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.
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).

#### 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 (10⁶ 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 KH₂PO₄, 137 mM NaCl, 10 mM Na₂HPO₄, 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 KH₂PO₄, 137
mM NaCl, 10 mM Na₂HPO₄, 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’-ATACCGTCTCTGCAACGGCA-3’; S2: 5’-GCCTTCTGTGCTGTATGGG-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 μg 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 washed 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 9×10⁷ to 9×10⁰ 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 10⁶ 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).

| mAbs | Isotype     | Ascites Titre | Ascites Concentration, mg.mL⁻¹ |
|------|-------------|---------------|-------------------------------|
| A3   | IgG1, κ chain | 10⁶           | 3.50                          |
| G5   | IgG2b, κ chain | 10⁶          | 2.38                          |
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).
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 $9 \times 10^7$ to $9 \times 10^0$ CFU mL$^{-1}$, and the Shigella was detected even at $9 \times 10^0$ 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 $9 \times 10^5$ to $9 \times 10^0$ 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 *ippA* 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 9×10⁶ 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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