serially diluted and each dilution was tested by the developed LFT. The least amount of the toxin that can be detected by these kits was recorded.

Specificity of LED: Each standard toxin was tested by opposite type of LFT.

Validity test of LFD for detection of different toxins: One hundred faecal samples were collected from a broiler poultry farm suspected to suffer from *Clostridium* infection. The chickens showed undigested food, diarrhoea and low conversion rate. The samples were tested with the three developed LFT (α, β and ε) and also with three toxin detection ELISA kits (Bio-X Diagnostic, Belgium, Europe. cat. No. for Alpha toxin BIO K 289/2, for Beta Toxin BIO-X K267/2 for Epsilon toxin BIO-X K 277/2).

Statistical analysis

Mean, standard error of the mean and standard deviation of LFD results were determined according to Thrusfield (2007).

RESULTS

The results of molecular identification of *C. perfringens* toxin producing strains used in the preparation of the LFT is shown in Fig 1.

The minimal amount of the *C. perfringens* α toxins that can be detected using the prepared LFT was 2 μg/mL. It was 250 ng/mL for the β toxin and 60 ng/mL for the ε toxin as shown in Table 2 and Fig. 2.

The developed kits gave positive results when tested with the corresponding

standard *C. perfringens* α, β and ε toxins. No cross reactivity was recorded (Fig. 3).

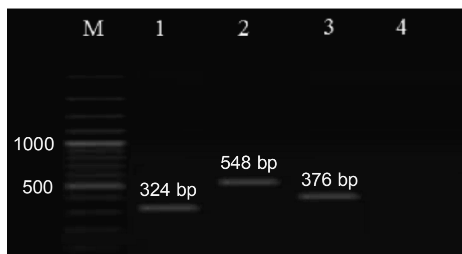

Fig. 1. Multiplex PCR for the three *Clostridium perfringens* strains used for detection of toxin genes. Lane M: 100 bp DNA ladder (Fermentas); lane 1: a 324 bp band specific for alpha toxin gene of *C. perfringens* type A; lane 2: 548 bp band specific for beta gene of *C. perfringens* type B; lane 3: 376 bp band at specific for epsilon toxin gene of *C. perfringens* type D; lane 4: negative control.

One hundred faecal samples from diseased chickens were tested by three types of ELISA kits and at the same time by three of the developed *C. perfringens* toxin detection LFT (α, β and ε). The results by both kits were compared and divided into four groups; true positive (T+), false positive (F+), false negative (F−) and true negative (T−) for each types of toxins. The T+, F+, F− and T− for alpha toxin were 30, 3, 7 and 60, respectively. In case of the beta toxin, the values were 23, 1, 7 and 60, respectively, while for epsilon toxin: 8, 1, 4 and 87, respectively (Table 3).

The validity test for LFT that depends on the sensitivity, specificity and accuracy determination was calculated and it was 81%, 95.2% and 90%, respectively, for alpha toxin, 76.6%, 98.5% and 72%, respectively, for beta toxin and 66.6%, 98.8% and 95%, respectively, for epsilon (Table 3).

DISCUSSION

The conventional diagnosis of *C. perfringens* infections and intoxication depends upon detection of the organism using bacteriological tools, detection of the toxin genes or the producing bacteria using PCR and/or detection of *C. perfringens* toxins in intestinal contents and other body fluids using any of the following procedures: the mouse neutralisation test (MNT), counter immune-electrophoresis

Table 2. Sensitivity of developed LFT for detection of *C. perfringens* alpha, beta and epsilon toxins

MLD* (µg/mL)	Dilutions						
	1	1/2	1/4	1/8	1/16	1/32	1/64
Alpha toxin							
4	+ 4 µg/mL	+ 2 µg/mL	–	–	–	–	–
Beta toxin							
1	+ 1 µg/mL	+ 0.5 µg/mL	+ 0.25 µg/mL	–	–	–	–
Epsilon toxin							
1	+ 1 µg/mL	+ 0.5 µg/mL	+ 0.25 µg/mL	+ 0.12 µg/mL	+ 0.06 µg/mL	–	–

* MLD=minimum lethal dose.

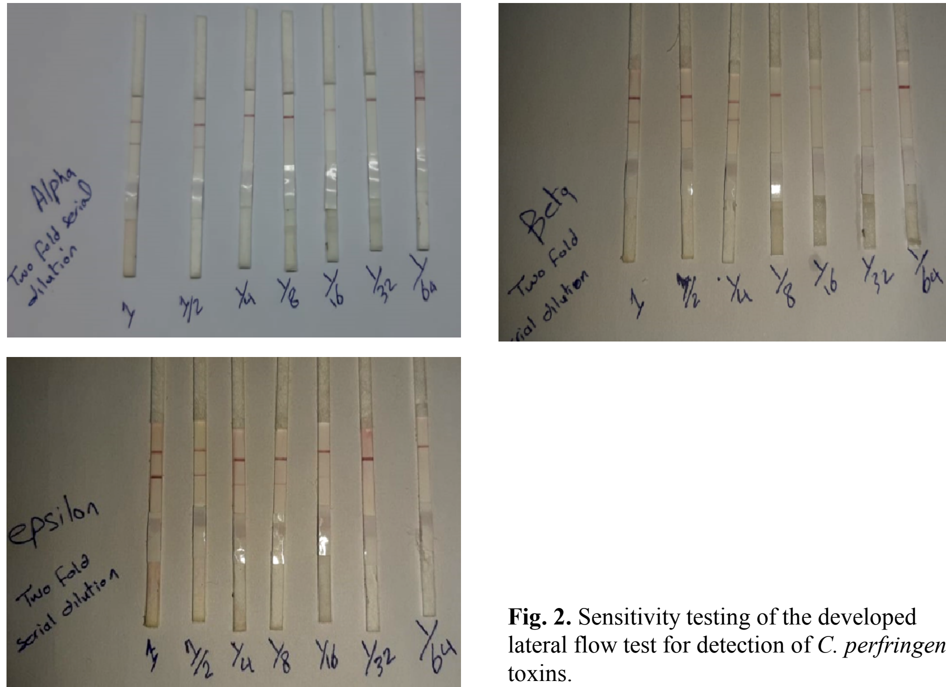

Fig. 2. Sensitivity testing of the developed lateral flow test for detection of *C. perfringens* toxins.

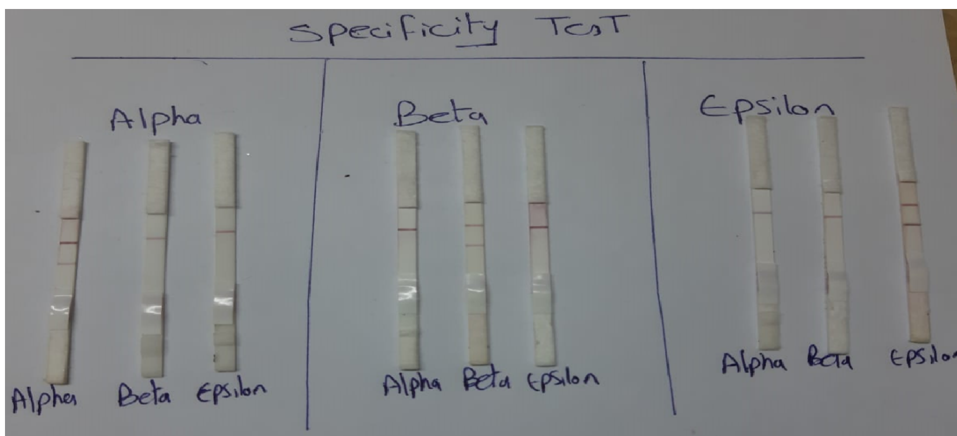

Fig. 3. Specificity test of the lateral flow test for detection of *C. perfringens* toxins.

(CIEP), latex agglutination, immunodiffusion and enzyme-linked immunosorbent assays (ELISAs) (Miyamoto, 2012). All of these tests, however, have diagnostic limitations (Rumah *et al*., 2013). Despite the fact that toxin detection is of paramount importance for diagnosis, the cytotoxicity assays are too complex and the conventional toxin immunoassays like ELISA are cumbersome requiring specialised equipped lab and well trained personnel.

Table 3. Validity of the lateral flow test (LFT) for detection of *C. perfringens* α, β and ε toxins compared with toxin standard ELISA kit

Test		ELISA			Sensitivity	Specificity	Accuracy
LFT		POS	NEG	Total			
Alpha toxin	POS	(T+) 30	(F+) 3	33	81.0%	95.2%	90%
	NEG	(F–) 7	(T–) 60	67			
	Total	37	63	100			
Beta toxin	POS	(T+) 23	(F+) 1	24	76.6%	98.5%	72%
	NEG	(F–) 7	(T–) 69	76			
	Total	30	70	100			
Epsilon toxin	POS	(T+) 8	(F+) 1	7	66.6%	98.8%	95%
	NEG	(F–) 4	(T–) 87	91			
	Total	12	88	100			

* POS: positive; NEG: negative; (T+): true positive; (F+): false positive; (T–): true negative; (F−): false negative.

Though these tests are widely available they are not sensitive enough to detect many infections; they miss up to 30% of cases, particularly for some *C. perfringens* toxins like epsilon toxin that breaks down at room temperature within two hours, therefore, a negative result may also indicate that the sample was not transported, stored, or processed promptly.

The lateral flow immunochromatographic assays, on the other hand, as one step test, have attracted the attention of many researchers due to their high specificity, accuracy, low cost, high sensitivity and easy application by non-specialised personnel. This test seems to be suitable as field and laboratory test and can be applied on large scale for examination of faecal samples, poultry products etc. This technique has been applied with high sensitivity and specificity for detection of *C. botulinum* A and B toxins (Tarisse *et al.,* 2017; Liu *et al.,* 2017), for identification of *C. difficle* toxins in clinical samples (Sharp *et al.,* 2010) and also for *C. perfringens* epsilon toxin (Tarisse *et al.,* 2017). However, there is no direct one step assay recorded in the literature for the detection of the other *C. perfringens* toxins.

Therefore, the present work was planned to develop a lateral flow immunochromatographic test (LFT) and to evaluate its sensitivity, specificity and accuracy for rapid detection of *C. perfringens* α, β and ε toxins as compared with ELISA.

The minimal amount of *C. perfringens* α, β and ε toxins that could be detected in clinical samples using the developed LFT kits were 2 µg/mL, 250 ng/mL and 60 ng/mL, respectively. In contrast, the single immunochromatographic test strip test that have been developed by Kathryn *et al.,* (2012) for detection of botulinum toxin A (BoNT/A) and B (BoNT/B) showed much higher sensitivity as it can

detect as little as 5 ng/mL of purified BoNT/A and 10 ng/mL of BoNT/B in 2% and 1% milk, respectively. Their kits could also detect 25 ng/mL of BoNT/A and 10 ng/mL of BoNT/B in undiluted apple juice. Also Liu *et al.* (2017) developed LFT that had excellent performance in the detection of botulinum neurotoxin using only 1 µL of simulated serum, and its sensitivity and specificity were comparable to those of mouse lethality assay.

The developed LFT manifested specificity in its reaction and showed no cross reactivity between the 3 types of *C. perfringens* toxins kits. The standard C. *perfringens* α, β and ε toxins gave positive reaction only when tested with the same toxin type of LFT. Compared with ELISA test, the sensitivity, specificity and accuracy of the developed LFT designed for detection of C. *perfringens* α toxin were 81%, 95.2% and 90%, respectively. In LFT kits developed for detection of beta toxin respective values were 76.6%, 98.5% and 72%, while in LFT kits developed for epsilon toxin: 66.6%, 98.8% and 95%, respectively. Similarly, Tarisse *et al.* (2017) evaluated a lateral flow test for detection of *C. perfringens* epsilon toxin in different matrices and the detection limits in these complex matrices were 0.10 to 2 ng/mL. Also Sharp *et al.* (2010) developed lateral flow assay for rapid, simple diagnosis of *Clostridium difficile* disease that, compared with PCR, showed high sensitivity (89.6 to 100%) and specificity (97.3 to 99.9%).

The developed lateral flow immunochromatographic test for detection of C. *perfringens* α, β and ε toxins is suitable as a screening field test that can be applied on large scale of samples (faecal samples, poultry product). The test is fast, requires no skilled personnel, is cheap, and gives results that are helpful in detection *C. perfringens* toxins and diagnosis of *Clostridium* diseases.

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