

Development and Application of a Multiplex PCR Assay for Detection of *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum* and *Clostridium chauvoei*

Ming Wei

Qingdao Agricultural University

Yan Feng

China Institute of Veterinary Drug Control

Ruirui Wei

Yutai County Agriculture and Rural Bureau

Xiaoxia Ren

China Institute of Veterinary Drug Control

Chenxiao Huang

China Institute of Veterinary Drug Control

Haoran Zhao

China Institute of Veterinary Drug Control

Jian Li

China Institute of Veterinary Drug Control

Liangquan Zhu

China Institute of Veterinary Drug Control

Wensheng Yao

China Institute of Veterinary Drug Control

Ming Zou

zoudnet@163.com

Qingdao Agricultural University

Yizhi Zhang

China Institute of Veterinary Drug Control

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optimized via gradient PCR and single-factor tests. The specificity, sensitivity and repeatability of the assay were systematically evaluated, and the validated method was applied to clinical tissue and fecal samples from cattle and sheep. The optimal annealing temperature was 55°C, with primer concentrations of 0.25, 0.25, 0.25 and 0.5 µmol/L for the four targets respectively, and 30 amplification cycles. The assay showed high specificity with no cross-reactivity with common non-target pathogens including *Clostridium tetani*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., bovine enterovirus (BEV), bovine astrovirus (BAstV), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV) and peste des petits ruminants virus (PPRV). The limits of detection for recombinant plasmids were 10², 10³, 10⁴ and 10³ copies/µL respectively. Intra- and inter-assay repeatability was high. Among 206 clinical samples, the positivity rates were 0%, 2%, 12% and 9% for the four pathogens, with 100% concordance with uniplex PCR and sequencing. This specific, sensitive and reproducible quadruplex PCR method enables rapid simultaneous detection and differentiation of four pathogenic *Clostridium* species, providing a reliable tool for clinical diagnosis and epidemiological investigation.

Key Words: *Clostridium perfringens*; *Clostridium novyi*; *Clostridium septicum*; *Clostridium chauvoei*; multiplex PCR

Nonstandard abbreviations used: BAstV = bovine astrovirus; BEV = bovine enterovirus; BCoV = bovine coronavirus; BVDV = bovine viral diarrhea virus; CVCC = National Center for Veterinary Culture Collection; ddH₂O = double distilled water; GAM = Gifu Anaerobic Medium; LOD = limit of detection; PPRV = peste des petits ruminants virus; RCM = Reinforced Clostridial Medium.

INTRODUCTION

Clostridial diseases, caused by pathogenic species of the genus *Clostridium*, are characterized by rapid onset, short disease courses, and high mortality, with a worldwide distribution [1,2]. *Clostridium perfringens* (*C. perfringens*),

Clostridium novyi (*C. novyi*), *Clostridium septicum* (*C. septicum*), and *Clostridium chauvoei* (*C. chauvoei*) are Gram-positive bacteria belonging to the family Clostridiaceae. Their spores are ubiquitous in soil, feces, and animal intestinal tracts, invading hosts through contaminated environments, wounds, or the digestive tract [1]. These highly resistant spores survive for extended periods in harsh conditions. Upon germination, the resulting exotoxins cause severe diseases such as gas gangrene and necrotic enteritis, leading to substantial economic losses in the global livestock industry, particularly in large-scale cattle and sheep farming [3,4].

The clinical signs and necropsy findings associated with these four *Clostridium* species are highly similar, and mixed infections are common, complicating differential diagnosis [5]. *C. perfringens* is the primary pathogen responsible for enterotoxemia in ruminants. Based on toxin production, *C. perfringens* was classified into seven toxinotypes (A–G) in 2018 [6]. In small ruminants, including sheep and goats, all toxinotypes of *C. perfringens* can cause gastrointestinal diseases [7]. In peracute cases, animals often die suddenly within hours of onset without obvious prodromal signs [8,9]. In type D infections, epsilon toxin causes specific damage to renal tubular epithelial cells, resulting in “pulpy kidney disease” [10]. Black disease (infectious necrotic hepatitis), caused by *C. novyi* type B, predominantly occurs in areas endemic for the liver fluke (*Fasciola hepatica*). Its pathogenesis is linked to liver tissue damage from fluke migration, which creates an anaerobic microenvironment conducive to *C. novyi* proliferation. The disease follows an acute course, with affected sheep often dying suddenly [11,12]. *C. septicum* primarily invades through skin or mucosal wounds to cause malignant edema marked by painful gaseous swelling and necrotizing myositis, with a fatal course of only 1–2 days [13,14]. *C. chauvoei* mainly affects large livestock such as cattle, sheep, and equines, causing blackleg. Lesions are typically localized to well-muscled areas (e.g., hips, shoulders, and loins), manifesting as localized gaseous swelling with dry, darkened skin [15]. Although each disease has characteristic features, they frequently occur as mixed infections and

can all present as sudden death and acute toxemia, further challenging differential diagnosis. Traditional diagnosis relies on bacterial isolation, culture, morphological staining, biochemical identification, and animal inoculation. However, as strict anaerobes with fastidious culture requirements, clostridia are prone to inactivation during sampling, and transport, and detection can take several days, failing to meet the demand for rapid diagnosis of acute cases [16,17]. Therefore, establishing a highly efficient, specific molecular diagnostic technique capable of simultaneously detecting multiple pathogenic *Clostridium* species is significant for early disease warning, outbreak tracing, and scientific prevention and control [18].

In this study, we designed and screened specific primer sets targeting conserved gene sequences of *C. perfringens*, *C. novyi*, *C. septicum*, and *C. chauvoei* to establish a quadruplex PCR assay. By systematically optimizing key reaction parameters including annealing temperature, cycle number, extension time, and primer concentrations, detection sensitivity and amplification efficiency were significantly improved. The sensitivity, specificity, and reproducibility of the assay were further validated. Although various singleplex and duplex PCR assays have been developed for clostridial pathogens affecting cattle and sheep [19], a multiplex PCR method targeting these four pathogens has not been reported. Compared with traditional methods and singleplex PCR, this assay enables the simultaneous detection of four *Clostridium* species in a single reaction system, reducing costs and shortening detection turnaround time. Thus, it provides technical support for rapid clinical diagnosis, epidemiological investigation, and vaccine development for clostridial diseases in cattle and sheep.

MATERIALS AND METHODS

Bacterial Strains and Sources

Clostridium perfringens strains CVCC 37, CVCC 60081, CVCC 60102, and CVCC 60201; *Clostridium novyi*

strains CVCC 60281 and CVCC 60282; *Clostridium septicum* strain CVCC 60022; *Clostridium chauvoei* strain CVCC 60002; Peste des petits ruminants virus (Clone 9 strain) RNA reference material Z359; Bovine viral diarrhea virus (inactivated) RNA reference material Z435; *Escherichia coli*; *Salmonella*; *Staphylococcus aureus*; *Clostridium tetani*; Bovine enterovirus; Bovine astrovirus; Bovine coronavirus and Bovine viral diarrhea virus; among others, were all obtained from the National Center for Veterinary Culture Collection (CVCC), China.

Main Reagents and Instruments

The pUCm-T ligation kit was purchased from Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China; DH5 α competent cells, 2 $\times$ Rapid Taq PCR Master Mix, and DL 2000 Plus DNA Marker were obtained from Vazyme Biotech Co., Ltd., Nanjing, China; the DNA Gel Extraction Kit was purchased from Omega Bio-Tek, Inc., Norcross, GA, USA; the Bacterial Genomic DNA Extraction Kit, Fecal Genomic DNA Extraction Kit, and Endotoxin-Free Plasmid Mini Kit were obtained from Tiangen Biotech (Beijing) Co., Ltd., Beijing, China; the Magnetic Bead-Based Genomic DNA Extraction Kit for Whole Blood/Saliva/Buccal Swabs (One-Step Method) was purchased from Sino-Link (Beijing) Biotechnology Co., Ltd., Beijing, China; the NanoDrop spectrophotometer was from Thermo Fisher Scientific Inc., Waltham, MA, USA; the PCR thermal cycler and ChemiDoc imaging system were from Bio-Rad Laboratories, Inc., Hercules, CA, USA; the electrophoresis apparatus was from Beijing Liuyi Biotechnology Co., Ltd. Beijing, China; the automatic nucleic acid extraction system was from Wuhan Nach Magnetic Biotechnology Co., Ltd., Wuhan, China; the 2.5 L anaerobic gas generating kits were from Qingdao Hi-Tech Industrial Park Haibo Biotechnology Co., Ltd., Qingdao, China; RNase-Free Water was purchased from Beijing Tsingke Biotech Co., Ltd., Beijing, China; and the 37°C incubator was from Sanyo Electric Co., Ltd., Osaka, Japan.

Main Reagents and Culture Media

Gifu Anaerobic Medium (GAM) broth and Reinforced Clostridial Medium (RCM) were obtained from Qingdao Hi-Tech Industrial Park Haibo Biotechnology Co., Ltd., Qingdao, China.

Nucleic Acid Extraction

C. chauvoei, *C. septicum*, and *C. perfringens* were inoculated into RCM, whereas *C. novyi* was cultured in GAM broth. The 2.5 L anaerobic gas generating kits were placed in sealed jars, and all cultures were incubated at 37°C for 24-48 h. Following incubation, genomic nucleic acids were extracted from bacterial cultures and clinical samples using the Bacterial Genomic DNA Extraction Kit, the Swab Genomic DNA Extraction Kit, and the Magnetic Bead-Based Viral DNA/RNA Extraction Kit, respectively, according to the manufacturers' instructions. The concentration of all extracted genomic DNA was determined using a NanoDrop spectrophotometer, and the samples were stored at -80°C until further use.

Preparation of Recombinant Plasmid Standards

PCR amplification was performed separately using the corresponding nucleic acids as templates. The PCR reaction mixture (25 µL) consisted of 12.5 µL of 2× Taq PCR Master Mix, 1 µL of template, 1 µL each of forward and reverse primers (10 µmol/L), and nuclease-free water to reach the final volume. The PCR cycling conditions were as follows: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 25 s; and a final extension at 72°C for 5 min. The amplification products were analyzed by 2.0% agarose gel electrophoresis, purified using a gel extraction kit, ligated into the pUCm-T vector, and transformed into DH5α competent cells. Positive clones were identified by colony PCR and sequencing. Confirmed positive clones were cultured for plasmid extraction using a plasmid mini kit. The OD_{260/280} values were measured using a NanoDrop spectrophotometer to calculate the plasmid copy

number, and the plasmids were subsequently diluted with nuclease-free water to 1×10^9 copies/ μ L. These dilutions served as positive standards for PCR reactions and were stored at -80°C .

$$\text{copy number(copies}/\mu\text{L}) = \frac{(6.02 \times 10^{23}) \times (X \times 10^{-9})}{(A + 2773) \times 660}$$

The pUCm-T vector used in this study is 2773 bp in size.

Primer Screening and Synthesis

Specific primers targeting the target genes, including *plc*, *fliC*, *α -toxin*, and *cctA*, were designed based on sequences retrieved from GenBank. The primer sequences are listed in **Table 1**. All primers were synthesized by Beijing Ruibo Xingke Biotechnology Co., Ltd., Beijing, China.

Table 1. Primers used in this method

Agent	Primer name	Sequence (5'→3')	Size (bp)	NCBI Accession No.	Reference
<i>plc</i>	plc-F	CCCGCTGTTCTTTTGGAGAG	731	MH900563.1	This study
	plc-R	TGGGCTGGGACATCAACTAA			
<i>fliC</i>	fliC-F	GCTTTAAACGAAACACACGC	529	AB264051.1	This study
	fliC-R	CTTGAGCTGCTTGTTGAAGT			
<i>α-toxin</i>	α -F	AATTCAGTGTGCGGCAGTAGT	230	EU482197.1	This study
	α -R	CGTTTGGATTAGCCGTTCCAC			
<i>cctA</i>	<i>cctA</i> -F	GCAGGGGGAGGAATAAATGGAT	117	JQ692583.1	This study
	<i>cctA</i> -R	GCAACTTCCCAAGATGCACTAT			

Optimization of Uniplex PCR Conditions

To determine the optimal reaction conditions for uniplex PCR, a temperature gradient ranging from 51.0°C to 59.0°C was established based on the melting temperatures (T_m) of the four primer pairs. The reaction system and other conditions identical were to those described above, to identify the optimal annealing temperature for each primer pair. After determining the optimal annealing temperatures, the specificity of each primer pair was evaluated via PCR amplification. Nucleic acids from the four target bacteria (*C. perfringens*, *C. novyi*, *C. septicum*, and *C. chauvoei*) and non-target pathogens, including *Clostridium tetani* (*C. tetani*), *Escherichia coli* (*E. coli*), *Salmonella* spp., and *Staphylococcus aureus* (*S. aureus*), were used as templates under the same reaction system and cycling conditions. The amplification products were analyzed by agarose gel electrophoresis to assess the specificity of each primer pair. Through these two optimization steps, the optimal annealing temperatures for uniplex PCR were determined and primer specificity was verified, laying the foundation for the subsequent development of the multiplex PCR system.

Optimization of Multiplex PCR Reaction System and Validation of Sensitivity, Specificity, and Repeatability

Nucleic acid concentrations were determined using a NanoDrop spectrophotometer and diluted to 5 ng/μL with ddH₂O. Equal volumes of nucleic acids from each target species were mixed to serve as the template for multiplex PCR. The annealing temperature range was initially predicted using Oligo7 bioinformatics software, followed by optimization using gradient temperature and checkerboard methods. The optimized parameters included annealing temperature (59, 58.6, 57.7, 56.1, 54.2, 52.6, 51.5, and 51°C), primer volumes (0.2, 0.5, 0.75, 1.0, and 1.5 μL), and extension time (5, 10, 15, 20, 25, and 30 s), followed by optimization of cycle numbers (20, 25, 30, 35, and 40). Amplification products (6 μL) were analyzed by electrophoresis on 2% agarose gels. Using the optimized reaction system and conditions, multiplex PCR amplifications were performed with nucleic acids from non-target pathogens including *C. tetani*, *E. coli*, *S. aureus*,

Salmonella spp., bovine enterovirus (BEV), bovine astrovirus (BAstV), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), and peste des petits ruminants virus (PPRV) as templates. A mixture of nucleic acids from the four target bacteria (*C. perfringens*, *C. novyi*, *C. septicum*, and *C. chauvoei*) was used as the positive control, and ddH₂O served as the negative control, to evaluate the specificity of the established method. Meanwhile, the constructed standard plasmids were serially diluted 10-fold with ddH₂O. Equal volumes of the diluted plasmids from each target species were mixed to serve as templates, with nuclease-free water used as the negative control. The optimized reaction conditions were applied to determine the limit of detection (LOD) of the multiplex PCR assay, thereby evaluating its sensitivity. Furthermore, using 5 ng/μL of the nucleic acid mixture as the template and ddH₂O as the negative control, the established multiplex PCR assay was performed once per month over a total of three independent runs. The results were assessed by agarose gel electrophoresis to determine the repeatability and reliability of the detection method.

Detection of Clinical Samples

A total of 206 clinical bovine anal swab samples were collected from different cattle farms in Jiangsu Province. Nucleic acids were extracted using an automated nucleic acid extraction system and detected with the established multiplex PCR assay. In parallel, singleplex PCR assays targeting *C. perfringens* [20], *C. novyi* [21], *C. septicum* [22], and *C. chauvoei* [19] were performed according to previously published methods, and the results were compared with those of the multiplex PCR for cross-validation.

RESULTS

Preparation of Recombinant Plasmid Standards

Based on conserved gene sequences, specific primer pairs were designed and screened for each target gene: *plc*, *fliC*, *α-toxin*, and *cctA*. PCR verification confirmed that all four primer pairs successfully amplified specific bands of the

expected sizes, with fragment lengths of 731 bp, 529 bp, 230 bp, and 117 bp, respectively(**Figure 1**). The target fragments were cloned into the pUCm-T vector. Positive clones were screened via colony PCR and subsequently sequenced by Beijing Ruibo Xingke Biotechnology Co., Ltd., Beijing, China for sequencing. The plasmids with correctly confirmed sequences were preserved as standard positive templates.

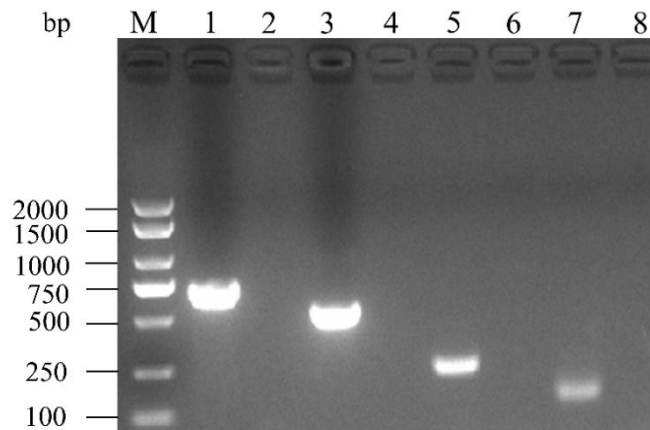


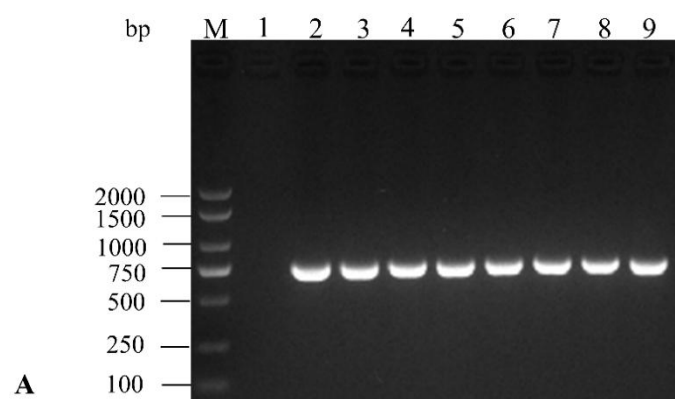
Figure 1. Amplification of *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*, and *Clostridium chauvoei*.

M: DNA molecular weight marker (DL 2000 Plus); Lane 1 = *Clostridium perfringens* (731 bp); Lane 2 = *Clostridium perfringens* negative control; Lane 3 = *Clostridium novyi* (529 bp); Lane 4 = *Clostridium novyi* negative control; Lane 5 = *Clostridium septicum* (230 bp); Lane 6 = *Clostridium septicum* negative control; Lane 7 = *Clostridium chauvoei* (117 bp); Lane 8 = *Clostridium chauvoei* negative control.

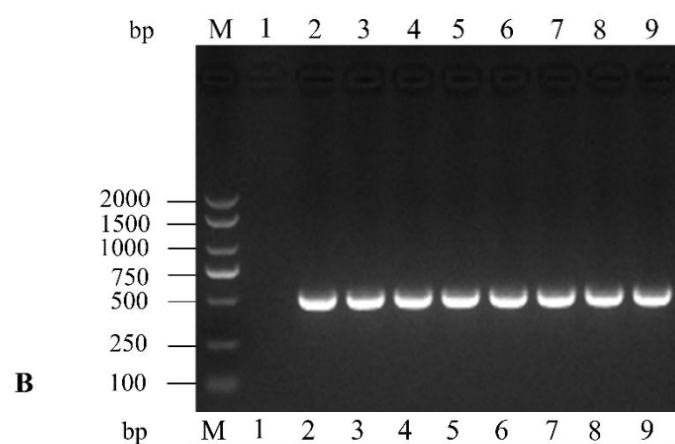
Optimization of Uniplex PCR Conditions

Based on the melting temperatures (T_m) of the four primer pairs, a temperature gradient ranging from 51.0°C to 59.0°C was established for annealing temperature optimization. PCR amplifications were performed for *C. perfringens*, *C. novyi*, *C. septicum*, and *C. chauvoei*. Specific target fragments of the expected sizes were successfully amplified across the entire temperature range of 51.0°C to 59.0°C(**Figure 2**).

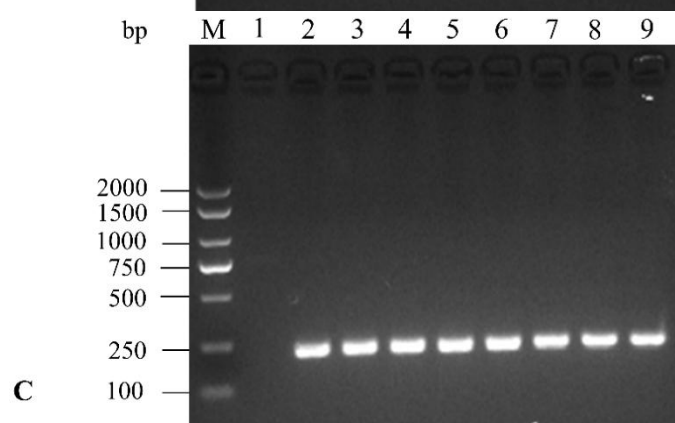
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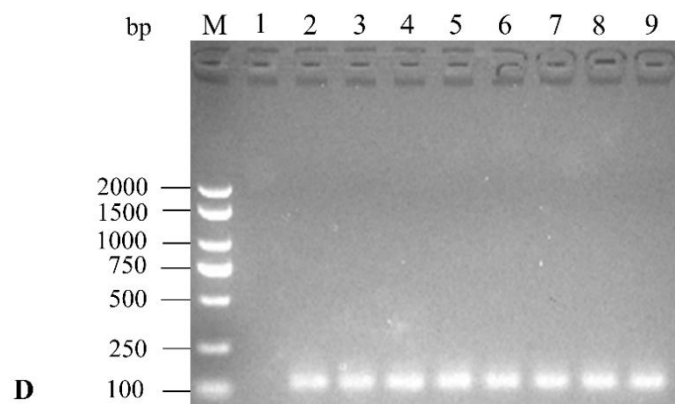
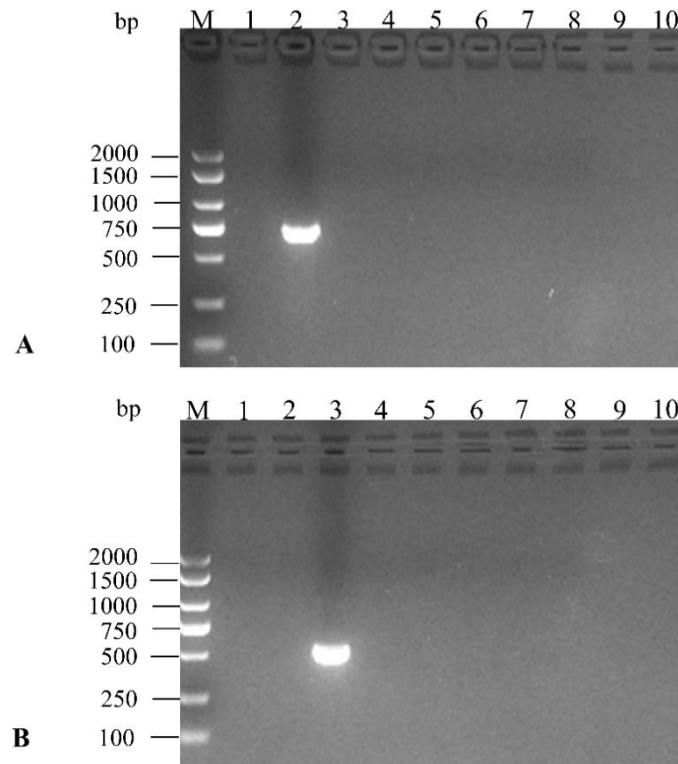


Figure 2. Amplification results of single PCR at different annealing temperatures.

A-D: *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*, and *Clostridium chauvoei*. M: DNA molecular weight marker (DL 2000 Plus); Lane 1: negative control; Lanes 2–8: 51 °C, 51.6 °C, 52.8 °C, 54.5 °C, 56.7 °C, 58 °C, 59 °C, and 60 °C.

Uniplex PCR specificity tests were performed using the four primer pairs designed in this study, with genomic DNA from *C. perfringens*, *C. novyi*, *C. septicum*, *C. chauvoei*, *C. tetani*, *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus* serving as templates(**Table 1**). Reaction conditions followed those described in the Methods section. Specific amplification bands were observed only when templates corresponded to their respective primers, whereas no amplification was detected for other templates(**Figure 3**).



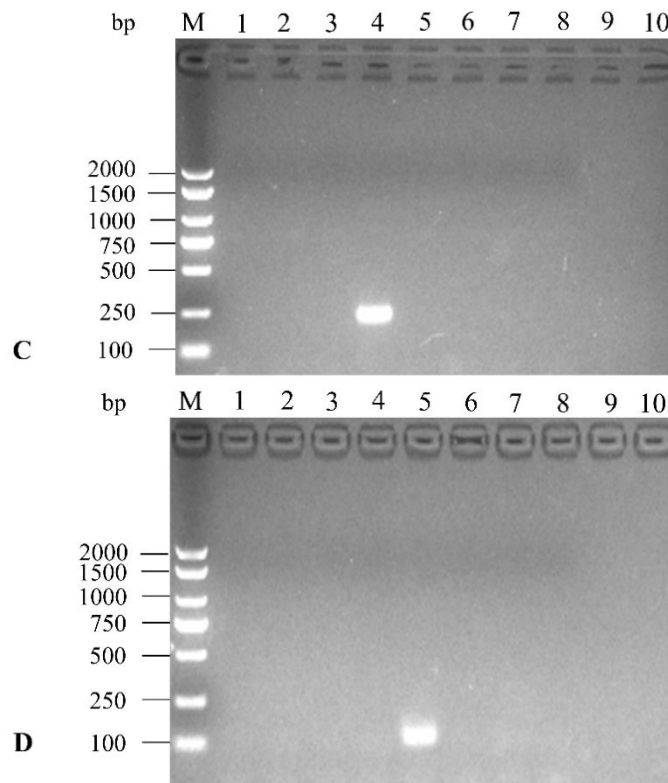


Figure 3. Specificity analysis of single PCR.

A-D: *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*, and *Clostridium chauvoei*. M: DNA molecular weight marker (DL 2000 Plus); Lanes 1 and 10: negative control; Lanes 2–9: *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*, *Clostridium chauvoei*, *Clostridium tetani*, *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*.

Optimization of the Multiplex PCR Reaction System and Conditions

To optimize the multiplex PCR assay, the annealing temperature was first evaluated across a gradient of 51.0°C to 59.0°C, based on uniplex PCR results. As shown in **Figure 4**, all four primer pairs targeting the *plc*, *fliC*, *α-toxin*, and *cctA* genes achieved effective amplification with specific bands within this range; consequently, 55°C was selected as the optimal annealing temperature for subsequent experiments. Subsequently, extension time was optimized using a gradient approach. Results (**Figure 5**) demonstrated that effective amplification with clear target bands and no nonspecific products was achieved across extension times of 5 s to 30 s. Considering both amplification efficiency and

detection time, the extension time was set at 5 s. Furthermore, the cycle number was optimized by testing values from 20 to 40 cycles at 5-cycle intervals. As shown in **Figure 6**, satisfactory amplification was achieved at 30 to 40 cycles; therefore, 30 cycles were selected to balance experimental efficiency. Based on this comprehensive optimization, the final multiplex PCR reaction system was established as follows: 12.5 μ L of 2 $\times$ Rapid Taq Master Mix, 3.5 μ L of ultrapure water, 0.5 μ L each of the forward and reverse primers (10 μ mol/L) for *plc*, *fliC*, and *α -toxin*, 1.0 μ L each of the forward and reverse primers (10 μ mol/L) for *CctA*, and 4 μ L of DNA template, in a total volume of 25 μ L. The final cycling program consisted of initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 5 s; followed by a final extension at 72°C for 5 min.

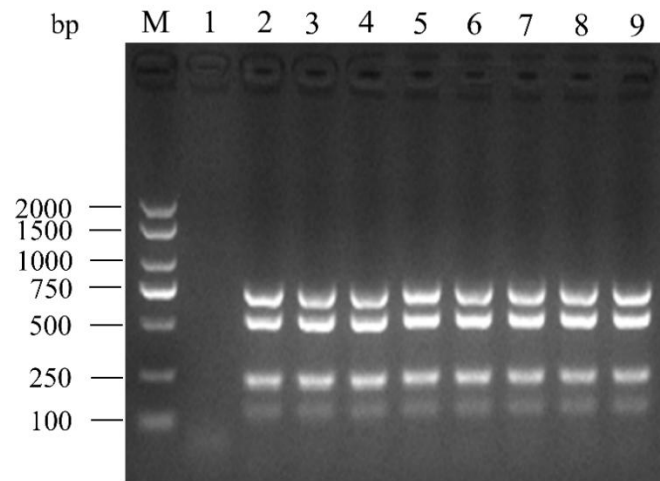


Figure 4. Optimization of annealing temperature for multiplex PCR.

M: DNA molecular weight marker (DL 2000 Plus); Lane 1: negative control; Lane 2: 51 °C; Lane 3: 52 °C; Lane 4: 53 °C; Lane 5: 55 °C; Lane 6: 56.7 °C; Lane 7: 58 °C; Lane 8: 59 °C; Lane 9: 60 °C.

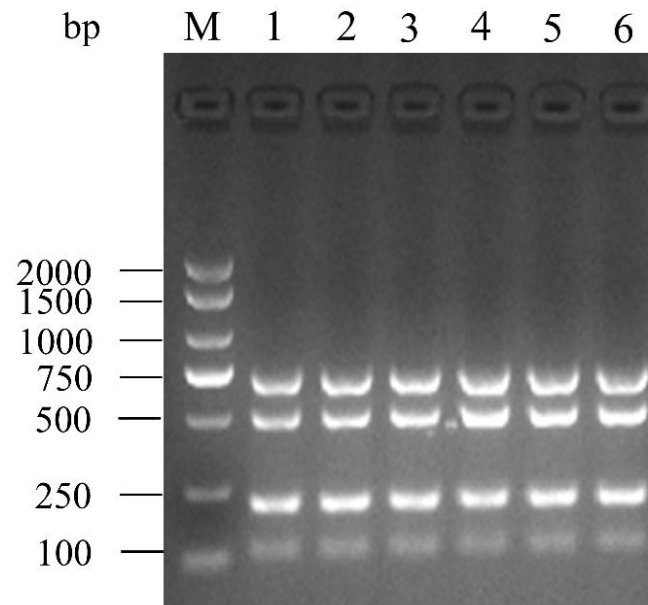


Figure 5. Amplification results of multiplex PCR with different extension times.

M: DNA molecular weight marker (DL 2000 Plus); Lane 1: 5 s; Lane 2: 10 s; Lane 3: 15 s; Lane 4: 20 s; Lane 5: 25 s; Lane 6: 30 s.

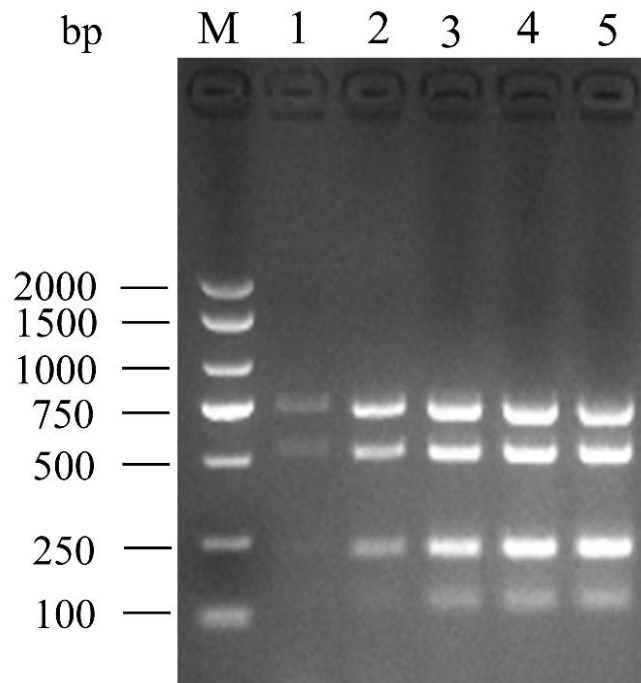


Figure 6. Optimization results of cycle number for multiplex PCR.

M: DNA molecular weight marker (DL 2000 Plus); Lane 1: 20 cycles; Lane 2: 25 cycles; Lane 3: 30 cycles; Lane 4: 35 cycles; Lane 5: 40 cycles.

Optimization of Multiplex PCR Reaction System and Validation of Sensitivity, Specificity, and Repeatability

The sensitivity, specificity, and repeatability of the established multiplex PCR assay were systematically evaluated. For the sensitivity assay, the recombinant plasmids constructed as described above were serially diluted 10-fold with nuclease-free water, and equal volumes of the diluted plasmids for each target gene were mixed to serve as PCR templates. Using the optimized reaction system and cycling conditions, results (**Figure 7**) revealed that the LOD for *plc* was 10^2 copies/ μ L, while the LOD for *fliC* and *cctA* was 10^3 copies/ μ L, and the LOD for *α -toxin* was 10^4 copies/ μ L. Subsequently, assay specificity was assessed by performing multiplex PCR amplifications under optimized conditions using nucleic acids from *C. perfringens*, *C. novyi*, *C. septicum*, *C. chauvoei*, *C. tetani*, *E. coli*, *S. aureus*, *Salmonella* spp., BEV, BAstV, BCoV, BVDV, and PPRV as templates, with ddH₂O as the negative control. Results(**Figure 8**) demonstrated that specific amplification was observed exclusively for the four target pathogens, with no cross-reactivity against any non-target pathogens. Furthermore, to verify repeatability, three independent multiplex PCR runs were conducted at different time points. Results (**Figure 9**) confirmed that the assay exhibited good repeatability. Finally, the interpretation criteria for multiplex PCR results were established as follows: the test run was considered valid when DNA marker bands were clearly resolved, the negative control showed no amplification, and the positive control yielded specific amplicons of the expected sizes. Under valid experimental conditions, a sample was deemed positive for a given pathogen when a band of the corresponding size was observed; co-detection of multiple bands indicated co-infection with the corresponding pathogens; and the absence of specific amplification bands was interpreted as a negative result.

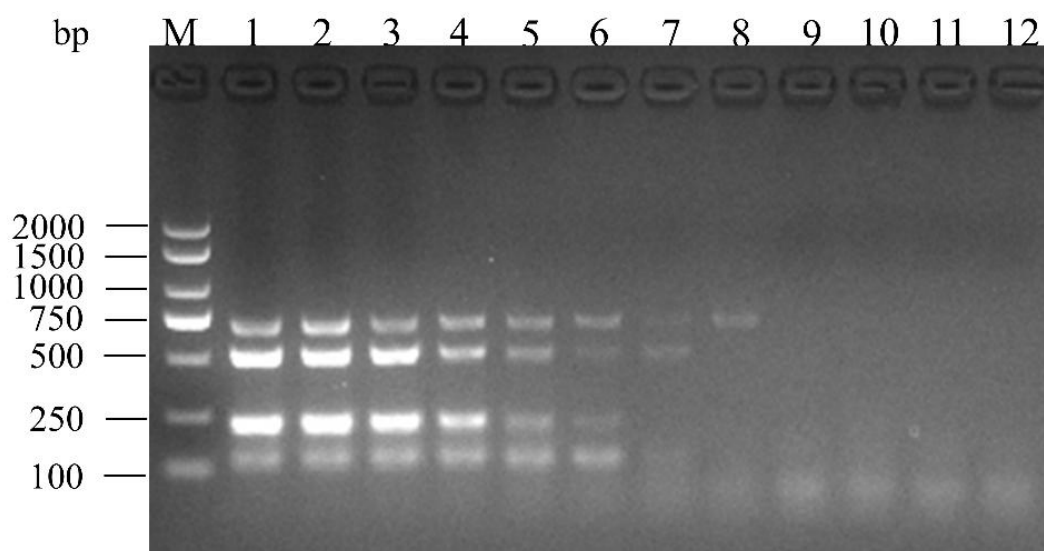


Figure 7. Sensitivity assay of multiplex PCR.

M: DNA molecular weight marker (DL 2000 Plus); Lanes 1–11: 10^9 to 10^{-1} ; Lane 12: negative control.

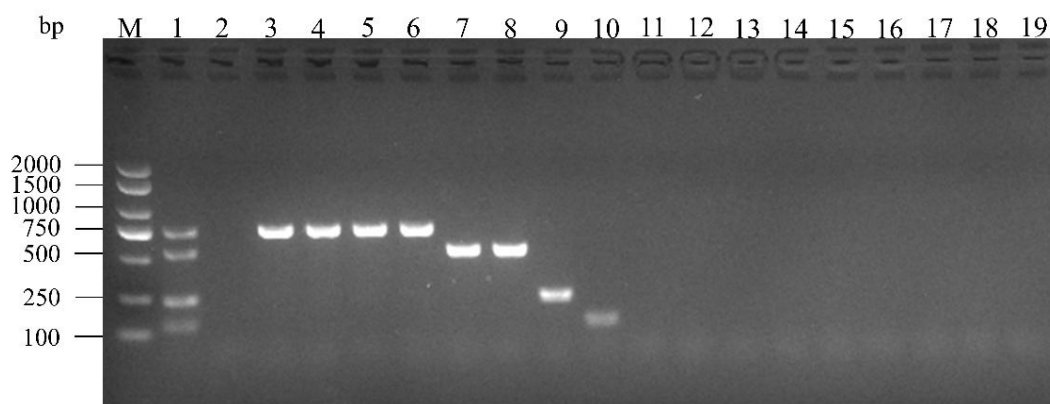
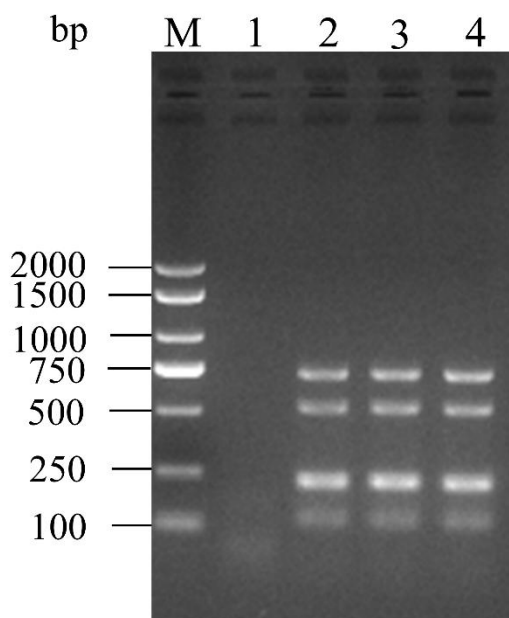


Figure 8. Specificity test of multiplex PCR.

M: DNA molecular weight marker (DL 2000 Plus); Lane 1: positive control; Lane 2: negative control; Lanes 3–6: *Clostridium perfringens* CVCC 37, *Clostridium perfringens* CVCC60081, *Clostridium perfringens* CVCC 60102, and *Clostridium perfringens* CVCC 60201; Lanes 7–8: *Clostridium novyi* CVCC 60281 and *Clostridium novyi* CVCC 60282; Lane 9: *Clostridium septicum* CVCC 60022; Lane 10: *Clostridium chauvoei* CVCC 60002; Lane 11: *Clostridium tetani*; Lane 12: *Escherichia coli*; Lane 13: *Staphylococcus aureus*; Lane 14: *Salmonella* spp.; Lane 15: bovine enterovirus ; Lane 16: bovine astrovirus ; Lane 17: bovine coronavirus ; Lane 18:

257 bovine viral diarrhea virus ; Lane 19: peste des petits ruminants virus .



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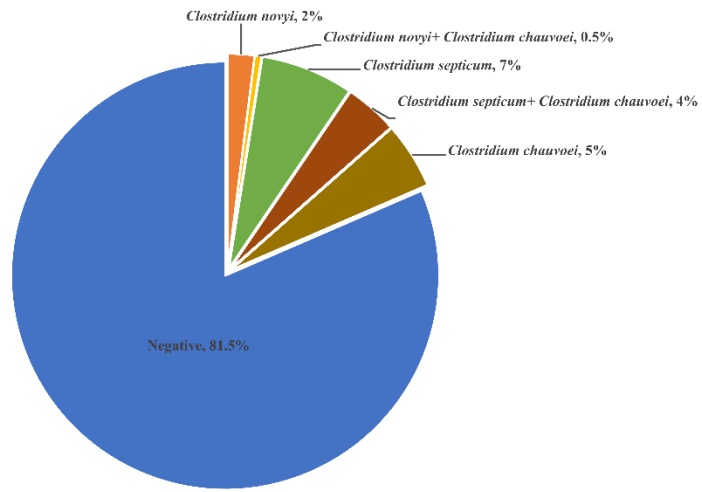
259 **Figure 9.** Repeatability analysis of multiplex PCR.

260 M: DNA molecular weight marker (DL 2000 Plus); Lanes 1–3: three replicates.

261 **Detection of Clinical Samples**

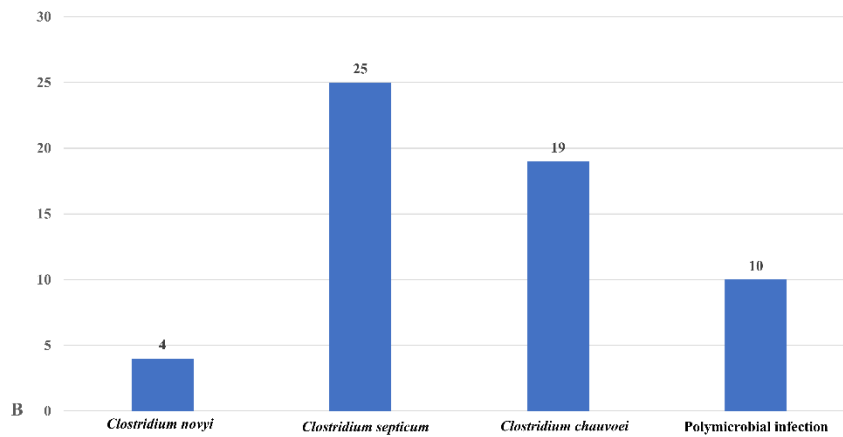
262 The optimized multiplex PCR assay was applied to detect 206 bovine and ovine fecal swab samples (**Fig. 10A**). Detection
263 results showed that *C. novyi* was identified in 4 cases (2%), *C. septicum* in 25 cases (12%), and *C. chauvoei* in 19 cases
264 (9%). Mixed infections were detected in a total of 10 samples (5%), comprising 1 case of co-infection with *C. novyi* and
265 *C. septicum*, and 9 cases of co-infection with *C. septicum* and *C. chauvoei* (**Fig. 10B**). After excluding duplicate counts
266 arising from mixed infections, the actual number of positive samples was 38, yielding an overall positivity rate of 18%
267 (38/206), with *C. septicum* exhibiting the highest rate among the four target pathogens. The multiplex PCR results were
268 fully concordant with those obtained by singleplex PCR, and the multiplex assay demonstrated advantages including
269 high detection efficiency, excellent sensitivity, time-saving operation, and low cost.

Positivity rates of four target *Clostridium* species in 206 clinical samples



A

Positivity rates of four target *Clostridium* species in 206 clinical samples



B

Figure 10. Detection of four pathogenic *Clostridium* species in clinical bovine samples by multiplex PCR assay.

A: Representative electrophoresis results of amplification products from clinical anal swab specimens; B: Counts of positive samples and mixed infections among 206 specimens: *Clostridium novyi* (4), *Clostridium septicum* (25), *Clostridium chauvoei* (19),

and polymicrobial infections (10).

DISCUSSION

During primer design, candidate targets were initially selected through literature review and genome alignment[23]: the *plc* gene of *C. perfringens*, the *Tcna* gene of *C. novyi*, the α -toxin gene of *C. septicum*, and the *cctA* gene of *C. chauvoei*. However, Primer-BLAST specificity evaluation and preliminary experiments revealed that the *Tcna* gene of *C. novyi* exhibited excessive homology and significant non-specific amplification. Consequently, this target was discarded and replaced with the *fliC* gene, for which new primers were designed. Subsequently, multiple candidate primer pairs were designed for each target gene. Following repeated screening, specificity assessment, and optimization of primer combinations, four optimal primer pairs were finalized. These yielded amplicons of 731 bp (*plc*), 529 bp (*fliC*), 230 bp (α -toxin), and 117 bp (*cctA*), which were clearly resolved on a 2.0% agarose gel. The assay exhibits favorable overall performance in specificity and reproducibility. Its detection sensitivity is comparable to or slightly better than the 7.5×10^3 copies/ μ L limit of detection reported for a *C. perfringens* multiplex PCR established by Jin et al. (2025) [24] and the multiplex PCR developed by Assis et al. (2008) [25] for detecting *C. chauvoei* and *C. septicum*. Furthermore, this assay enables simultaneous detection of all four Clostridium species, thereby substantially improving detection efficiency.

The established multiplex PCR assay was applied to detect 206 clinically collected bovine and ovine anal swab samples. The results indicated an overall positivity rate of 18% (38/206). Mixed infections were identified in 10 samples (5%), comprising one case of co-infection with *C. novyi* and *C. septicum*, and nine cases of co-infection with *C. septicum* and *C. chauvoei*. The presence of mixed infections suggests that clinical clostridial disease may arise from the synergistic pathogenicity of multiple pathogens. Consequently, detecting a single Clostridium species alone could lead to

misdiagnosis of the etiology. Therefore, the multiplex detection capability of this assay is valuable for comprehensively assessing pathogen distribution and elucidating mixed-infection patterns [25,26]. Notably, some positive samples were obtained from cattle and sheep without overt clinical signs, which may be associated with subclinical carriage of clostridia in the host. When host resistance declines or environmental stressors occur, clostridia can proliferate extensively and produce toxins, thereby triggering disease [3,27]. Multiplex PCR results should be integrated with clinical history, husbandry practices, and clinical signs for comprehensive evaluation, enabling the implementation of targeted prevention and control strategies for clostridial diseases. South Asia, East Asia, and Southeast Asia are regions with the highest *C. perfringens* infection rates in sheep and goats. An analysis based on 29 studies involving a total of 8,386 sheep and goat samples revealed an estimated pooled prevalence of 38.8% (95% confidence interval: 30.9%-46.9%), with toxinotype A being the most common type. The detection rates in healthy and diseased populations were 20.7% and 28.7%, respectively [28]. Subgroup analyses indicated that infection rates are significantly influenced by multiple factors. Regarding sample type, the detection rate in intestinal samples (40.6%) was higher than that in fecal samples (36.8%), which may be attributable to the fact that *C. perfringens*, as an anaerobe, grows more readily in the intestinal environment. The detection rate was highest in young animals aged 3-12 months (48.4%), whereas it was markedly lower in adult animals over one year of age (32.9%), suggesting that young animals are more susceptible. In terms of vaccination, the detection rate in unvaccinated groups (63.0%) was far higher than that in vaccinated groups (23.9%), confirming the key role of vaccines in reducing infection risk. The zero detection of *C. perfringens* in this study contrasts somewhat with the previously reported high prevalence. This discrepancy may be attributable to the following factors: first, the sampled populations may have been widely vaccinated against clostridial diseases, thereby reducing the carrier rate; second, single-point sampling and limited sample size may have resulted in an underestimation of the

detection rate. In summary, these findings highlight the urgency and necessity of implementing vaccination and continuous surveillance to reduce the risk of *C. perfringens*-associated outbreaks.

With the accelerated development of large-scale and intensive cattle and sheep farming in China, multiple clostridial vaccines have been licensed for market distribution. Traditional sheep clostridial triple- or quadruple-component inactivated vaccines have played an important role in clinical prevention and control [4]. The substantial annual batch-release volumes indicate a sustained strong demand for clostridial disease prevention in the livestock industry (Fig. 11). For example, from 2021 to 2025, both the production batches and the number of doses of the hexavalent lyophilized inactivated vaccine against braxy, struck, lamb dysentery, enterotoxemia, black disease, and *Clostridium botulinum* type C toxemia increased rapidly. In contrast, the number of doses of the tetravalent lyophilized inactivated vaccine against braxy, struck, lamb dysentery, and enterotoxemia showed a slight declining trend over the same period. This indicates that the Chinese clostridial vaccine market is gradually replacing the tetravalent vaccine with a more convenient and efficient hexavalent vaccine. Although the upgrading of multivalent inactivated vaccines has improved the convenience of prevention and control, traditional inactivated vaccines still suffer from limitations such as unstable toxin production, difficulties in manufacturing processes, and challenging quality control. These limitations make it difficult to fully meet the precision prevention and control requirements of large-scale farming [21]. In the area of novel vaccine development, genetically engineered subunit vaccines have become an important direction. For instance, the genetically engineered subunit vaccine against bovine *C. perfringens* type A produced by Jinyu Baoling Biological Pharmaceutical Co., Ltd. has obtained a national new veterinary drug certificate in China [29]. based on immunoinformatics approaches, designed a recombinant fusion protein vaccine, rFPD, targeting both *C. perfringens* type D epsilon toxin and *C. novyi* type B α toxin. However, this remains at the theoretical design stage, and no specific animal protection efficacy data have been provided.

These developments indicate that clostridial vaccines are evolving from traditional multivalent inactivated vaccines toward genetically engineered subunit vaccines[30].

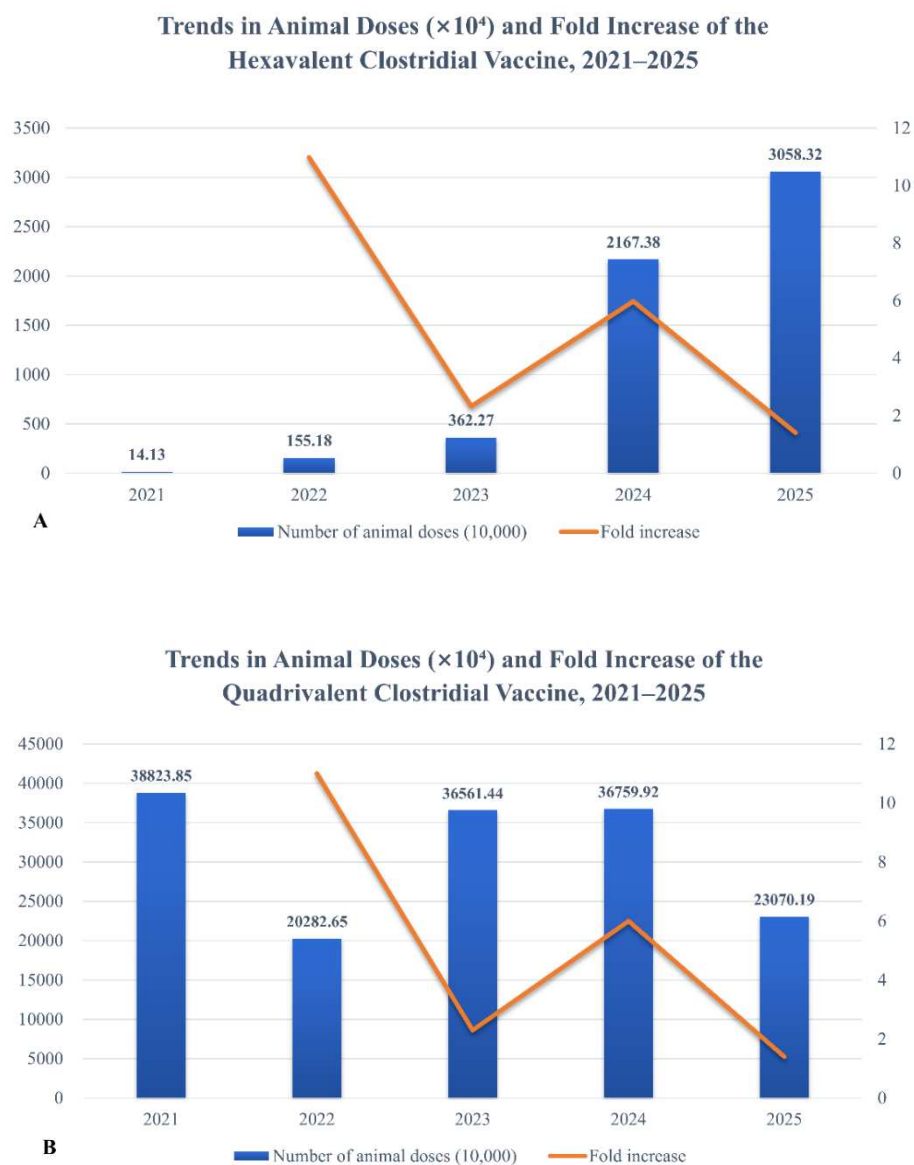


Figure 11. Changes in the number of doses of multivalent clostridial vaccines in China, 2021–2025.

A: Number of animal doses of the hexavalent freeze-dried inactivated vaccine against braxy, struck, lamb dysentery, enterotoxemia, black disease, and *Clostridium botulinum* type C toxicosis, 2021–2025.

B: Number of animal doses of the quadrivalent freeze-dried inactivated vaccine against braxy, struck, lamb dysentery, and enterotoxemia, 2021–2025.

Although the established method can simultaneously detect four major pathogenic *Clostridium* species, it has certain limitations. The genus *Clostridium* contains numerous pathogenic species; besides the four species detected in this study, *C. botulinum* and *C. haemolyticum* are also important zoonotic pathogens [31]. Because of the limited genomic sequence divergence among different *Clostridium* species, simple primer design often struggles to distinguish highly homologous species. For example, the 16S rRNA gene sequences of *C. novyi* type B and *C. haemolyticum* share >99.9% similarity. Furthermore, the N-terminal and C-terminal amino acid sequences of their *fliC* proteins are highly conserved, with variation confined to the middle region [32]. Moreover, optimal amplification conditions may differ across clostridial species, posing considerable technical challenges for achieving efficient co-amplification of additional targets. Therefore, future studies can further expand the range of detection targets on this basis to enable high-throughput detection of a broader spectrum of clostridial pathogens.

In conclusion, the one-step multiplex PCR assay developed in this study demonstrates high specificity, sensitivity, rapidity, and reproducibility. It is suitable for the rapid clinical diagnosis, epidemic surveillance, and molecular epidemiological investigation of pathogenic *Clostridium* species in cattle and sheep, thereby holding practical value for safeguarding the healthy development of the livestock industry.

CONCLUSIONS

In this study, a multiplex PCR assay was developed for the simultaneous detection of four pathogenic *Clostridium* species in ruminants: *C. perfringens*, *C. novyi*, *C. septicum*, and *C. chauvoei*. Based on a literature review and preliminary primer screening, the *plc*, *fliC*, *α -toxin*, and *cctA* genes were selected as target markers. Following stringent primer design, Primer-BLAST specificity analysis, and combinatorial optimization, four optimal primer pairs were identified. These yielded amplicons of 731 bp, 529 bp, 230 bp, and 117 bp, which were clearly resolved on a 2.0% agarose gel. Reaction conditions were optimized to an annealing temperature of 55°C, primer concentrations of 0.25, 0.25, 0.25, and 0.50 μ mol/L, respectively, and 30 amplification cycles, enabling efficient and specific amplification without nonspecific products. The assay exhibited excellent specificity, showing no cross-reactivity with *C. tetani*, *E. coli*, *Salmonella spp.*, *S. aureus*, BEV, BAsV, BCoV, BVDV, or PPRV. Both intra- and inter-assay repeatability tests yielded consistent results, confirming high reproducibility. The limits of detection for the four recombinant plasmid standards were 10^2 , 10^3 , 10^4 , and 10^3 copies/ μ L, respectively. These values are comparable to or slightly better than those of previously reported singleplex PCR assays, while enabling the simultaneous detection of all four targets. When applied to 206 clinical tissue and fecal samples from cattle and sheep, the positivity rates for *C. perfringens*, *C. novyi*, *C. septicum*, and *C. chauvoei* were 0%, 2%, 12%, and 9%, respectively, with an overall positivity rate of 18% (38/206). Mixed infections were identified in 5% (10/206) of the samples, comprising one co-infection with *C. novyi* and *C. septicum*, and nine co-infections with *C. septicum* and *C. chauvoei*. Collectively, the developed multiplex PCR method offers high specificity, sensitivity, and detection efficiency, providing a valuable tool for the clinical diagnosis and epidemiological investigation of these four clostridial pathogens.

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Declarations

Ethics approval and consent to participate

All clinical samples were collected from cattle during routine health monitoring with the informed consent of the farm owners/managers. The study involved no invasive animal procedures or human subjects, thus ethical approval from the Institutional Animal Care and Use Committee or Institutional Review Board was not required.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The primer sequences used in this study are listed in Table 1. The recombinant plasmid standards and bacterial strains are available from the corresponding authors upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

M.W. conceived and designed the study, performed the multiplex PCR experiments, analyzed the data, and drafted the manuscript. Y.F. participated in primer design, PCR optimization, and data analysis. R.W. collected clinical samples and

performed epidemiological data recording. X.R., C.H., H.Z., J.L., L.Z., and W.Y. assisted with bacterial culture, nucleic acid extraction, PCR validation, and data collation. M.Z. and Y.Z. supervised the study, acquired funding, and critically revised the manuscript. All authors read and approved the final manuscript.

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