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Research article

Development of a novel polymerase spiral reaction (PSR) assay for rapid and visual detection of *Clostridium perfringens* in meat

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ABSTRACT

C. perfringens is a widespread foodborne pathogen and one of the major concerns in the meat industry. There is a need for a simple, rapid and equipment free detection system for *C. perfringens* as conventional anaerobic culture method is labour and resource intensive. Here, we applied a novel polymerase spiral reaction phenomenon to develop and evaluate an assay for effortless and visual detection of *C. perfringens* in meat foods employing pork as a representative model. Specificity of the assay was determined using 51 *C perfringens* and 20 non- *C. perfringens* strains. Analytical sensitivity of the developed test was 80 fg DNA per tube indicating 100 times more sensitivity than end-point PCR assay. The detection limits were 980 CFU/g and 9.8×10^4 CFU/g of pork for PSR and PCR assays, respectively. The operation time of the PSR assay including DNA extraction was 120 min. The developed PSR assay was accurate and effective in comparison to culture method, in detecting *C. perfringens* in 38 of 74 pork samples. Therefore the specificity, sensitivity, negative predictive value, positive predictive value and accuracy rate of the developed PSR assay were 100%. The developed PSR assay is easy to perform, rapid, affordable, permitting sophisticated-equipment free amplification and naked eye interpretation. This is the initial report in which the PSR assay was optimized for the detection of *C. perfringens*.

1. Introduction

Clostridium perfringens illnesses are one of the most common foodborne illnesses worldwide. In the United States (US) and the United Kingdom, *C. perfringens* is the second and third most common cause of foodborne illnesses, respectively (Grass et al., 2013; Dolan et al., 2016). In the US, in spite of many unreported sporadic illnesses, *C. perfringens* causes 1 million foodborne illness episodes or it contributes to 10% of all illnesses annually (Dailey et al., 2012). In India, *C. perfringens* has been reported in several human diarrhoeic episodes and frequently isolated in animal origin foods (Gupta and Gulati, 1974; Chakrabarty et al., 1977; Singh et al., 2005; Joshy et al., 2006; Yadav et al., 2017).

Clostridium perfringens, a prolific toxin-producing foodborne anaerobe is a Gram positive and spore-forming bacterium (Hu et al., 2018). It has got ubiquitous distribution in the environment including the alimentary tract of healthy human and animals (Grass et al., 2013). Based on its toxin production (α, β, ε, ι, enterotoxin & netB) ability, it is categorized into 7 (A-G) toxinotypes (Songer, 1996; Rood et al., 2018). All the seven toxinotypes produces alpha toxin (encoded in *cpa* gene), which is a phospholipase C and a key mediator for gas gangrene and other histotoxic and myonecrotic infections that lead to amputation of the affected part or death (Sakurai et al., 2004; Oda et al., 2015). Food poisoning, diarrhoea and enteritis are usually mediated by the enterotoxin (CPE) carrying *C. perfringens* type F (Carman et al., 2008; Daset al., 2018a, b; Rood et al., 2018). However, enterotoxin (encoded in *cpe* gene) negative isolates have been involved in many food poisoning cases. Recently in France, 43.26% of *cpe*-negative *C. perfringens* strains were implicated in foodborne outbreaks and it was not detected in strains associated with 13 outbreaks (Abdelrahim et al., 2019). *C. perfringens* spores are exceptionally resistant to conditions generally employed in the food industry to control bacterial growth, including chemical preservatives, low or high temperature or pH, osmotic pressure, desiccation and radiation (Garcia et al., 2019). Protein rich foods like meat are ideal for the growth of *C. perfringens* as it requires several amino acids and vitamins. In the US, meat and poultry were responsible for 92% of *C. perfringens* outbreaks with recognized single food commodity (Grass et al., 2013).

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Table 1. Bacterial strains used in the study.

Bacterial Species	Strain/Source[#]
***Clostridium perfringens* reference strain**	ATCC 13124
***Clostridium perfringens* field strains isolated from food samples (n = 20)**	Foods of animal origin
***Clostridium perfringens* field strains isolated from faecal samples (n = 30)**	Animal faeces
***non- Clostridium perfringens* strains**	
Clostridium sporogenes	ATCC 11437
Clostridium septicum	ATCC 12464
Shigella boydii	ATCC 9207
Shigella sonnei	ATCC 25931
Klebsiella oxytoca	ATCC 43863
Klebsiella pneumoniae	ATCC 700608
Enterococcus faecalis	ATCC 51299
Escherichia coli	ATCC 25922
Staphylococcus epidermidis	ATCC 12228
Campylobacter jejuni	ATCC 33291
Salmonella Typhimurium	ATCC 51812
Pseudomonas aeruginosa	ATCC 10145
Mycobacterium bovis	AN5
Mycobacterium smegmatis	ATCC 607
Listeria monocytogenes	ATCC 13119
Staphylococcus xylosus	ATCC 29971
Staphylococcus aureus	ATCC 33591
Salmonella Enteritidis	NSC 2478
Salmonella Uccle	NSC 60a
Staphylococcus sciuri	ATCC 29061

[#] ATCC-American Type Culture Collection (USA).

Another worrying fact is that the generation time of *C perfringens* is very short, i.e., <8 min (Garcia and Heredia, 2011). This allows the pathogen to quickly reach large quantities to cause food poisoning. And this also emphasizes the need for a rapid on-site technique to detect this pathogen in food particularly in meat. Many laboratories do not regularly test *C. perfringens* as it requires anaerobic culturing, which is labour and resource intensive (Garcia et al., 2019). While a range of DNA based detection techniques like end-point PCR (Baez and Juneja, 1995), real-time PCR (Kaneko et al., 2011; Chon et al., 2012) are existing, they are not appropriate for on-site testing as they entail technical expertise, costly equipments, and complex operation protocols, etc (Hara-Kudo et al., 2005; Milton et al., 2020a). Of late, quite a lot of isothermal amplification technologies such as loop mediated isothermal amplification (LAMP) were put to use to detect foodborne pathogens, particularly *C. perfringens* (Kaneko et al., 2011; Hong, 2017; Priya et al., 2018). Although these tests were simple and equipment free, some constraints like the requirement of 4–6 primers, complex optimization process, etc have limited their extensive adoption in resource-compromised laboratories (Momin et al., 2020; Milton et al., 2020a).

The constant pursuit to develop novel isothermal assays has resulted in the application of PSR phenomenon for development of detection assays (Liu et al., 2015), which is a distinctive blend of end-point PCR (one primer pair) and LAMP (isothermal amplification/equipment free). PSR has found its application for detecting many pathogens of medical and veterinary importance (Dong et al., 2015; Jiang et al., 2016; Gupta et al., 2017; Das et al., 2018a, b). Recently, PSR assays have been developed to detect foodborne pathogens like *Salmonellla* (Xu et al., 2019; Momin et al., 2020), *Staphylococcus aureus* (Milton et al., 2020a,b) and *Vibrio parahaemolyticus* (He et al., 2020).

There is a demand for a simple, user-friendly, rapid and equipment free detection system for *C. perfringens* that is suitable for application at the resource limited or field settings. Accordingly, the current study was planned to develop a PSR assay to detect *C. perfringens* in meat foods. Artificial spiking analysis and real-world (naturally contaminated pork) sample testing were done to validate and compare the developed assay with conventional PCR and culture method. The developed assay was rapid and easy to perform, permitting direct detection of *C. perfringens* from meat without any additional enrichment step.

2. Materials and methods

2.1. Bacterial strains

C. perfringens ATCC 13124 strain, 50 *C perfringens* field strains isolated from food and faecal samples and 20 other reference (non-*C. perfringens*) strains were employed to study the specificity of the developed assay (Table 1). All the stored bacterial strains (as 20% glycerol stock) were cultured on their appropriate growth media and incubated at 37 °C. Genomic DNAs were isolated following the manufacturer's directions of QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) after growing single colonies in Luria–Bertani broth for overnight at 37 °C. Extracted DNAs were stored at −20 °C.

2.2. Design of primers

Primers for PSR (as elucidated by Liu et al. (2015)) and end-point PCR assays were designed to amplify the conserved region of the *cpa* gene sequence (Gen Bank Accession number CP000246.1) of *C. perfringens* with the Primer3 platform of NCBI. The specificity of the primers (Table 2) was assessed *in silico* with BLAST-N program and custom synthesized (Imperial Life Sciences, India).

2.3. PSR standardization and visualization

The PSR assay was standardized for the detection of *cpa* gene of *C. perfringens*. Different concentrations of reagents and range of incubation time/temperature were used to standardize the assay. They were 5μM–15μM concentrations of each primer, 0.5–1.4 mM of betaine (Sigma-Aldrich, USA), 6–12 U of *Bst* 2.0 Warm start polymerase (New England BioLabs (NEB), USA), 0.5–1.6 mM of dNTP (Thermo Scientific, USA), 2.0–10.0 mM of MgSO4 (NEB, USA), 62–70 °C of temperature and 15–90 min of time. The standardized PSR reaction mixture contained 2.5 μL of Isothermal amplification buffer (10X, NEB, USA) and 1.0 μL of DNA template with the concentration of 80 ng/μL. The amplified PSR products were visually interpreted by adding 1 μL of 1:10 diluted SYBR Green I dye (Sigma-Aldrich, USA) and 120 μM (added before incubation) hydroxyl naphthyl blue (HNB) dye (Sigma Aldrich, USA). Results were also interpreted using agarose gel (2.5%) electrophoresis. The end-point PCR assay was accomplished in a 25 μL reaction blend containing 12.5 μL 2x Dream Taq Master Mix (Thermo Scientific, USA), 9.5 μL of nuclease-free water (Thermo Scientific, USA), 1 μL (10 pmol) of each primer and 1 μL

Table 2. Primer sequences used in the study.

Assay	Primer	Sequences	Product size	Source
PSR	CPAPSRF	5′-acgattcgtacatagaagtatag GCTTATTTGTGCCGCGCTA -3′	variable	This study
	CPAPSRR	5′-gatatgaagatacatgcttagca CATAGCATGAGTTCCTGTTCCA -3′		
Conventional PCR	CPAF	5′-GCTTATTTGTGCCGCGCTA-3′	100bp	This study
	CPAR	5′-CATAGCATGAGTTCCTGTTCCA-3′		

of DNA template. The PCR amplification condition was 94 °C for 10 min, 35 cycles of 94 °C, 62 °C and 72 °C for 1 min each and 72 °C for 7 min. PCR products were electrophoresed in 1.5% agarose gel.

2.4. Specificity and analytical sensitivity

The specificity of the PSR assay was analyzed employing genomic DNA of 51 *C perfringens* and 20 non-*C.perfringens* strains. The analytical sensitivity was estimated using serial tenfold (10−1 to 10−8) dilutions of the DNA of *C. perfringens* ATCC 13124 and compared with the end-point PCR assay.

2.5. Estimation of detection limit using C. perfringens-spiked pork

The detection limit of the developed PSR assay was estimated by spiking pork with *C. perfringens*. A single *C. perfringens* (ATCC 13124) colony was selected from a 7% sheep blood agar plate and was grown overnight (37 °C) in a 10 mL of Robertson's cooked meat media broth (HiMedia, India). Following overnight incubation, the bacterial cells were pelleted by centrifugation (10,000×g for 10 min) followed by washing and resuspension in 2 mL PBS. This bacterial suspension was serially diluted (ten-fold, 10^{-1} to 10^{-8}) in 1X PBS. The bacterial concentration of the serially diluted bacterial suspensions was estimated by spreading (100 µL) onto sheep blood agar (7%) followed by overnight incubation (37 °C). The raw pork was procured from a retail shop and was established to be *C. perfringens* free by culture and PCR method. Pork piece (25 g) was homogenized in 225 mL of tryptic soy broth (HiMedia, India) and the homogenates were dispersed in test tubes (9 mL in each tube). Already prepared bacterial suspension (1 mL) dilutions were added to respective 9 mL meat homogenate tubes. One ml of sterile PBS was added in place of bacterial culture to maintain a negative control. DNAs were extracted (without any enrichment) with the DNeasy blood and tissue kit. PSR and PCR assays were accomplished using extracted DNA. The experiment was repeated thrice.

2.6. Testing of naturally contaminated samples

To evaluate the field applicability of the established PSR assay, it was used to test naturally contaminated pork samples. For this purpose, 74 raw and processed pork samples were collected from the local meat shops. Genomic DNAs were directly extracted from the pork samples and PSR and conventional PCR assays were deployed to detect the presence of *C. perfringens* genome. The samples were also inoculated in Robertson's cooked meat broth (Himedia, Mumbai, India) and incubated at 37 °C for 48 h. A loopful of the enriched culture was then plated on to 7% sheep blood agar for isolation by incubating the plates at 37 °C under anaerobic conditions (Gaspak EZ Anaerobic container sachet, BD, New Jersey). Suspected colonies were further confirmed by biochemical tests and PCR assay targeting *cpa* gene (van Asten et al., 2009; Milton et al., 2017). The results of the PSR assay were related to the results of the traditional isolation method and PCR.

2.7. Statistical analysis

Using the outcomes of the PSR, PCR and culture methods, the specificity, sensitivity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) were computed (Momin et al., 2020). According to the results of the culture method, true positives and true negatives were decided.

3. Results

3.1. Standardized PSR assay

PSR optimization was achieved for different reagents, time and temperature. The 25 µl reaction mixture contained 2.5 µl of Isothermal amplification buffer (10X), 8.0 U of *Bst* 2.0 WarmStart DNA polymerase, 1.4 mM dNTP's, 6.0 mM MgSO4, 0.8 M betaine, 13 µM forward primer, 13 µM reverse primer and 1.0 µl of DNA template. The incubation temperature and time were optimized at 65 °C for 60 min. By resolving in agarose gel electrophoresis, a ladder pattern could be visualized in positive samples (Figure 1A). Visual detection was enabled by SYBR Green I under white (Figure 1B) and UV light (Figure 1D) and HNB dye under white light (Figure 1C). In SYBR Green dye, the positive samples produced a green (fluorescent) colour, whereas a negative sample stayed orange. Under UV light, intense fluorescence was observed. With HNB dye, the positive results were shown by a colour change from purple to blue.

3.2. Specificity analysis

The PSR assay developed to detect *C. perfringens* displayed 100% specificity with no amplification or colour change observed in non-*perfringens* strains. The specificity results are shown in Figure 2.

3.3. Sensitivity analysis

The analytical sensitivity of the established PSR assay was experimentally computed and related to the PCR assay. The stock concentration of the DNA extracted from the *C. perfringens* ATCC 13124 was 80 ng/µl.

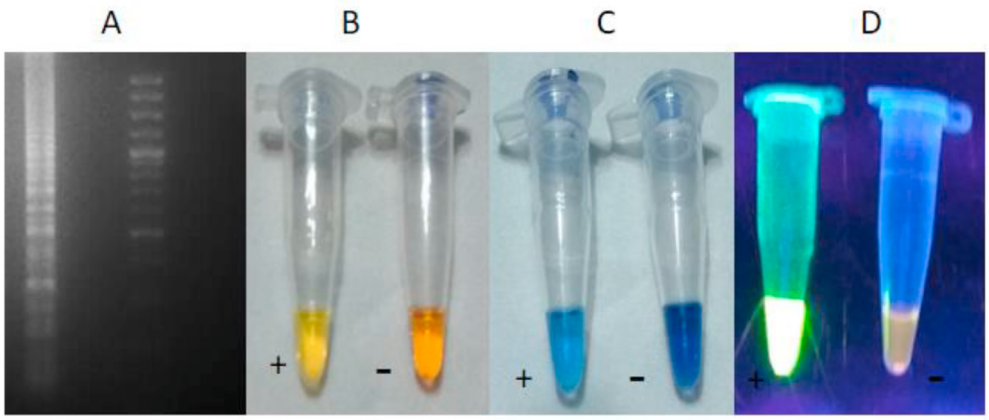

Figure 1. Detection of the PSR products by different methods (A) PSR products on 2.5% agarose gel (B) using SYBR Green I dye in white light, (C) using HNB dye in white light and (D) using SYBR Green I dye in UV light (Supplementary Figures 1–4).

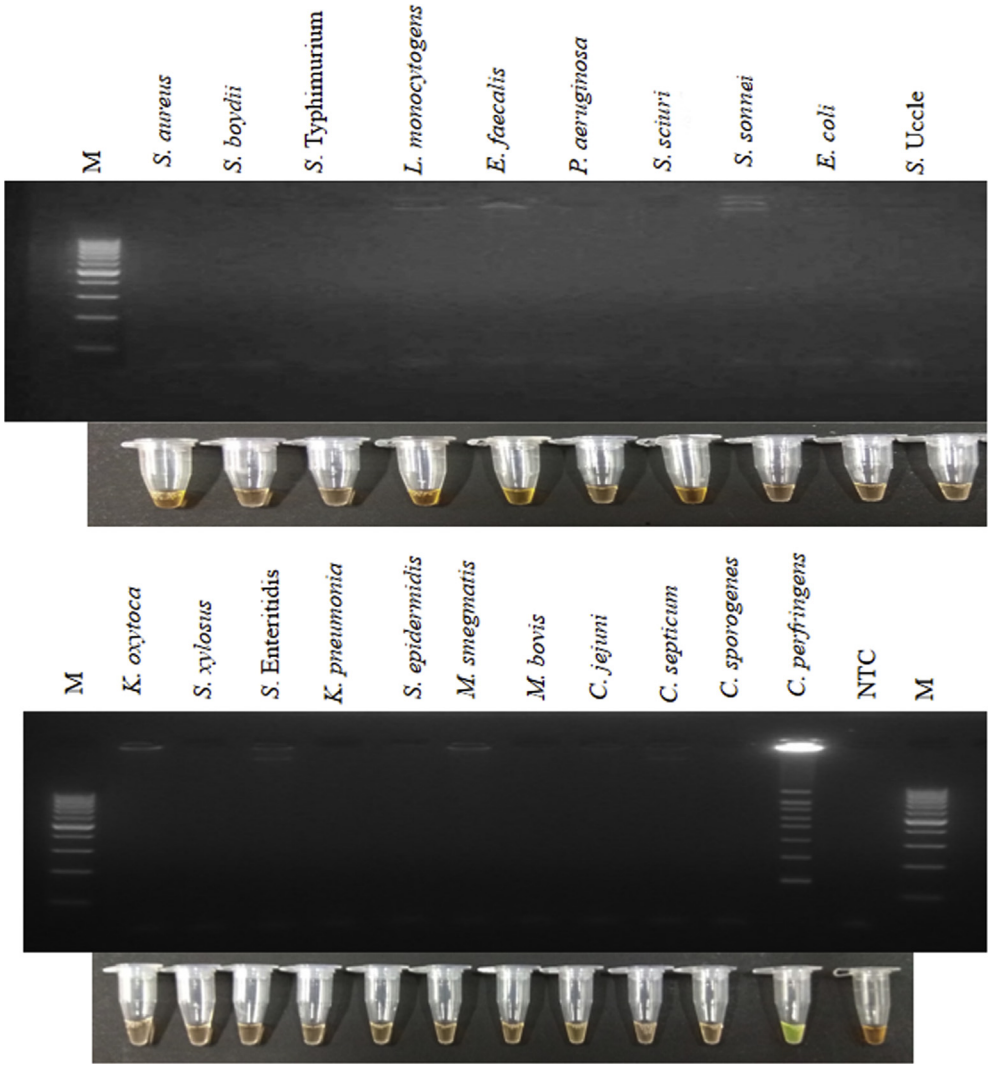

Figure 2. Specificity of PSR assay. First row- Electrophoretic pattern of PSR products (Lane 1–20) without amplification in non- *C.perfringens* DNA and amplification in *C.perfringens* DNA (Lane 21). Second row- Visual detection with SYBR Green I dye (corresponding tube numbers 1–20) showing orange colour in non- *C.perfringens* DNA and green fluorescence in *C.perfringens* DNA (Tube 21). (NTC- Non-template control; Lane M – 100 bp plus ladder) (Supplementary Figures 5–8).

The ten-fold serially diluted DNA had concentration ranging from 80 ng/µl to 0.8 fg/µl. In each reaction tube, 1 µl of DNA template was added, total genomic DNA per tube ranged from 80 ng to 0.8 fg. The analytical sensitivity of the PSR and PCR assays was 80 fg and 8 pg, respectively demonstrating PSR to be 100 fold sensitive than PCR assay (Figure 3).

3.4. Detection limit of the PSR assay

The initial suspension of *C. perfringens* ATCC 13124 contained a bacterial concentration of 9.8×10^6 CFU per mL. One ml of ten-fold serial dilutions (in 1X PBS) of *C. perfringens* ATCC13124 culture were added to 9 ml of the pork homogenate tubes. Hence, inoculated meat was carrying *C. perfringens* ranging from 9.8×10^6 CFU–0.098 CFU. The LoD of PSR and PCR was 980 CFU per g and 9.8×10^4 CFU per g of meat, respectively (Figure 4). No amplification was observed in the negative control, further confirming the exclusivity of the developed assay.

3.5. Testing of naturally contaminated samples

To evaluate the field applicability of the PSR assay, it was applied to detect *C. perfringens* from the raw and processed pork samples (n = 74) collected from the local meat shops. The samples were tested with cultural methods, PSR and conventional PCR assays. Out of 74 samples, employing the culture method, *C. perfringens* could be isolated from 38 samples. PSR and conventional PCR assays could detect *C. perfringens* from 38 and 29 culture positive samples, respectively.

3.6. Statistical analysis

The specificity, sensitivity, NPV, PPV and accuracy of the developed PSR for detection of *C. perfringens* was calculated to be 100%.

4. Discussion

The extensive presence of *C. perfringens* in the environment, human and animal excreta leads to the easy contamination of food (Grass et al., 2013). The attack rate of food poisoning outbreaks due to *C. perfringens* can be as high as 70% (Garcia et al., 2019). The rapid movement of meat foods in the food chain does not permit testing with time-taking conventional methods (Momin et al., 2020). Anaerobic culture based detection of *C. perfringens* is time consuming and very labor-intensive. Hence, culture independent and rapid detection of *C. perfringens* in foods using a simple and sensitive method is the need of the hour from the food safety and public health viewpoint. Timely and on-site testing of

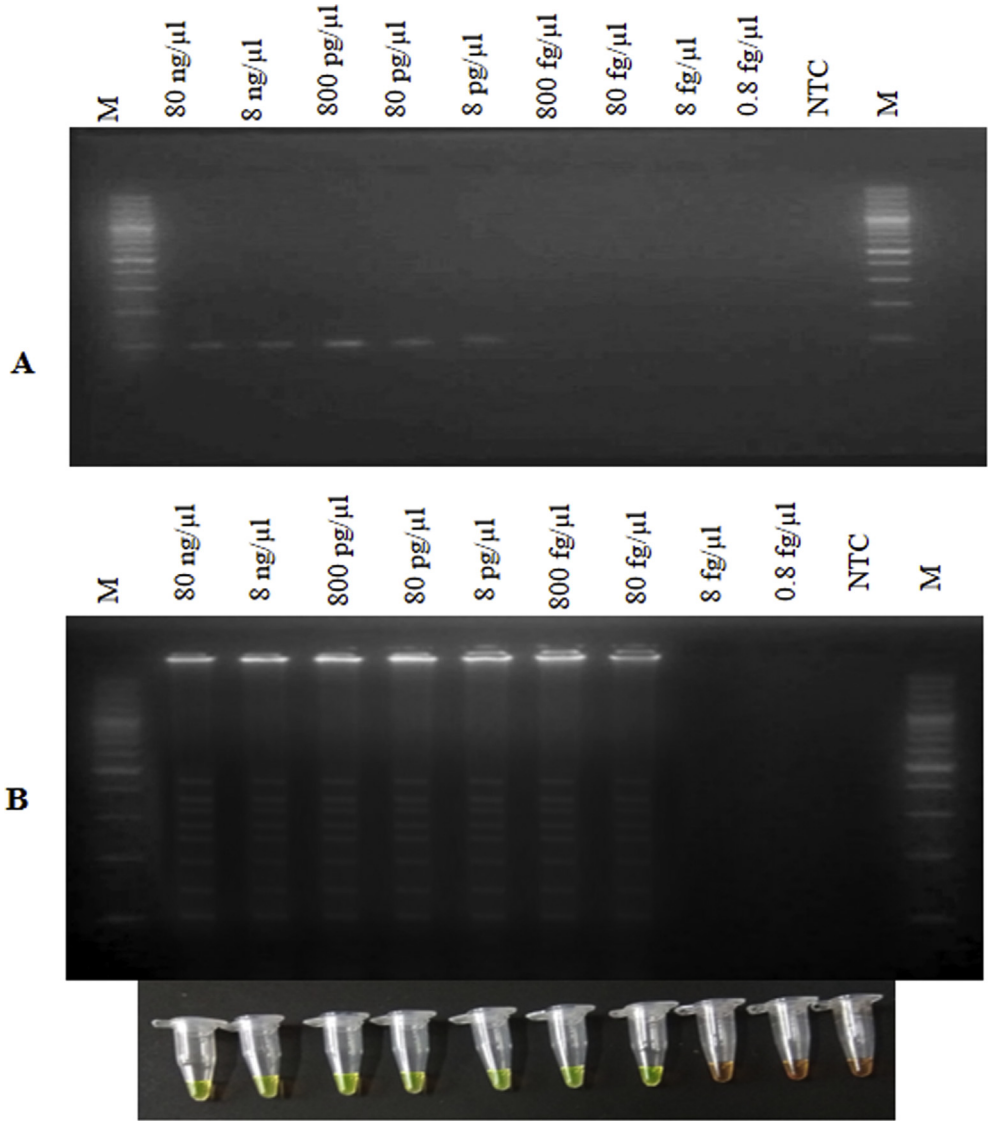

Figure 3. Analytical sensitivity A) Analytical sensitivity of conventional PCR showing amplification till 8 pg/µl, B) Analytical sensitivity of PSR assay showing amplification till 80 fg/µl (NTC- Non template control; Lane M –100bp plus ladder) (Supplementary Figures 9–11).

foods for the presence of pathogens not only prevents death or hospitalization but aids in source identification (Milton et al., 2020a). For that reason, a culture-independent and easy to perform PSR assay to detect *C. perfringens* in meat foods was developed.

Amongst isothermal amplification based pathogen detection techniques, LAMP is typically known for its equipment-free nature and rapidity. A number of LAMP tests have been established for detecting *C. perfringens* in foods (Kaneko et al., 2011; Hong, 2017; Priya et al., 2018). The novelty of the developed PSR assay is the prerequisite of one enzyme and one pair of primer, isothermal amplification (65 °C) and result interpretation by the naked eye. Besides, the *Bst* polymerase used is sturdier to the Taq DNA polymerase's inhibitors (Milton et al., 2020a). *cpa* gene, employed as the target gene has been the most frequently used in previously reported assays for the detection of *C. perfringens* in foods (Hong, 2017; Priya et al., 2018). However, some assays have used *cpe* (enterotoxin), as target gene (Kaneko et al., 2011). Furthermore, *cpe*--negative strains have been implicated more in recent foodborne outbreaks (Abdelrahim et al., 2019). The developed PSR assay has displayed 100% specificity as amplifications were observed only in 51 *C perfringens* strains and no false amplification or cross reactivity was noticed with 20 non-*C.perfringens* strains.

The analytical sensitivity of the established PSR assay (80 fg) was 100 fold higher than the PCR assay (8 pg). The developed assay is also sensitive than the LAMP assays published earlier for the detection of *C. perfringens* by Priya et al. (2018) and Kaneko et al. (2011) who demonstrated an analytical sensitivity of 0.34 pg and 1 pg, respectively. Similar to our observation, recently developed PSR assays for detecting *Salmonella* (Momin et al., 2020), *S. aureus* (Milton et al., 2020a) and *V. parahaemolyticus* (He et al., 2020) have shown 100 times higher sensitivity than end-point PCR assays. However previously developed LAMP assays for detecting *C. perfringens* in foods have revealed only 10 times higher sensitivity than conventional PCR assays (Hong, 2017; Priya et al., 2018). Similarly, the detection limit of the established PSR assay (980 CFU/g) in artificially contaminated pork was 100 fold better than the end-point PCR (9.8×10^4 CFU/g). This result is noteworthy as 10^5 CFU/g of food is the infectious dose of *C. perfringens* reported in foods linked to foodborne outbreaks (Garcia et al., 2019). Thus our PSR assay may serve as a handy tool for rapid and culture independent detection of *C. perfringens* in food products and recall of contaminated ones. Also, the detection efficiency of our assay is far superior to the recently developed LAMP test which showed the detection limit of 10^7 CFU per g of spiked goat meat without enrichment (Priya et al., 2018). Our assay is also

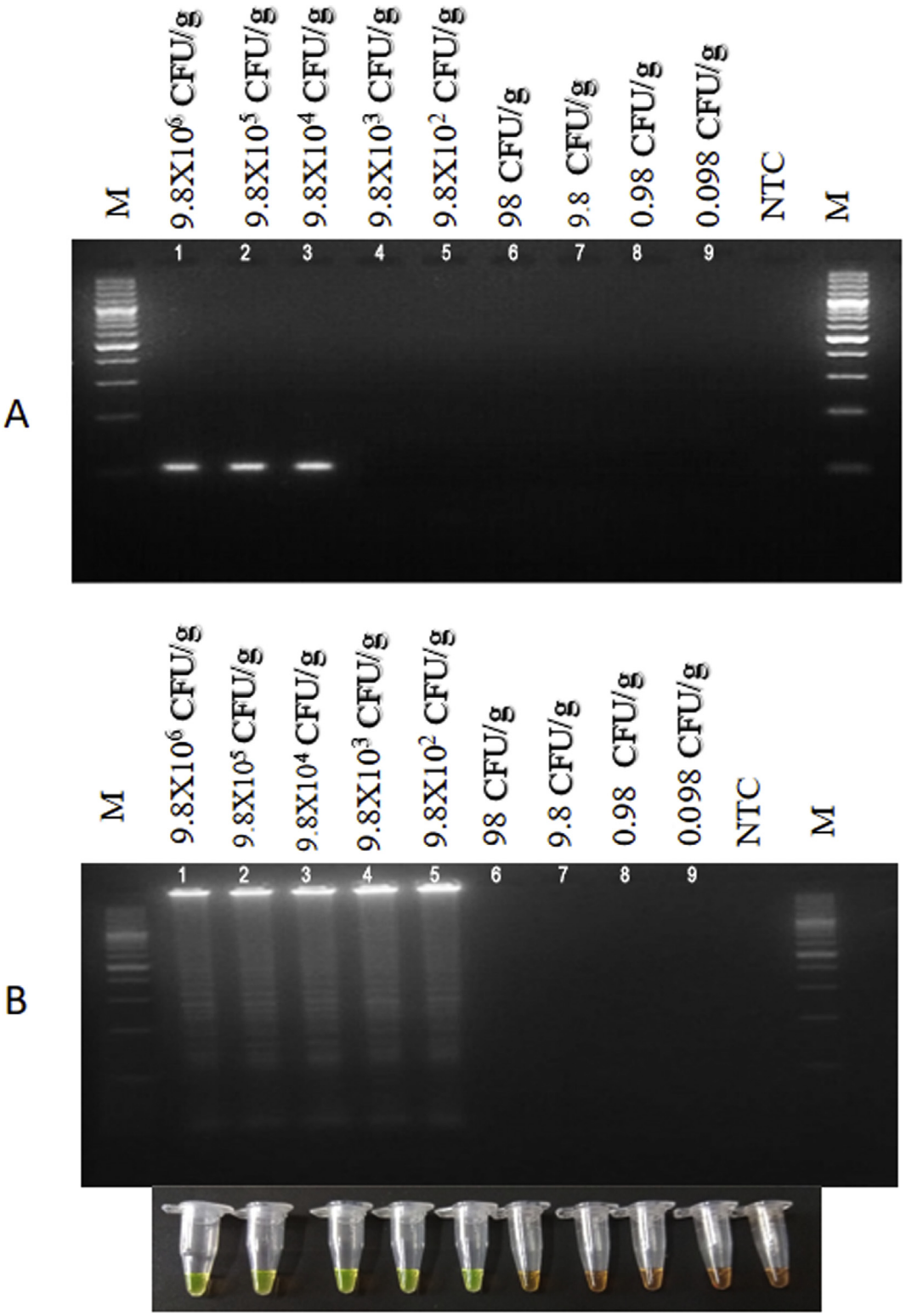

Figure 4. Limit of detection (LoD) in artificially spiked pork. A) Conventional PCR showing LoD (Lane 1–9: 9.8×10^6 CFU/g, 9.8×10^5 CFU/g, 9.8×10^4 CFU/g, 9.8×10^3 CFU/g, 9.8×10^2 CFU/g, 98 CFU/g, 9.8 CFU/g, 0.98 CFU/g, 0.098 CFU/g; Lane 10: NTC; Lane M −100bp plus ladder), B) Electrophoretic pattern of PSR products showing LoD (Lane 1–9: 9.8×10^6 CFU/g, 9.8×10^5 CFU/g, 9.8×10^4 CFU/g, 9.8×10^3 CFU/g, 9.8×10^2 CFU/g, 98 CFU/g, 9.8 CFU/g, 0.98 CFU/g, 0.098 CFU/g; Lane 10: NTC; Lane M −100bp plus ladder) and in second row- SYBR Green dye based visual detection of PSR products (Tube 1–9: 9.8×10^6 CFU/g, 9.8×10^5 CFU/g, 9.8×10^4 CFU/g, 9.8×10^3 CFU/g, 9.8×10^2 CFU/g, 98 CFU/g, 9.8 CFU/g, 0.98 CFU/g, 0.098 CFU/g; Tube 10:NTC) (Supplementary Figures 12–14).

efficient than the LAMP assays published earlier with an LoD of 10^4-10^5 cells per gram of meat (Kaneko et al., 2011) and 10^4 CFU per ml of samples (Hong, 2017). Further, Taqman-based real-time PCR assays developed earlier to detect *C. perfringens* required more than 10^3 bacterial cells/g of meat for detection (Kaneko et al., 2011; Chon et al., 2012). The detection limit of the present PSR assay can also be further improved if a short enrichment step is included as reported by Priya et al. (2018). But a bacteriological lab set up is required, which may limit its wider

application as an on-site tool. On testing field samples, PCR, PSR and culture method could detect *C. perfringens* from 29, 38 and 38 samples, respectively. The operation time of the PSR assay was 120 min, which is only 2% of the time required for the culture method. While, the operation time of PCR and culture methods for detection and isolation of *C. perfringens* are 5–6 h and 3 days, respectively. This emphasizes the suitability of PSR assay as a rapid on-site test. Further, four detection methods were compared in this study. Among SYBR green and HNB dyes, HNB under white light is more preferred as it is a pre-addition dye which obviates contamination resulting in false positives (Ali et al., 2017). The PSR standardized in the present study can be adapted for other purposes such as detection of *C. perfringens* in faecal or tissue samples of animal and human being as it causes diseases like food poisoning, necrotic enteritis, myonecrosis, gas gangrene, antibiotic-associated diarrhea, etc (Milton et al., 2020b).

5. Conclusion

In this study, a novel PSR assay was developed for culture-independent and direct detection of *C. perfringens* in meat within an hour. A comparison of performance with end-point PCR demonstrated that PSR assay had higher sensitivity than PCR for detection of *C. perfringens* in the pork samples. The developed PSR assay is easy to perform, rapid, affordable, permitting sophisticated-equipment free amplification and naked eye interpretation. This is the initial report in which the PSR assay was optimized for the detection of *C. perfringens.*

Declarations

Author contribution statement

A.A.P. Milton: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

K.M. Momin, M. Angappan: Performed the experiments.

S. Ghatak: Analyzed and interpreted the data; Wrote the paper.

G.B. Priya: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

K. Puro, I. Shakuntala, A. Sen: Contributed reagents, materials, analysis tools or data.

S. Das, R.K. Sanjukta, B.K. Kandpal: Analyzed and interpreted the data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e05941.

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