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Research Article

Development and Application of an Immunocapture PCR Diagnostic Assay Based on the Monoclonal Antibody for the Detection of *Shigella*

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Background: *Shigella* is among the most important human pathogenic microorganisms, infecting both humans and nonhuman and causing clinically severe diarrhea. *Shigella* must be enriched before detection, which is time-consuming.
Objectives: To develop a sensitive, rapid, and specific method for *Shigella* detection.
Materials and Methods: *Shigella* was used as an antigen to generate monoclonal antibodies (mAbs). mAbs were screened via indirect enzyme-linked immunosorbent assay (ELISA) and western blot, and two mAbs were selected. The mAb A3 showed high affinity and specificity and was used to develop immune magnetic beads (IMBs) for *Shigella* enrichment. An immunocapture (IC)-PCR primer was designed from the *ipaH* gene, and IC-PCR was developed based on the IMBs and PCR.
Results: This system shortened the *Shigella* detection time to 70 min. The sensitivity of the IC-PCR was 9 colony-forming units.mL^{-1} in artificial milk. The accuracy of the IC-PCR was confirmed using 46 clinical samples collected from monkeys. The IC-PCR results were consistent with the serological and biochemical assays.
Conclusion: The IC-PCR described herein accurately detected *Shigella* from milk samples, monkeys and can thus be used to complement classical detection methods.
Keywords: Magnetic Immunocapture; Monoclonal Antibodies; PCR; *Shigella*

1. Background

As an important bacterial pathogen worldwide, *Shigella* causes foodborne gastroenteritis, resulting in severe bacterial diarrhea. *Shigella* has caused serious public health problems in many countries (1). More than 1.8 million patients worldwide have died from acute diarrhea, and many cases of acute diarrhea (160 million annually) are caused by *Shigella* infections (2) Numbers of *Shigella*-associated foodborne diseases continue to increase in China (3). Numerous studies have reported that *Shigella* causes most cases of bacterial dysentery, especially in developing countries (4, 5, 7).

Humans and other primates are the main natural hosts of *Shigella*. However, a recent report showed that some new hosts, such as piglets, calves, chickens, and rabbits, can also be infected with *Shigella* (5). In China, a serum epidemiological survey of *Shigella*-infected animals revealed that *Shigella* has a 28.3%–33.7% seroprevalence, suggesting that *Shigella* has an important etiology among animals in China and that the *Shigella* epidemic in China is serious. Thus, *Shigella* must be rapidly and accurately detected to protect the food industry and animal health (6). Culture-based techniques are the widely used gold standards of *Shigella* detection; however, they are time-consuming, labor-intensive, and require multiple subculturing steps with subsequent biochemical and serological tests (7). PCR is a highly sensitive technique that requires few steps. PCR is used to detect various pathogens, including fungi, parasites, viruses, and bacteria (8, 9). However, PCR cannot quickly target single cells, thus necessitating *Shigella* to be enriched before detection as well as requiring a bacterial genome extraction kit to extract the genome or plasmid (10).

Immune magnetic separation technology is a new biological detection method used to enrich bacteria (11, 12). Immune magnetic beads (IMBs) are coated with a specific antibody that can recognize a specific antigen. These IMB-antibody-cell complexes are isolated from the environment under the action of an outside magnetic field to rapidly enrich cells without bacterial culturing or centrifugation (13). However, this technology can only separate the bacteria; thus, other methods are needed to further identify the bacteria. IMB sensitivity must also be improved.

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In this study, the hybridomas, A3 and G5, which stably secrete antibodies against *Shigella*, were screened. PCR combined with a magnetic immunocapture (IC) assay was developed and applied to screen for *Shigella*. IC-PCR does not require extracting plasmids or genomic DNA, making it faster, more convenient, and efficient in pathogen cell enrichment. Therefore, the IC-PCR developed in this study efficiently and rapidly screened for *Shigella* and showed high specificity in pure cultures, artificial milk, and clinical samples.

2. Objectives

This study was conducted to develop a simple, rapid, and visual detection method employing the IC-PCR based on the *ipaH* gene.

3. Materials and Methods

3.1. Strains Used

Eight strains were used in this study (**Table 1**). Forty-six clinical samples (12 containing the *Shigella* strain and 34 containing non-*Shigella* strains) were obtained from Kunming Biomed International (Table 3). All strains were refreshed on Luria-Bertani (LB) solid plates (1% tryptone, 1% NaCl, 0.5% yeast extract, 1.5% agar, pH 7.0) for overnight at 37 °C, and a single clone was cultured in LB liquid medium (1% tryptone, 1% NaCl, 0.5% yeast extract, pH 7.0) for 6 h at 37 °C, in a shaking incubator at 160 rpm (10).

Table 1. Bacterial strains used in this study

Bacterial	Bacterial Strains Source
Shigella	Isolated from monkey
P. aeruginosa	1606SE0010, isolated from secretion substance
E. coli	ATCC25922
S. aureus	ATCC29213
Salmonella	ATCC13076
A. baumannii	Isolated from clinical samples
K. pneumoniae	1412SP0200, isolated from sputum
Listeria	1608SE0080, isolated from secretion substance

3.2. Production of Monoclonal Antibodies (mAbs) and Polyclonal Antibodies (pAbs) Against Shigella

mAbs and pAbs against *Shigella* were produced and characterized as previously described (10, 14). After a booster immunization, spleen cells were collected from immunized BALB/c mice, and then fused with SP2/0 cells. The fused cells were maintained in Roswell Park Memorial Institute (RPMI 1640) medium containing 20% fetal bovine serum (FBS) and 1% hypoxanthine-aminopterin-thymidine (Sigma-Aldrich) (15, 16). Five days later, half of the medium was replaced with RPMI 1640 containing 20% FBS and 1% hypoxanthine-thymidine (Sigma-Aldrich). The hybridoma supernatants were examined via indirect enzyme-linked immunosorbent assay (ELISA) to detect *Shigella*-specific antibodies (14, 17).

3.3. Preparation and Purification of Ascites

Two positive hybridoma cells were prepared via the limited dilution method after three cycles of subcloning. The hybridoma cells (10^6 cells) were suspended in RPMI 1640 then injected into each female mouse that had been intraperitoneally inoculated with 0.5 mL of sterile paraffin oil one week prior. Ascites was collected from the mice and purified with protein A Sepharose (GE Healthcare, Chicago, IL, USA) (14, 18). The purified ascites titer was determined via ELISA and identified via dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) (14, 19). A BCA assay was used to determine the mAb concentrations.

3.4. Antibody Isotype Determination

The immunoglobulin isotypes of the A3 and G5 mAbs were determined using a mouse mAb isotyping kit (SouthernBiotech, SBA clonotyping System-HRP Kit), according to the manufacturer's instructions.

3.5. Western Blot

Western blot was used to evaluate the mAb reactivity against *Shigella*. *Shigella* was collected and suspended in 25 mL of phosphate-buffered saline (PBS; 2.7 mM KCl, 2 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4, pH 7.4), then sonicated on ice. The lysate and supernatant were harvested after centrifugation. Proteins in the supernatant and lysate were subjected to SDS-PAGE and transferred onto nitrocellulose (NC) membranes. The NC membrane was blocked with 5% (w/v) skim milk in PBS containing Tween-20 (PBS-T; 2.7 mM KCl, 2 mM KH_2PO_4, 137

mM NaCl, 10 mM Na_2HPO_4, 0.05% Tween-20, pH 7.4) for 2 h at 37 °C. Next, mAb A3 against *Shigella* (1:2,000 dilutions) was added and maintained for 2 h at 37 °C, then washed 5 times with PBS-T. Goat anti-mouse IgG (H+L) (1:5,000; GenScript, USA) was added and incubated for 1 h at 37 °C. Finally, the NC membrane was washed as described above and analyzed using the western blot kit, Easy See (TransGen, Beijing, China).

3.6. Genomic DNA Extraction

All bacterial genomes used were extracted via the Bacteria Genomic DNA kit (Zomanbio, China). Concentrations of the extracted genomes were determined using an ultraviolet spectrophotometer (A260/280).

3.7. Standard PCR Reaction

A specific primer pair was designed according to the *ipaH* gene of *Shigella*(S1:5'-ATACCGTCTCTGCACGCA-3'; S2: 5'-GCCTTCTGATGCCTGATGG-3') and used in the PCR reaction. The 25 µL volume contained 10 µM of the S1 and S2 primers, 2 µL genomic DNA, and 12.5 µL of 2×TSINGKE Master Mix. The reaction conditions were as follows: predenaturing at 95°C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. The products were analyzed with 1% agarose gel via electrophoresis.

3.8. IMB Preparation

IMBs were prepared as previously described (10). The purified mAb A3 was diluted in binding buffer (50 mM Tris, 150 mM NaCl, 0.5% Tween-20, pH 7.5) to a final concentration of 400 µg mL^{-1}, and 1 mL of the primary mAb A3 was mixed with 10 mg of protein A/G-coated magnetic beads (Biotool, USA) for 30 min at room temperature in a shaking incubator at 100rpm. The supernatant was removed and washed three times with PBS-T after magnetic separation. Thereafter, the IMBs were dissolved in PBS and stored at 4 °C for further use.

3.9. Standard IC-PCR Assay

IMBs captured the *Shigella* from the samples for 30 min at 37 °C in a shaking incubator at 80 rpm. The samples were magnetically separated, and the IMB-antibody-cell complexes were harvested and washed three times with PBS. Thereafter, the mixtures were used for PCR amplification as described above.

3.10. Practical Application of IC-PCR for Shigella Detection in Milk

Applicability of the IC-PCR assay was further confirmed using milk samples. First, the IC-PCR sensitivity was evaluated by serially diluting *Shigella* in milk for the final concentrations ranging from 9×10^7 to 9×10^0 colony-forming units CFU.mL^{-1}. The IMBs were added to the sample and stirred for 30 min at 37 °C. After enriching the cells, the IMB-antibody-cell complexes were washed three times with PBS. The mixture was used for the PCR assay. Milk samples without *Shigella* but containing *Klebsiella pneumoniae* (*KPN*), *Listeria*, *Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), Acinetobacter baumannii (A. baumannii),* or *Pseudomonas aeruginosa (P. aeruginosa)* were used as negative controls.

3.11. IC-PCR Assay for Detecting Shigella in Clinical Samples from Monkeys

To export laboratory monkeys, *Shigella* was prohibited. Thus, the monkeys were rigorously inspected for *Shigella* before export. The 46 monkey samples were collected from different monkey feeds at Kunming Biomed International. The IC-PCR assay was used to directly detect *Shigella* in these samples. The excreta and/or anal swabs were dissolved in a tube with LB liquid medium, then IMBs were added to each tube to capture *Shigella* as described above. Thereafter, the bacteria were magnetically separated, and the IMB-antibody-cell complexes were harvested and used for IC-PCR. Clinical samples from healthy monkeys without *Shigella* were used as negative controls.

4. Results

4.1. Shigella-Specific mAb Preparation

Serum antibody titers from each immunized mouse were determined using ELISA after booster immunizations (**Fig. 1A**), and the results showed that the titers were high enough to prepare the mAbs. After the cell fusion, the antibodies against *Shigella* in the hybridoma cell supernatants were screened using ELISA. After three rounds of subcloning, two stably positive hybridomas (A3 and G5) were harvested and used to produce ascites. Ascites against *Shigella* was prepared after injecting the hybridomas at 10^6 cells/mouse. The immunoglobulin isotypes of the A3 and G5 mAbs were IgG1 and IgG2b; the light chains were kappa chains (**Table 2**). The purified mAb A3 was identified via SDS-PAGE (**Fig. 2A**).

Table 2. Characterization of the mAbs Against *Shigella*

mAbs	Isotype	Ascites Titre	Ascites Concentration, mg.mL^{-1}
A3	IgG1, κ chain	10^6	3.50
G5	IgG2b, κ chain	10^6	2.38

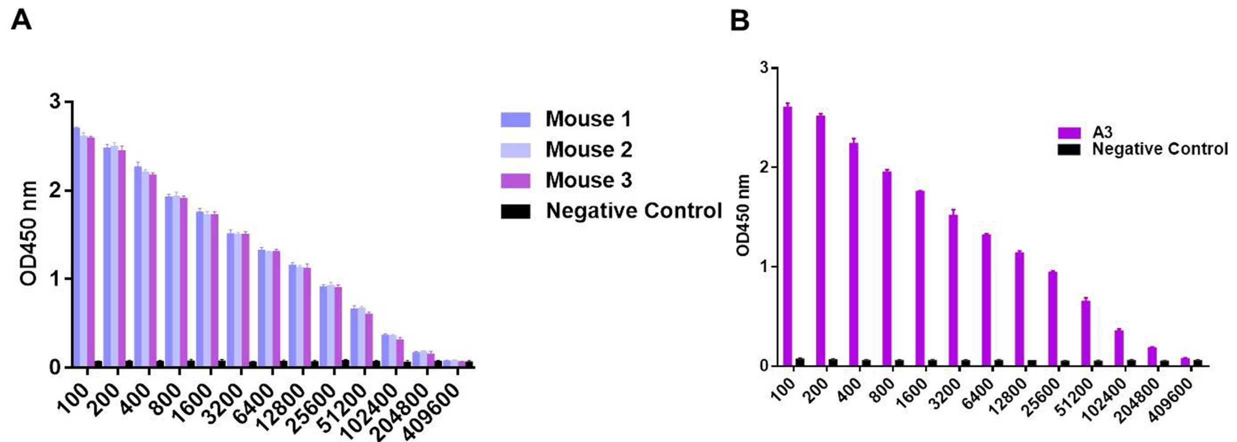

Fig 1. **(A)** The titer of mice serum and ascites **(B)** at different dilution ratios were determined by ELISA

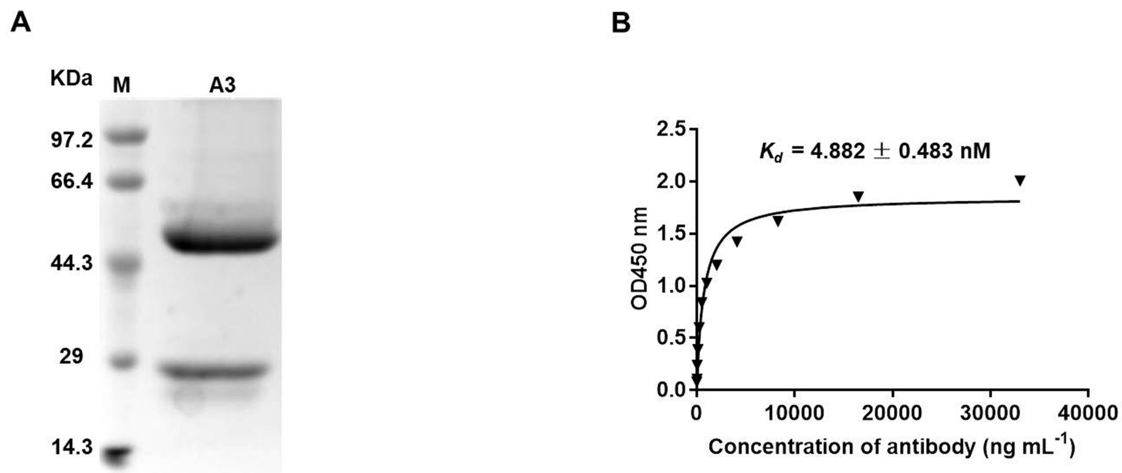

Fig 2. SDS-PAGE analysis of purified mAb A3 and the binding affinity of mAb A3. **(A)** 12% SDS-PAGE gels of the purified mAb A3; lane M, protein marker; lane 1, the purified mAb A3; **(B)** The saturation curves for the determination of the dissociation constants of mAb A3

4.2. ELISA Measurement of the Titer and mAb Affinity

Anti-*Shigella* antibody-positive hybridoma cells were prepared and used to generate ascites. The reactivity of the ascites diluted from 1:200 to 1:409600 was determined via ELISA (20). The mAb A3 titer was higher than 1:204800 (**Fig. 1B**). The mAb A3 K_D value was measured via affinity testing and analyzed via nonlinear regression analysis using GraphPad Prism 5 software (14, 18). The K_D value was calculated as 4.882±0.483 nM (**Fig. 2B**).

4.3. Characterization of the mAb Against Shigella

The mAb A3 reactivity and specificity against *Shigella* were evaluated via western blot and ELISA. Both western blotting and ELISA showed that mAb A3 only recognized *Shigella* (**Fig. 3A, lanes 1, 2**) and not the other common bacteria (*KPN*, *Listeria*, *S. aureus*, *E. coli*, *A. baumannii*, and *P. aeruginosa*; (**Fig. 3B, 3C**).

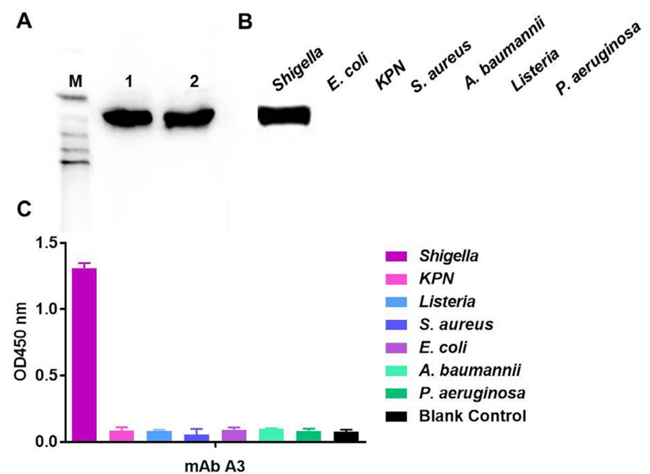

Fig 3. The reactivity and specificity of the mAb A3. **(A)** The reactivity of the mAb A3; the supernatant (lane 1) and lysate (lane 2) of *Shigella* were detected by western blot; The specificity of the mAb A3 was detected by western blot **(B)** Western blot and **(C)** ELISA were performed to evaluate the specificity of the mAb A3

4.4. IC-PCR Specificity and Sensitivity

To determine the IC-PCR specificity in processed milk, *Shigella*, *KPN*, *Listeria, S. aureus*, *E. coli*, *A. baumannii*, and *P. aeruginosa* were mixed and captured by IMBs. Bacteria without *Shigella* were also mixed and captured by IMBs. The IC-PCR assay only showed green fluorescence and fragments when the reaction contained *Shigella* (**Fig. 4A and B**). Sensitivity of the IC-PCR assay was tested with the *Shigella* serially diluted in milk from 9×10^7 to 9×10^0 CFU. mL $^{-1}$, and the *Shigella* was detected even at 9×10^0 CFU mL^{-1} (**Fig. 4C and D**). *Shigella* was also serially diluted for the standard PCR assay. The number of *Shigella* in each standard PCR reaction was adjusted from 9×10^5 to 9×10^0 CFU. The standard PCR assay detected *Shigella* at up to 10^2 CFU; *Shigella* was undetectable below 10^2 CFU (**Fig. 5B**).

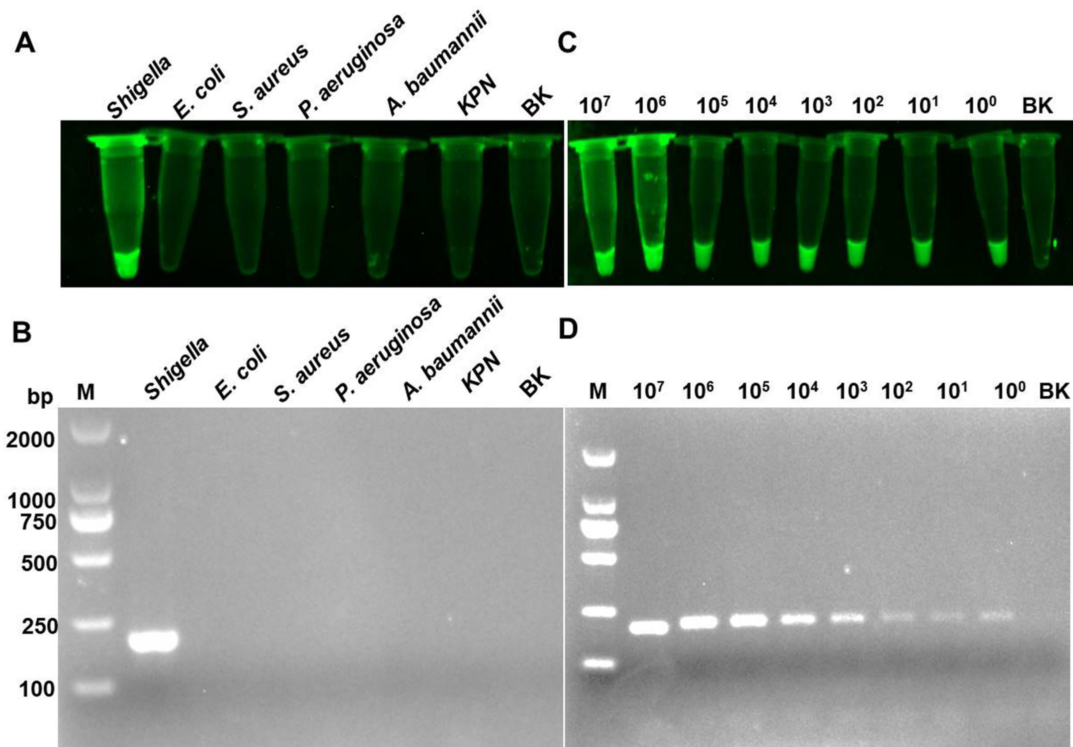

Fig 4. Specificity and sensitivity of the IC-PCR assays. **(A)** Specificity of the IC-PCR based on SYBR Green I and **(B)** agarose gel by electrophoresis detection; **(C)** Sensitivity of the IC-PCR based on SYBR Green I and **(D)** agarose gel by electrophoresis detection

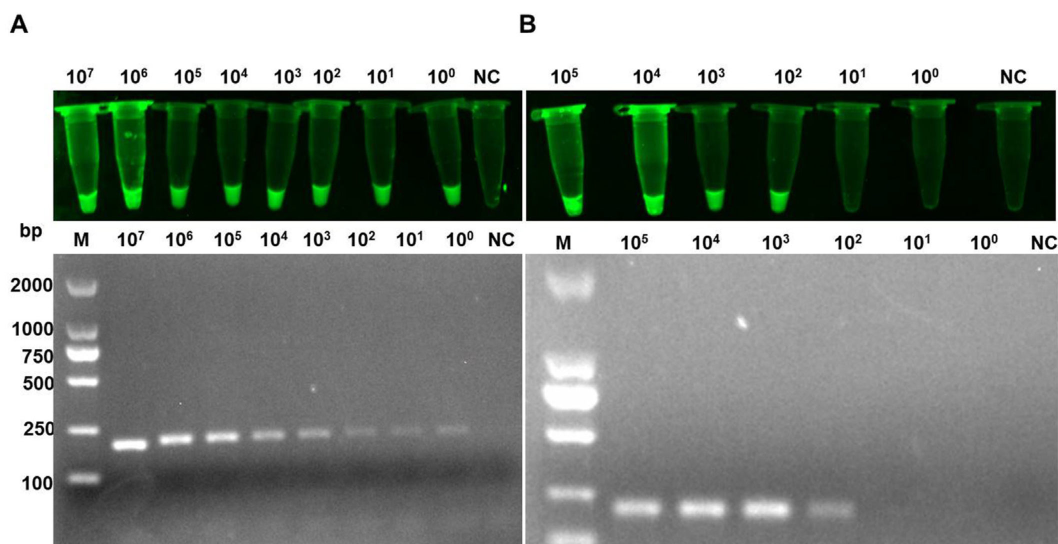

Fig 5. Comparison of the sensitivity between IC-PCR and PCR. **(A)** Sensitivity of the IC-PCR based on SYBR Green I and agarose gel by electrophoresis detection; **(B)** Sensitivity of PCR based on SYBR Green I and agarose gel by electrophoresis detection

4.5. Clinical Sample Detection Using the IC-PCR Assay

The IC-PCR application was verified using 46 clinical samples from monkeys, of which, 12 were positive and 34 were negative (**Fig. 6A and B**). Traditional biochemical identification and serum agglutination testing were also performed on the 46 samples (**Table 3**) and yielded results that were consistent with those of the IC-PCR, thus demonstrating that the IC-PCR can accurately, rapidly, and specifically detect *Shigella* in clinical samples (**Table 4**).

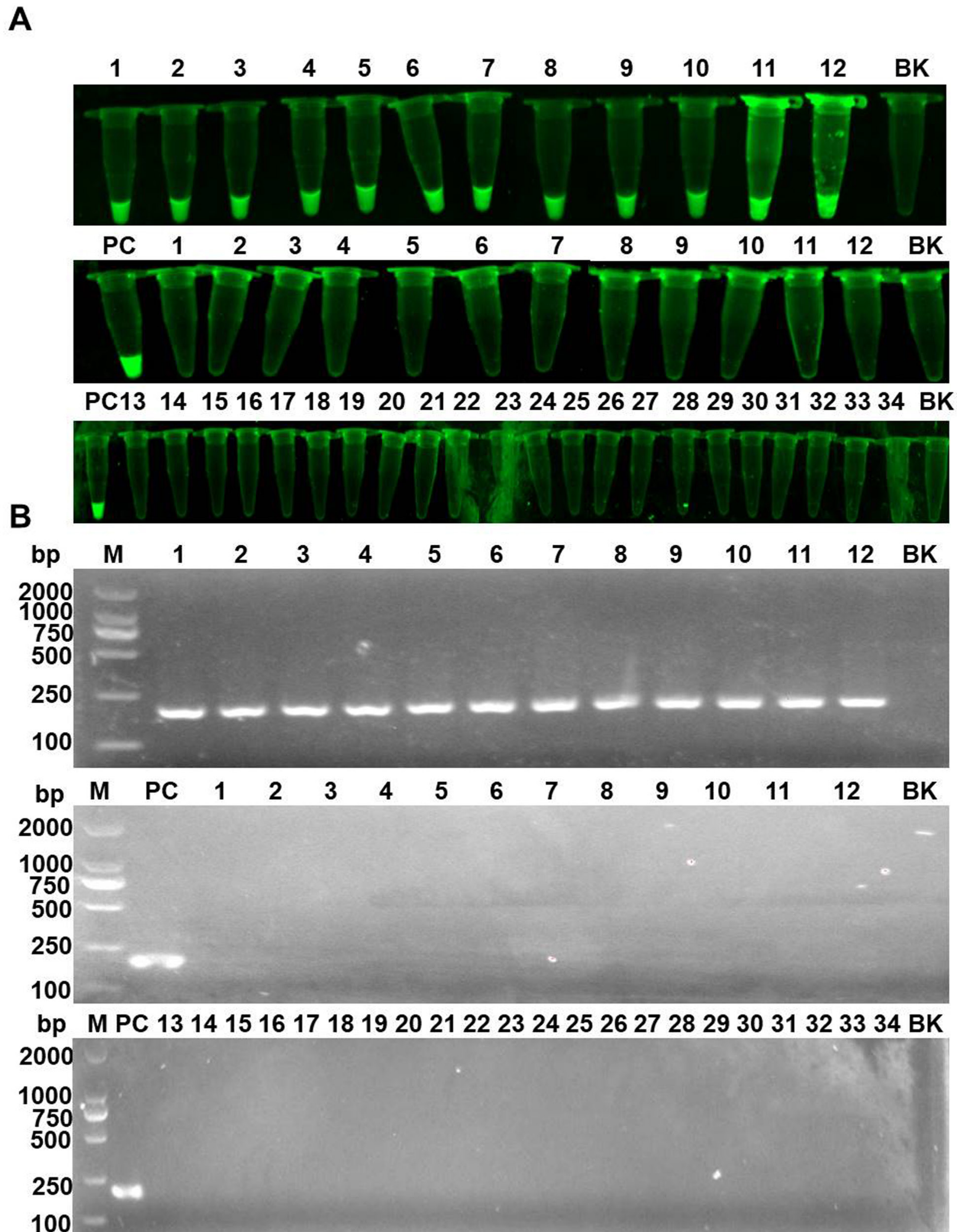

Fig 6. Detection of the forty-six monkey clinical samples by IC-PCR. **(A)** Twelve positive clinical samples and thirty-four negative clinical samples were detected by IC-PCR; **(B)** Twelve positive clinical samples and thirty-four negative clinical samples were applied for agarose gel by electrophoresis assay

Table 3. The results of the biochemical test and serum agglutination test for clinical samples.

Number of Sample	Source of the Sample	Type	Biochemical Test	Serum Agglutination Test
36	cynomolgus monkey	excreta	+	+
66	cynomolgus monkey	excreta	+	+
77	cynomolgus monkey	excreta	+	+
85	cynomolgus monkey	excreta	+	+
90	cynomolgus monkey	excreta	+	+
95	cynomolgus monkey	excreta	+	+
96	cynomolgus monkey	excreta	-	-
107	cynomolgus monkey	excreta	+	+
127	cynomolgus monkey	excreta	+	+
128	cynomolgus monkey	excreta	+	+
142	cynomolgus monkey	excreta	+	+
152	cynomolgus monkey	excreta	+	+
193	cynomolgus monkey	excreta	-	-
195	cynomolgus monkey	excreta	-	-
196	cynomolgus monkey	excreta	-	-
198	cynomolgus monkey	excreta	+	+
199	cynomolgus monkey	excreta	-	-
200	cynomolgus monkey	excreta	-	-
202	cynomolgus monkey	excreta	-	-
203	cynomolgus monkey	excreta	-	-
204	cynomolgus monkey	excreta	-	-
207	cynomolgus monkey	excreta	-	-
208	cynomolgus monkey	excreta	-	-
209	cynomolgus monkey	excreta	-	-
212	cynomolgus monkey	excreta	-	-
214	cynomolgus monkey	excreta	-	-
216	cynomolgus monkey	excreta	-	-
217	cynomolgus monkey	excreta	-	-
219	cynomolgus monkey	excreta	-	-
220	cynomolgus monkey	excreta	-	-
221	cynomolgus monkey	excreta	-	-
222	cynomolgus monkey	excreta	-	-

Table 4. Comparison between IC-PCR and traditional biochemical identification and serum agglutination test.

The Gold Standard Test*	IC-PCR		
	Positive	Negative	Total
Positive	12	0	12
Negative	0	34	34
Total	12	34	46

* Selective medium isolation and commercial *Shigella* multivalence diagnostic serum identification as the gold standard test.

5. Discussion

Shigella is among the most important human pathogenic and foodborne infectious bacteria. *Shigella* causes acute intestinal tract infections, accounting for approximately 1.7 million deaths annually (21). Many studies have reported that *ipaH* can be present in multiple copies on both the invasion plasmid and the chromosome of *Shigella*, but most detection methods are time-consuming (22). Culture-based techniques and biochemical assays are the most common detection methods but require a long enrichment time and subsequent identification (22, 23). PCR-based technologies can detect *Shigella* from various food products and environmental samples (24). However, these techniques cannot effectively enrich cells, especially in some special samples, which significantly limits the wider application of PCR in under-resourced settings or field laboratories (25). Moreover, PCR equipment is essential but is expensive and unavailable in some areas such as developing countries.

IC-PCR is a fast and accurate alternative for detecting pathogenic infections, including bacterial, viral, and parasitic infections (26, 27). Importantly, IMB separation is simple, easy, and rapid, with high separation purity, retention of the target material activity, and high efficiency (28-30). IMB separation has been widely used in cell separation and purification and in immune detection, purification, and precipitation (31-33).

Many studies use pAbs; however, although pAb preparation is relatively easy, simple and rapid, pAbs have disadvantages. For example, differences between batches cannot be controlled, and pAbs are limited, making their large-scale use impossible. In addition, pAb production results in many unneeded nonspecific antibodies; thus, they are unsuitable for large-scale detection. Conversely, mAbs have many advantages that pAbs lack. The chemical structures of mAb can have a defined specificity for a specific analyte (target molecule), and mAbs can be produced in unlimited quantities (34).

In this study, two mAbs against *Shigella* were prepared and confirmed via ELISA and western blot. Using mAb A3, we developed and validated an IC-PCR assay to detect *Shigella*. This method rapidly, sensitively, and specifically screened *Shigella* from contaminated milk and clinical samples. The IC-PCR detection limit was 9×10^{0} CFU. mL^{-1}, and IC-PCR does not require enriching the *Shigella* before detection or using a bacterial genomic DNA kit to extract genomic DNA or a plasmid as a template. Compared with standard PCR and culture-based techniques, IC-PCR can easily and rapidly separate bacteria from the environment without requiring specialized equipment. This method is more convenient, faster, and more specific than standard PCR and culture-based detection methods. Establishment of the IC-PCR improves options for *Shigella* detection and has far-reaching significance.

6. Conclusions

Here, we developed the IC-PCR, a convenient, faster, and specific method for monitoring *Shigella*, based on PCR combined with a magnetic immunocapture assay. The practicality and accuracy of the IC-PCR method were demonstrated using processed milk and clinical samples. With the one-step-visible procedure, *Shigella* can be screened within 70 min. In addition, a magnetic immunocapture assay and portable PCR equipment enable convenient on-site detection. The rapid, sensitive, and specific IC-PCR assay can be used to detect *Shigella*.

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Ethical Approval

The animal study was approved by the Kunming Biomed International with permit number: KBIk001117020-01,01. All experimental procedures were performed in accordance with the regulations prescribed by the Administration of Laboratory Animals.

Author Contributions

LZ designed and drafted the work. LZ, XD, QW, QH, QC and MZ performed the experiments, analyzed the data, and interpreted the results. JZ, YS and XX designed the work and revised it critically.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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