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Comprehensive Investigation of *Salmonella* spp.: Isolation, Characterization and PCR Primer Set Evaluation for Enhanced Detection and Identification

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ABSTRACT

Background: Salmonellosis is a serious health concern for both animals and humans. According to a report of the Centers for Disease Control and Prevention, 40,000 human infections occur annually; however, the number of unconfirmed cases is as high as approximately 800,000-4000,000. These organisms gain entry into the gastrointestinal cells and cause serious diseases, resulting in intestinal damage and even death.

Methods: In this research, 115 *Salmonella* isolates from different sources were tested. Using morphological, cultural, and biochemical methods, they were classified into 30 serovars. Plasmid profiling was used for quick identification, which showed similarities among the isolates.PCR primer sets targeting specific genes, such as *invA, hilA, iroB, oriC, fimA, hitJ* and *stn,* demonstrated reliability, accuracy and specificity for identifying *S. enterica* serovars.

Result: This study recommends the use of specific primer sets in PCR-based methods for rapid and reliable identification and detection of locally isolated *Salmonella* serovars/strains. This research adds to the continuing efforts for improving the diagnostic techniques to tackle the challenges presented by *Salmonella* spp.

Key words: Food, PCR, Plasmid, Morphological, *Salmonella*.

INTRODUCTION

Salmonella bacteria can cause food poisoning, which is a big deal for public health. It's important to quickly and accurately identify the bacteria to prevent outbreaks and keep people healthy (Ehuwa *et al*., 2021). Conventional methods for bacterial culture are usually dependable but not always quick enough for immediate diagnosis (Helmick *et al.,* 1994; Aladhadh, 2023). They may also lack the precision needed to identify low levels of Salmonella in complex samples. Molecular techniques like polymerase chain reaction offer enhanced accuracy and sensitivity in pinpointing Salmonella (Ndraha *et al*., 2023; Liu *et al*., 2023; Sabina *et al.,* 2015), transforming microbiology by swiftly identifying small amounts of Salmonella DNA within hours, regardless of whether it comes from viable or non-viable cells-a significant upgrade over the days required for culture-based methods. This study thoroughly examined characteristics of Salmonella isolates based on cultural, morphological and biochemical aspects, offering essential insights for developing trustworthy molecular detection methods.

This research paper emphasizes the importance of thoroughly assessing the precision and efficiency of PCR primer sets for identifying and analyzing Salmonella strains in specific local areas. Customized primer sets adapted to different regions are vital for precise detection due to geographical and environmental differences, as well as evaluating their sensitivity when working with samples containing small amounts of Salmonella found in complex substances such as food, water and clinical specimens.

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Recent advances in genomics have led to the discovery of promising genetic markers for using PCR to detect Salmonella. However, there is still a lot to learn about how well these primer sets can distinguish Salmonella strains from other common bacterial species in the area (Zhang *et al.*, 2021; Malorny *et al*., 2003; Ward *et al.,* 2005; Rabins *et al.,* 2018). That's why this study carefully looked at how specific and sensitive certain PCR primers are especially when dealing with locally isolated Salmonella strains. The goal is to provide valuable insights that can help develop accurate and effective methods for detecting and identifying Salmonella (Chiramboo *et al.,* 2021; Brasher *et al.,* 1998; Vantarakis *et al.,* 2000; Desai *et al.,* 2005; Singh *et al.,* 2018), ultimately improving food safety measures and public health protection.

MATERIALS AND METHODS

Salmonella isolates were collected in 2021-2022 from different sources, including human feces, eggs, poultry, fish, milk and various feed and food samples. As per the World Health Organization guidelines (2001), fecal swabs, liquid samples and solid samples were kept in sterilized buffered peptone water. The suspensions collected after incubation at 37°C were used to inoculate selective enrichment media, including selenite cystine broth and tetrathionate broth. After streaking on xylose lysine desoxycholate (XLD), Salmonella - Shigella agar (SSA), and brilliant green agar (BGA) plates, they were incubated overnight at 37°C. The research took place at the University of Khartoum in Sudan.

Characterization of Suspected *Salmonella* Isolates

Various characterization methods were used for tentative *Salmonella* isolates. Stereo microscope was utilized to observe the shape, cultural characteristics and Gram staining (Sulmiyati, 2018). Thioglycolate broth was used to determine aerobiosis, while motility tests were performed on SIM medium. Biochemical tests included lysine iron agar, triple sugar iron agar (TSI), urease, oxidase tests, Voges Proskauer (VP) and indole.

Specificity of PCR Primer Sets for the Detection of *Salmonella* Isolates

For identifying locally isolated *Salmonella* serovars, 10 primer sets (*Invitro*gen), each targeting a different gene were assessed for sensitivity and specificity.

DNA was extracted as per the boiling–centrifugation method outlined by Soumet *et al.* (1994). Pure nutrient agar culture was used to grow a single colony overnight at 37°C in 1 mL of Luria–Bertani (LB) broth. The bacterial cells were collected and DNA was extracted by boiling followed by centrifugation. The collected DNA was then stored at 4°C until used as a template for PCR amplification.

PCR

The PCR amplification method proposed by Sambrook *et al.* (1989) was performed in a reaction volume of 25 µL. The PCR mixture contained template DNA, PCR master mix (Promega) and specific primer pairs.

Agarose gel electrophoresis

Standard agarose gel electrophoresis using 1.2% agarose gel was used to observe the PCR products. Subsequently, ethidium bromide was used to stain the gel and the PCR products were loaded into wells alongside a DNA ladder of 1 Kb (Invitrogen). The gel was visualized in a transillumination cabinet by performing electrophoresis at 75 volts for 45 min with subsequent image capture using a gel documentation system.

Sensitivity of PCR primers

The sensitivity of each primer set was determined using dilutions ($10\text{-}10^{-9}$) of a pure culture of *S. typhi*. Plate count agar was used to count the viable cells. DNA was extracted from each dilution and used as a PCR template for each primer set.

Plasmid Profiling Analysis

The alkaline detergent methodology was used to extract plasmid DNA. The cultures were grown overnight in LB broth and then subjected to sequential treatments with lysozyme, NaOH–SDS solution, and sodium acetate-ethanol. The extracted plasmid DNA was resuspended in distilled water and then stored at -20°C until it was utilized for plasmid profiling analysis.

Plasmid DNA gel electrophoresis

The extracted plasmid DNA of each isolate was diluted and electrophoresed on a 0.8% agarose gel. For performing gel analysis, diluted DNA, bromophenol blue dye and 1 Kb DNA ladder were used. Electrophoresis was conducted at 50 volts for 2 h and the gel was visualized using ultraviolet transillumination and gel documentation system capture.

This thorough approach guaranteed the comprehensive evaluation of the specificity and sensitivity of the PCR primer sets for plasmid profiling to detect and characterize the *Salmonella* isolates.

RESULTS AND DISCUSSION

Isolate Recovery and Preliminary Identification

A total of 115 isolates of *Salmonella* spp. were obtained from various sources, including poultry, eggs, animal and human feces, meat, fish, milk, and different food and feed items. Those exhibiting growth characteristics consistent with *Salmonella*, such as a slightly transparent zone of reddish color and black center on XLD medium (Plate 1a), gray reddish and slightly convex colonies on BGA (Plate 1b) and colorless colonies with dark black centers on SSA (Plate 1c) were considered tentative *Salmonella* isolates. These colonies were picked, purified and reserved for further characterization.

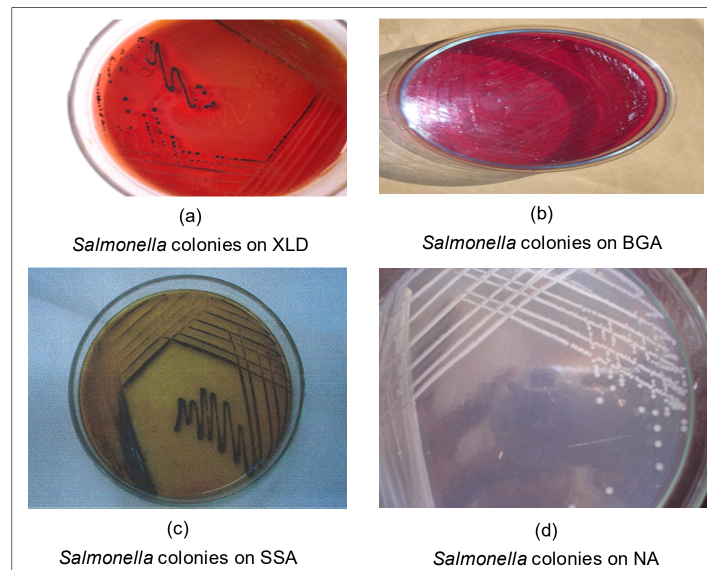
Cultural characteristics of the tentative *Salmonella* isolates on nutrient agar included slimy, convex, circular, and transparent colonies with entire margins and smooth surfaces (Plate 1d).

Biochemical and microscopic characterization

Biochemical and microscopic tests were performed, which revealed that all tentative *Salmonella* isolates produced H_2S gas in TSI medium, displayed an alkaline red slope and acidic yellow butt and were able to produce lysine decarboxylase. The isolates were found to be facultatively anaerobic, motile gram-negative rods.

Plasmid profiling analysis

Thirty-two *Salmonella* serovars were subjected to plasmid profiling analysis, which revealed seven distinct profile types with plasmids ranging from 2.3 kb to 12.2 kb (Table 1). Of the serovars, 21 had 1-4 plasmids, whereas 11 did not have plasmids. Notably, some similarities were observed in the detected profiles across different *Salmonella* serovars.



(a) *Salmonella* colonies on XLD

(b) *Salmonella* colonies on BGA

(c) *Salmonella* colonies on SSA

(d) *Salmonella* colonies on NA

Plate 1: Growth characteristics of *Salmonella* isolates on different media.

Table 1: Results of plasmid profiling analysis.

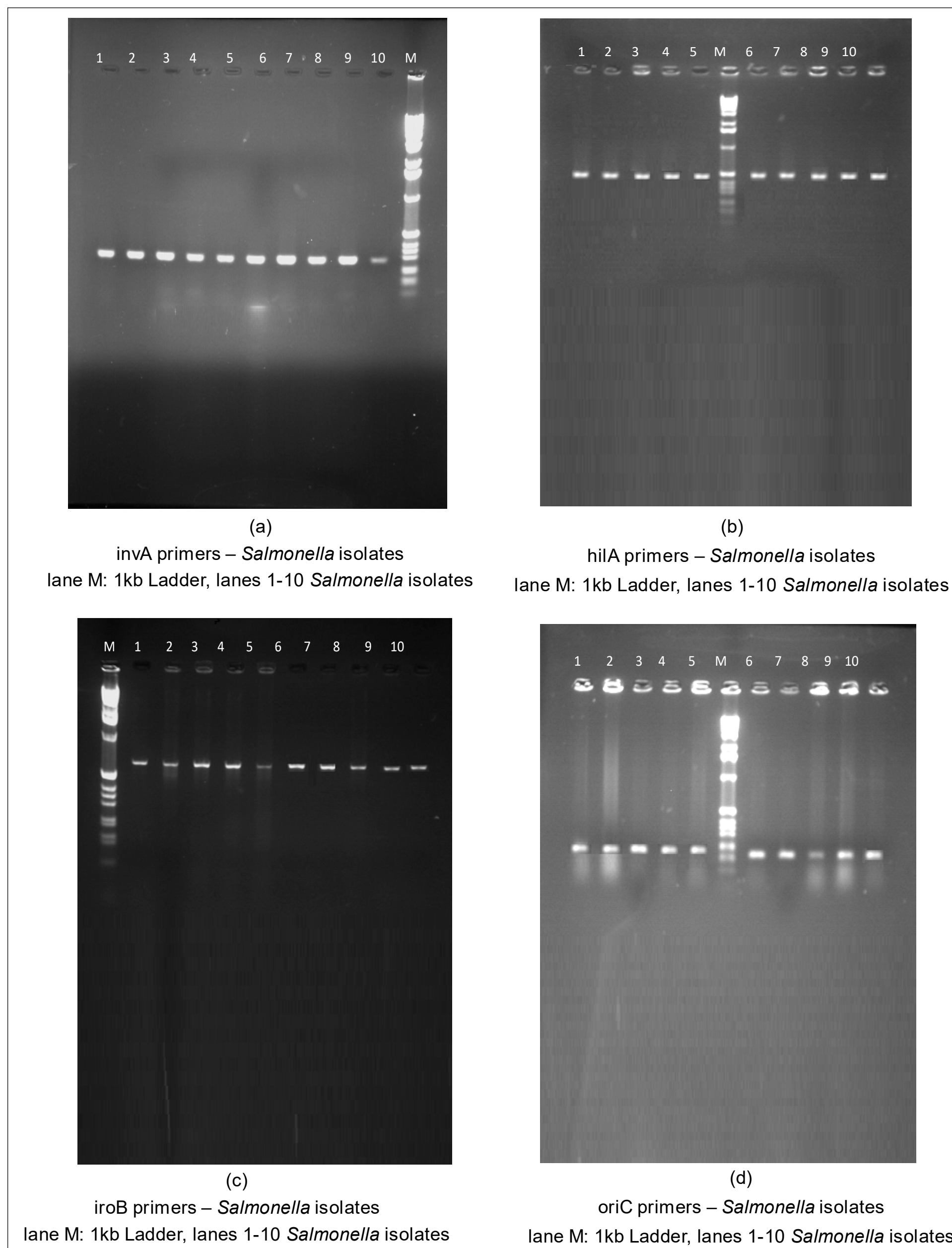
Serotype	No. of plasmids	Plasmid profile type	Size of plasmids (kb)
Hadar	1	Type 1	12.2
1:4:12:r,-:enz15	1	Type 1	12.2
1:Rough-O:1,z13:1,1	1	Type 1	12.2
Alachua	2	Type 1	12.2
Enteritidis	1	Type 1	12.2
Havana	1	Type 1	12.2
Livingstone	2	Type 1	12.2
Risen	1	Type 1	12.2
1:4,12:eh:-	4	Type 2	12.2, 5, 4, 3
Poona	1	type 3	12.5
1:6,7:-:enx	0	Type 4	-
Adelaide	0	Type 4	-
Agona	0	Type 4	-
Johannesburg	0	Type 4	-
Kentucky	0	Type 4	-
Mbandaka	0	Type 4	-
Meleagridis	0	Type 4	-
Pending	0	Type 4	-
Schwarzengrund	0	Type 4	-
Senftenberg	0	Type 4	-
Virchow	0	Type 4	-
Blockely	3	Type 5	12.5, 12.2, 2.3
1:rough-O:z29:-	2	Type 6	12.5, 12.2
Infantis	2	Type 6	12.5, 12.2
Molade	2	Type 6	12.5, 12.2
Montevideo	2	Type 6	12.5, 12.2
Muenster	2	Type 6	12.5, 12.2
Para TyphiB	2	Type 6	12.5, 12.2
Typhi	2	Type 6	12.5, 12.2
Typhimurium	2	Type 6	12.5, 12.2
Uganda	2	Type 6	12.5, 12.2
Stanleyville	3	Type 7	12.5, 12.2, 1.2

Specificity and Sensitivity of the Primer Sets

PCR amplification using specific primer sets (*invA, hilA, iroB, oriC, fimA, hitJ*, and *stn*) demonstrated high specificity for *Salmonella* strains. These primer sets detected DNA from all tested *Salmonella* serotypes, yielding specific amplicons. Sensitivity tests revealed detection limits ranging from 6.07 × 101 to 6.07 × 102 CFU/mL for different primer sets. Results of PCR amplification are shown in Plates 2, 3 and 4.

Salmonellosis is a significant public health concern globally, affecting both humans and animals. The conventional method of bacterial culturing remains the gold standard for *Salmonella* detection. In this study, the recovery and identification of 115 *Salmonella* isolates from diverse sources emphasize the widespread prevalence of this pathogen.

The cultural characteristics of the tentative *Salmonella* isolates on nutrient agar, including slimy, convex, circular, and transparent colonies, provided valuable insights into their growth patterns. Subsequent biochemical and microscopic tests revealed key characteristics, such as the production of H_2S gas in TSI agar, an alkaline red slope and



(a)
invA primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

(b)
hilA primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

(c)
iroB primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

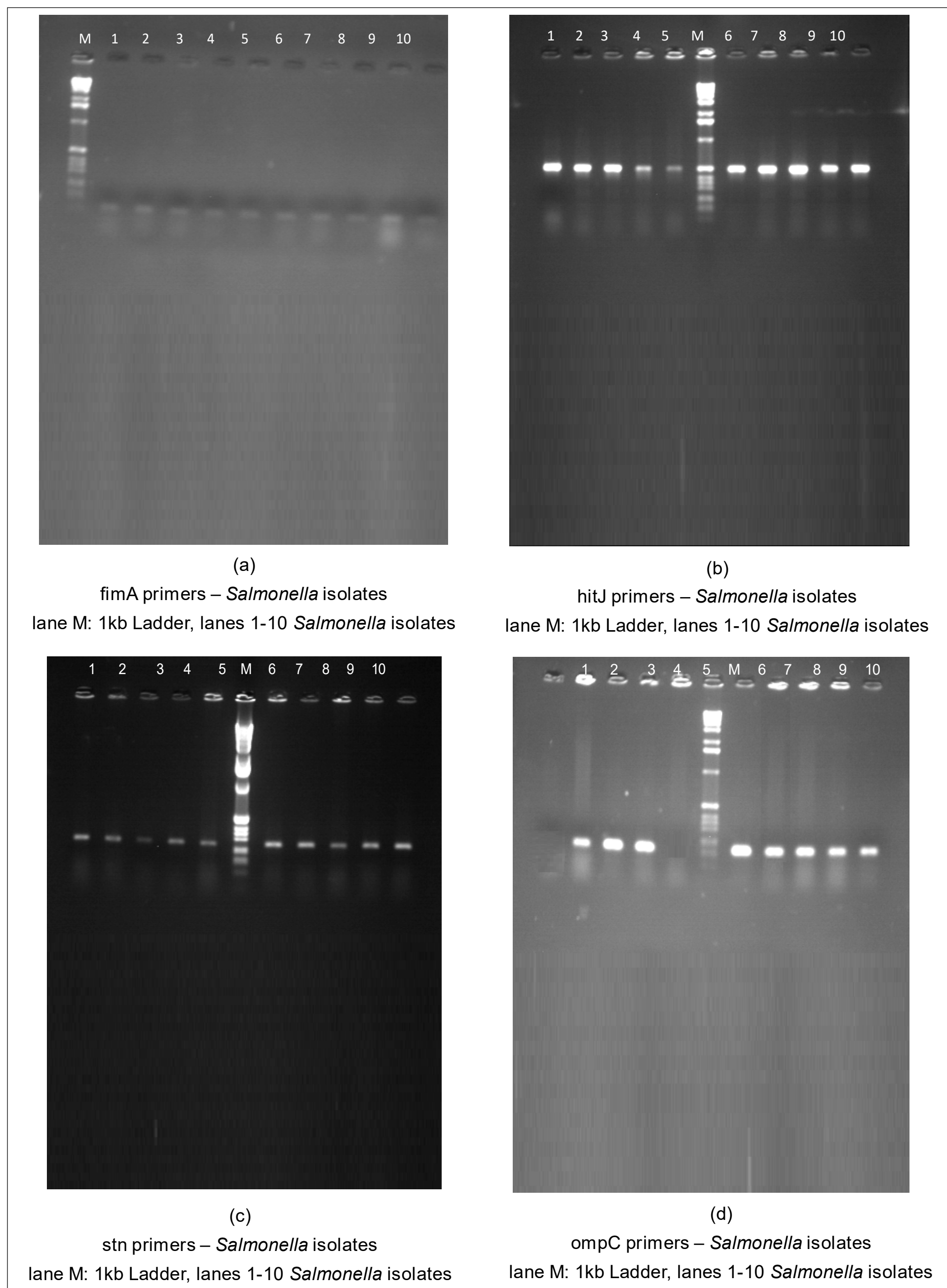
(d)
oriC primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

Plate 2: PCR amplification products detected for the primer sets invA , hilA, iroB and oriC.

acidic yellow butt, lysine decarboxylase production, gram-negative rods, facultative anaerobic behavior, and motility (Cheesbrough, 2006).

Plasmid profiling offered insights into genetic similarities among isolates; however, its limitations necessitate supplementary molecular typing techniques (Brown *et al.,* 1991; Liebana, 2002; Pan *et al.,* 2022). This study evaluated PCR-based detection using seven primer sets targeting specific genes. The *invA, hilA, iroB, oriC, fimA, hitJ*, and *stn* primer sets demonstrated high specificity, accurately detecting *Salmonella* strains with varying sensitivities. Nonetheless, three primer sets (opmC, 16S rDNA and repetitive DNA fragment) exhibited limitations, underscoring the need for careful primer selection.

This study recommends the use of *invA, hilA, iroB, oriC, fimA, hitJ* and *stn* primer sets in PCR-based protocols for the reliable, simple, and rapid detection and identification of locally isolated *Salmonella* serovars/strains. Further investigations with a larger sample size, including additional *Salmonella* serovars and genetically related genera, are warranted to validate these primers and enhance their applicability in diverse settings. Additionally,



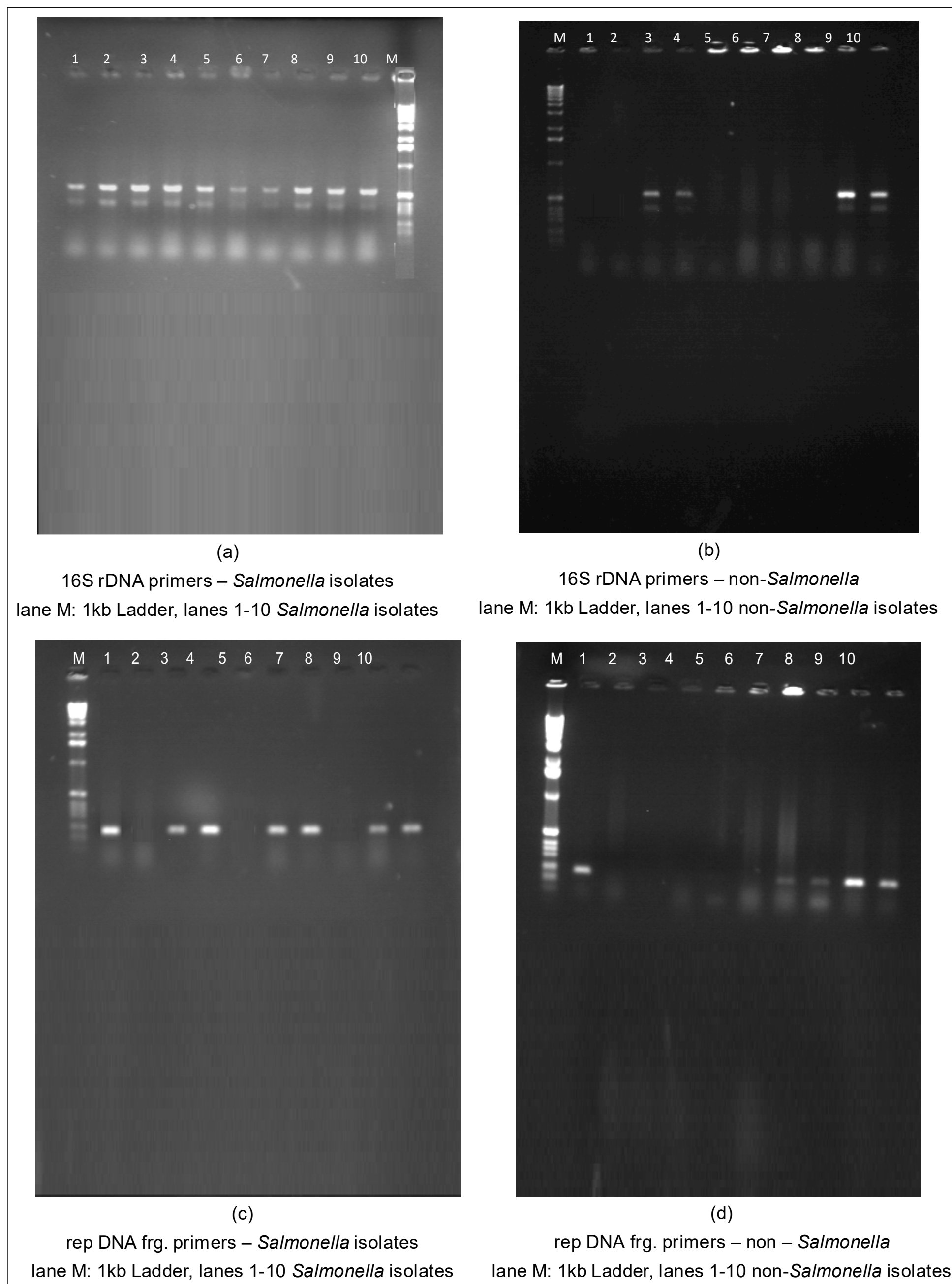
(a)
fimA primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

(b)
hitJ primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

(c)
stn primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

(d)
ompC primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

Plate 3: PCR amplification products detected for the primer sets fimA, hitJ, stn and ompC.



(a)
16S rDNA primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

(b)
16S rDNA primers – non-*Salmonella*
lane M: 1kb Ladder, lanes 1-10 non-*Salmonella* isolates

(c)
rep DNA frg. primers – *Salmonella* isolates
lane M: 1kb Ladder, lanes 1-10 *Salmonella* isolates

(d)
rep DNA frg. primers – non – *Salmonella*
lane M: 1kb Ladder, lanes 1-10 non-*Salmonella* isolates

Plate 4: PCR amplification products detected for the primer sets 16S rDNA and rep DNA frg.

Testing the primers directly on samples without isolating them first could speed up the analysis process.

CONCLUSION

This study offers valuable insights into Salmonella detection methods, emphasizing the effectiveness of PCR techniques and targeted primers for quick and precise identification. These findings have potential to enhance food safety measures and strengthen public health responses to evolving threats from Salmonella strains.

Conflict of interest

The authors have no conflict of statement to declare.

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