invA Gene to Detect *Salmonella Enterica* Serovar *Typhimurium* Supported by Serum Anti-*Salmonella* Antibodies and Protein Profiles for Chicken Carcass Isolates

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Research article

Keywords: invA gene, Salmonell typhimurium, Serum Anti S. typhimurium, Protein profile

Posted Date: October 9th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-87131/v1

Abstract

Background: *Salmonella typhimurium* causes salmonellosis in humans. This and other serotypes are among the most common food-borne pathogens affecting human and animals The ability of this serovar to infect birds and contaminate chickens during slaughter and processing provides an opportunity for the bacterium to infect people. *S. typhimurium* is, thus, a food-borne pathogen, and detection and identification of contamination in chickens are critical for developing strategies needed to improve control of the bacterium. Chicken samples display a high contamination load of *Salmonella spp.,* and the aim of this study is to isolate *S. typhimurim* from chicken carcasses and identify strains genetically using *invA* gene primers and relevant sequences in GenBank. Further, serological tests were completed using serum anti-*S. typhimurium* antibodies. Protein profiles of strains positive for both *invA* gene and anti-*S. typhimurium* sera in Widal tests separated on sodium *dodecyl sulfate-polyacrylamide* gel electrophoresis (SDS-PAGE) for final verification.

Results: The positive isolates by polymerase chain reaction (PCR) for the *invA* gene showed a specific molecular size of 244 bp. Sequencing of the *invA* gene and comparisons published sequences on GenBank allowed isolate identification of *Salmonella enterica* subsp. *enterica* serovar *typhimurium* strain ST45 with a similarity of 100%. Multidrug resistance to antibiotics was also surveyed for assessment of evolution in strains relative to some standard strains.

Conclusions: use of the *invA* gene as indicator gene for *Salmonella* spp. supported by serological tests with specific serum anti-*S. typhimurium* or anti sera for any other *Salmonella* spp. and protein profile are considered to be an accurate method for identification for *Salmonella* spp.

Background

Salmonella are Gram-negative rod-shaped cells with flagella. These bacteria are facultative anaerobes in the family Enterobacteriaceae. *Salmonella* ferment glucose but not lactose and are urease negative, in contrast to *Proteus sp*. *Salmonella* spp likely cause more food-related illness than any other bacteria worldwide. Symptoms of *Salmonella* infection range from gastroenteritis to sepsis [1]. The bacterium is a zoonotic agent that infects a variety of animal species [2]. *Salmonella typhimurium* is infectious to humans and can cause severe gastro-intestinal pathology and typhoid fever [2].

The virulence of *Salmonella* is linked to a combination of chromosomal and plasmid factors. *invA*, *spv*, *fimA*, and *stn* genes are major virulence factors underlying salmonellosis. The *invA* gene is located in the genome and codes for a protein on the inner membrane that is necessary for invasion of epithelial cells [3]. PCR for the *invA* gene is a rapid and reliable technique with possible diagnostic application for the identification of *Salmonella* spp [4, 5]. The *invA* virulence gene is the most common and clinically significant genetic marker for the serovar that causes salmonellosis globally. The marker is found in both *S. typhimurium* and *S. enteritidis* [6].

Our study was designed to investigate the prevalence of *S. typhimurium* and *S. enteritidis* in chicken carcasses using PCR for detection and presence of the *invA* gene. Serological tests using control serum anti-*S. typhimurium* and anti-*S. entertidis* antibodies were used for confirmation of *invA* gene presence. Positive isolates were verified with protein profiles on SDS-PAGE. Finally, multidrug resistance (MDR) of *Salmonella* isolates was determined using an array of antibiotics from different classes.

Results

Salmonella colonies on xylose lysine desoxylate agar (XLD) medium show black centers circular white halos. Microscopic examination of *Salmonella* colonies shows Gram-negative rod-shaped cells.

Amplification results for all *Salmonella* isolates showed 23 isolates are positive for *invA* gene, displaying a DNA marker of 244 bp (Figure 1). Submission of this 244 bp sequence to GenBank for BLAST alignment analysis indicated a location in the genome of *S. enterica* subsp. *enterica* serovar *typhimurium* strain ST45, Sequence ID: CP050753.1, for 10 isolates and 13 identified as *Salmonella sp.*

Results of Widal tests for visible agglutination between *invA* positive *Salmonella* isolates and control sera are provided in Table 1. Anti-*Salmonella typhimurium* and anti S. *entertidis* were applied for colonies grown on brain heart infusion agar for 24 h at 37°C. Out of 10 isolates (20%) positive for the *invA* gene were also positive in Widal tests. Remaining isolates 13 (26%) were positive for the gene but negative in Widal assays. Fourteen of 27 isolates (54%) were negative for the *invA* gene and in Widal tests.

Table 1: Results of *Salmonella* sp., *invA* gene and serum anti-*typhimurium* and -*entertidis* detection.

	Chicken samples	Serum Anti-*Salmonella typhimurium + invA gene*	Serum Anti-*Salmonella entertidis*	Not identified	*invA* gene
Total number	50	10	0	27	13
%	100	20	0	54	26

Protein profiles for ten isolates (lanes 2–9, 12, 13) on SDS- PAGE showed similar major bands compared with *S. typhimurium* ATCC 14028 (Figure 2). The presence of the *invA* gene with positive agglutination in control sera with anti-*Salmonella typhimurium* and protein similarity for the isolates, provides assurance for accurate identification for *S. typhimurium*.

Conversely, isolates positive for the *invA* gene but negative in Widal assays do not show the same degree of similarity in major protein bands on SDS-PAGE with standard strains *S. typhimurium* ATCC 14028 and *S. enetrtidis* ATCC 13078 (Figure 3).

Lane M: the molecular weight standard. Lanes 1, 11 and 21 *Salmonella Typhimurium* ATCC 14028, Lanes 2, 12and 22 *Salmonella entertidis* ATCC 13078. Lanes (3-9), (13-19) and (23-29), isolates were similar to

standard strains.

S. *typhimurium* 19 and 25 showed resistance to 60% of antibiotics. *S. typhimurium* 26 showed resistance to 55%, and *S. typhimurium* 8 and 20, 50%. Standard strain *S. typhimurium* ATCC 14028 is resistant to 45% of antibiotics (Table 2). *S. typhimurium* 1, 3 and 5 showed similar resistance as the standard strain. *S. typhimurium* 8 was resistant to fewer antibiotics (40%). Resistance ratios reached 70% in isolates 23 and 24. Infection with such strains could be difficult to treat.

Resistance to antibiotics reached 100% for β-lactams (cefoxitin and carbencillin), and were high for sulfonamides (sulfamethoxazole trimethoprim), macrobids (erythromycin), glycopeptides (vancomycin) and lincosamides (lincomycin).

Discussion

PCR with the *invA* gene in a single reaction is a rapid method for identifying *Salmonella sp*. that also addresses a key virulence factor. Early detection of a virulence gene provides many benefits for public health, especially for rapid diagnosis and control of contamination and infection. Foods such as chicken can show a high incidence of *Salmonella* sp. that may suggest a need for development of vaccines, use of antibiotics for birds during breeding or the design of prophylactic or other strategies for control. Using of PCR assay by invA gene primers for *Salmonella* sp. considered to decrease identification problems which always happened in diagnosis Oliveira et al. (2003) [7]. Use of the invA gene is recognized as an international standard procedure for detection of the genus, *Salmonella* (Malorny et al., 2003)[8]. The importance of using molecular methods for *Salmonella* detection is increasing. Traditional serological methods are being replaced with molecular techniques [9]. The most sensitive, rapid and specific method for the detection of the *invA* gene is PCR [10]. The *invA* gene is used because it contains sequences specific to the genus, *Salmonella* [8].

Conversely, the *invA* gene is used as a target for the diagnosis of *Salmonella* at the genus level [11]. PCR amplification with *Salmonella* primers selected from invasive species followed by SDS-PAGE accurately identifies *S. typhimurium* (Fig. 1) as previously reported by Darwin and Miller [12].

These results are consistent with research by Pererat and Murray [13] using PCR on various serotypes of *Salmonella* with positive results, while PCR for non-*Salmonella* strains, such as *E. coli*, *Klebsiella, Proteus*, and *Shigella*, were negative. Invasion of intestinal epithelial cells is the first step in the pathogenesis of *Salmonella*, and this step is supported and controlled by the *invA* gene [12, 14]. The DNA of *Salmonella* contains a region called the pathogenicity island I (SPI), that includes the *invA* gene [15]. SPI adds complex virulence to bacteria strains for host infection [13].

Invasion genes encoding the SPI are activated by expression of the HilA gene; transcription factors are also encoded in SPI. The role of *Salmonella* invasion genes in mice after intragastric inoculation indicated that bacteria with mutated invasion genes were found in intestinal tissue cells and systemic tissue compared to strains without such mutation (Murray and Lee [15].

Salmonella invasion of epithelial cells requires *invA* gene expression, and this gene is the first of at least two additional genes attack it, but now there is an *invA* gene that has mutations [16]. Mutants of *S. typhimurium invA* are less capable of invasion the cultured epithelial cells. *invA* mutants do not change the structure of microvilli of the epithelial cells and do not alter the distribution of actin microfilaments in cells. Mutations of *invA* genes do not show invasion gene bands on agarose gels because cells with mutant genes cannot replicate on the intestinal mucosa surface. Such bacteria can be removed by host defenses such as phagocytosis. *invA* gene of *Salmonella* is associated with the ability of bacteria to kill phagocytes *in vitro* [16].

The Widal test measures antibody binding and agglutination of lipopolysaccharides (LPS) and flagella in the serum of individuals with suspected *S. typhimurium* or *S. entertidis* infection; the test was widely used over a century ago [17]. It is simple to prepare, inexpensive and specific. Only a few minutes is required to obtain results.

Conventional methods for identification and typing of *Salmonella* serovars are still important for microbiological diagnosis [18]. Whole protein extracts of bacterial cells is a reflection of the genomic of different strains and can support identification, classification and typing of the bacteria. Comparative SDS-PAGE is also an important molecular technique used for identification at the species level [18].

Microbiological analysis related to an epidemiological investigation of outbreaks requires accurate identification and characterization of causative organisms. Many authors have been used total protein extracts of *Salmonella* serovars on SDS-PAGE to evaluate whole cell lysates [19–23].

Our study is in agreement with Nakamura et al., (2002) [19], who reported that total protein profile of *S. typhimurium* ATCC 14028 and *S. enteritidis* 13076 showed major similarity in the pattern of bands on SDS-PAGE. Major bands were noticed at 71.4, 67.7, 44.0, and 30.3 kDa [19] while Ngwai et al. (2005) [21], noted that total protein of *S. typhimurium* strains using SDS-PAGE, detected 36.5 and 65 kDa proteins in all strains. Hassanain (2008) [23], that whole protein analysis of *Salmonella* on SDS-PAGE shows many bands between 11.4 and 77.5 kDa and bands at 77.5, 55.2, 33.1 and 16.2 kDa are common. Total protein profiles of 54 *Salmonella* serovars, including *S. typhimurium, S. enteritidis, S. agona, S. anatum, S. virchow*, and *S. corvallis*, have also been compared using SDS-PAGE [22]. On SDS-PAGE and at 37.8 kDa there was no any difference between all serovars. Acik et al. (2005) [20] declare that protein banding profile on SDS-PAGE are not accurate to detect the difference between *Salmonella* species.

Our data is consistent with extensive resistance is some strains of *S. typhimurium.* This was agreed in results with another studies [24] in its resistance against some of antibiotics used as amoxicillin-clavulanic acid, cefoxitin, and ceftiofur, while decreased in susceptibility to ceftriaxone (MIC ≥ 4 μg/ml). In 1998, it was confirmed using of ceftiofur in animals as an antibiotic belonged to third generation of cephalosporin [24].

Resistance to antibiotics can be 96.42% and 57.14% for aminoglycosides (streptomycin and neomycin, respectively), 78.57% for the oxazolidone, linezolid and 64.28% for the cyclicpeptide, tetracycline). These

data indicate that many *Salmonella* isolates are resistant to multiple antibiotics and this help distribute these organisms and conform a serious problem to human public health.

S, typhimurium, S. entertidis and all unidentified isolates remain sensitive to amoxycillin/clavulanic acid, cefotaxime, norfloxacin and ofloxacin with sensitivity ratios of 100%.

Salmonella strains were found to be multidrug-resistant (MDR) and have been detected in many serotype, such as *S. enterica* serotype typhimurium [25, 26], *S. enterica* serotypes agona, anatum, choleraesuis, dublin, Heidelberg, Kentucky, Newport, Schwarzengrund, Senftenberg and Uganda among others [27–29].

Commonly, multidrug resistant (MDR) *S. typhimurium* are no longer sensitive to ampicillin (AMP), chloramphenicol (CHL), streptomycin (STR), sulfonamides, and tetracycline (TET), and termed as ACSSuT (AMP/CHL/STR/SMX/TET) and these refereed to this strain carrying the blaCMY gene and others [30]. Recently, many other strains belonged to family Enterobacteriaceae strains exhibiting the ACSSuT pattern also have acquired MDR plasmids carrying the blaCMY gene [27].

Some strains may also display resistance to gentamicin (GEN), kanamycin (KAN), and trimethoprim-sulfamethoxazole ([SMX] COT) as well as resistance to disinfectants and heavy metals. Cephalosporin is an important antibiotic for treating salmonellosis in children, so the high resistant of Salmonella to it is not desired specially because the prohibition of using fluoroquinolones in the treatment for such diseases [24].

Methods

Sample collection

Fifty samples from chicken carcasses were collected from Al-Riyadh markets, Saudi Arabia.

Isolation and identification of *Salmonella* strains

Fifty-five g of chicken was added to 225 mL of lactose broth and incubated at 37°C for 24 hours. One ml of lactose broth culture was added to selenite cysteine broth and incubated at 37°C for 24 h. One loop of selenite cysteine broth was streaked on Xylose Lysine Desoxychlate Agar (XLDA) (Oxoid code, CM0469) and incubated at 37°C for 24 h. Suspected *Salmonella* spp. appeared as colonies with black centers surrounded by white halos. Such colonies were used for further analysis.

Molecular detection of the *invar* gene

DNA extraction from Salmonella isolates

DNA of *Salmonella* isolates was extracted using a QIAamp DNA Mini Kit (50) Cat No./ID: 51304 (QIAGEN GmbH-Bezirksregierung Düsseldorf, Germany) following the manufacturer's protocols.

PCR amplification and electrophoresis

Invasive encoding gene (*invar* gene) was detected in *Salmonella* isolates using PCR. A final volume of 50 µl contained *invar* gene primer F: ACAGTGCTCGTTTACGACCTGAAT and
R: AGACGACTGGTACTGATCGATAA (Chiu and Ou (1996) [31]. Amplification used incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing at 56 °C for 30 s, and elongation at 72°C for 30 s, followed by a final extension at 72°C for 10 min. Amplified PCR products separated on 1% agarose gels with ethidium bromide at 100 v for about 1 h. DNA bands were observed under ultraviolet light. DNA bands at about 244 bp were identified using a 100 bp DNA ladder run concomitantly. Positive isolates were then sequenced, and the Gen Bank BLAST program used to ensure that the proposed primers were consistent with target species.

Serological tests

Anti-*S. typhimurium* and anti-*S. enteritis* control sera are intended for use in system control and assessing agglutination *Salmonella* test antigens in Wimal tests. Quantitative bacterial agglutination is assayed by interactions between specific *Salmonella* antibodies and test antigens. The goal of these tests is to determine the level of dilution (titer) of sera with clearly visible agglutination. This test was performed on glass slides following manual instructions.

Salmonella isolates were tested serologically using anti-*S. typhimurium* and anti-*S. enteritis* control sera (SIFIN, Institüt fur Immunpräparate und Nährmedien GmbH, Berlin, Germany REF., TS 1624 and TS 1625, LOT., 880910 and 1090712, respectively). A positive reaction is indicated by the degree of agglutination compared with agglutination with standard strains of *S. typhimurium* ATCC 14028 and *S. enteritis* ATCC 13076

Preparation of cell extracts of *Salmonella* isolates for sodium *dodecyl sulfate-polyacrylamide* gel electrophoresis (SDS- PAGE)

Overnight cultures (100 µL) were inoculated into 10 ml of fresh Brain heart infusion (Oxoid, CM1135) and incubated for 3 to 4 h to an optical density (OD_{620}) of 0.6 to 0.8. Cells were collected and weighed, and 250 mg of cells was then suspended in 100 µL of a TES buffer (50 mM tris HCl, pH 8, 1 mM EDTA, 25% sucrose). Twenty microliters of lysozyme (50 mg/mL) and 5 µL mutanolysin (5000 u/mL) were added to suspended cells in TES buffer and incubated at 37°C for 30 min. Five to 10 microliters of 20% SDS were added mixed until suspensions became visibly clear. The contents were stored at −20°C for 1 to 2 d. Fifty-microliter extracts (standard and isolated bacteria) were loaded on SDS-PAGE using 12% polyacrylamide for separation gels and 4% for stacking gel (Yehia and Al-Dagal, 2014) [32].

Electrophoresis was performed at room temperature in a vertical chamber (Biometra, Germany), using a constant voltage of 100 v, until the bromophenol blue tracking dye reached the bottom of the gel. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Marnesla-Coquette, France) at a concentration of 0.25% in water:methanol:acetic acid (6.5:2.5:1) overnight at room temperature. Gel destaining was performed by continuous agitation in methanol:glacial acetic acid:water (20:10:70 v/v/v) until obvious bands of proteins were obtained. Whole-cell protein profiles of presumptive *Salmonella* isolates were

compared with standard *Salmonella typhimurium* strain ATCC 14028. A high degree of similarity with standard strains was further confirmed with positive anti-*S. typhimurium* agglutination tests.

Antibiotic susceptibility profile for *Salmonella spp.,*

The results of susceptibility tests for *S. typhimurium* ATCC 14028 and *S. enteritis* ATCC 13076 were compared with results using *Salmonella* isolates. The bacterial isolates were obtained after overnight incubation following inoculation of single colonies into BHI media (Oxoid, U.K.). Cultures were spread on Mueller Hinton agar (Oxoid, U.K.), and individual plates were used for agar disk diffusion assays. Twenty different antibiotic-impregnated disks (Oxoid, U.K), with agents belonging to 7 different classes–b-lactam, aminoglycoside, cyclicpeptide, sulfonamide, quinolone, fluoroquinolone, and macrobid–were tested. Antibiotics were: cefoxitin (FOX, 30 µg, CT0119B), carbenicillin (CAR, 100 µg, CT0006B), ticarclline (TIC 75 µg, CT0167B), amoxicillin/clavulinic.acid (AMC, 30 µg, CT0223B), cefotaxime (CTX, 30 µg, CT0166B), streptomycin (S, 10 µg, CT0047B), gentamicin (CN, 10 µg, CT0024B), kanamycin (K, 30 µg, CT0025B), neomycin (N, 30 µg CT0033B), tetracycline (TE, 30 µg, CT0054B), sulphamethoxazole (RL, 25 µg CT0051B), naldioxic acid (NA, 30 µg, CT0031B), norfloxacin (NOR, 5 µg, CT0668B), ofloxacin (OFX, 5 µg, CT0446B), linezolid (LZD, 30 µg, CT1650B), nitrofurantoin (F, 300 µg, CT0036), erythromycin (E, 15 µg, CT0020B), chloramphenicol (C, 30 µg, CT0013B), vancomycin (VA, 30 µg, CT0058B) and lincomycin (MY, 2 µg, CT0027B). Diameters of zones of inhibition (mm) for each antibiotic and isolate were recorded using criteria recommended for Enterobacteriaceae [33]. Diameter measurements were used to classify isolates as sensitive (S) or resistant (R).

Conclusions

This study shows the prevalence of *S. typhimurium* more than *S. entertidis* in chicken carcasses distributed in markets in Saudi Arabia. The study found that use of the *invA* gene as indicator gene for *Salmonella* spp. is generally supported by serological tests with specific serum anti-*S. typhimurium* or anti sera for any other *Salmonella* spp. Subsequent confirmation using total protein profiles on SDS-PAGE combine for accurate identification, classification and typing of *Salmonella*. Multidrug resistance (MDR) to antibiotics was observed for *Salmonella typhimurium* strains in comparison with a standard strain. Such resistance can exacerbate public health risks associated with food-borne contamination.

Abbreviations

BHI, Brain Heart Infusion; BLAST, Basic Local Alignment Search Tool; PCR, Polymerase Chain Reaction; TPP, Total Protein Profile.

Declarations

Acknowledgments

We would like to express our sincere thanks to Deanship of Scientific Research at Princess Nourah bint Abdulrahman University, Saudi Arabia.

Author Contribution

H.M.Y. and M.M.A.-D. conceived and designed the experiments. H.M.Y. and A.H.A. performed the experiments. H.M.Y. and M.M.A and M.F.E. wrote the paper.

Funding

This work was supported by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University through the Fast-Track Research Funding Program.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Tables

Due to technical limitations, table 2 is only available as a download in the Supplemental Files section.

Figures

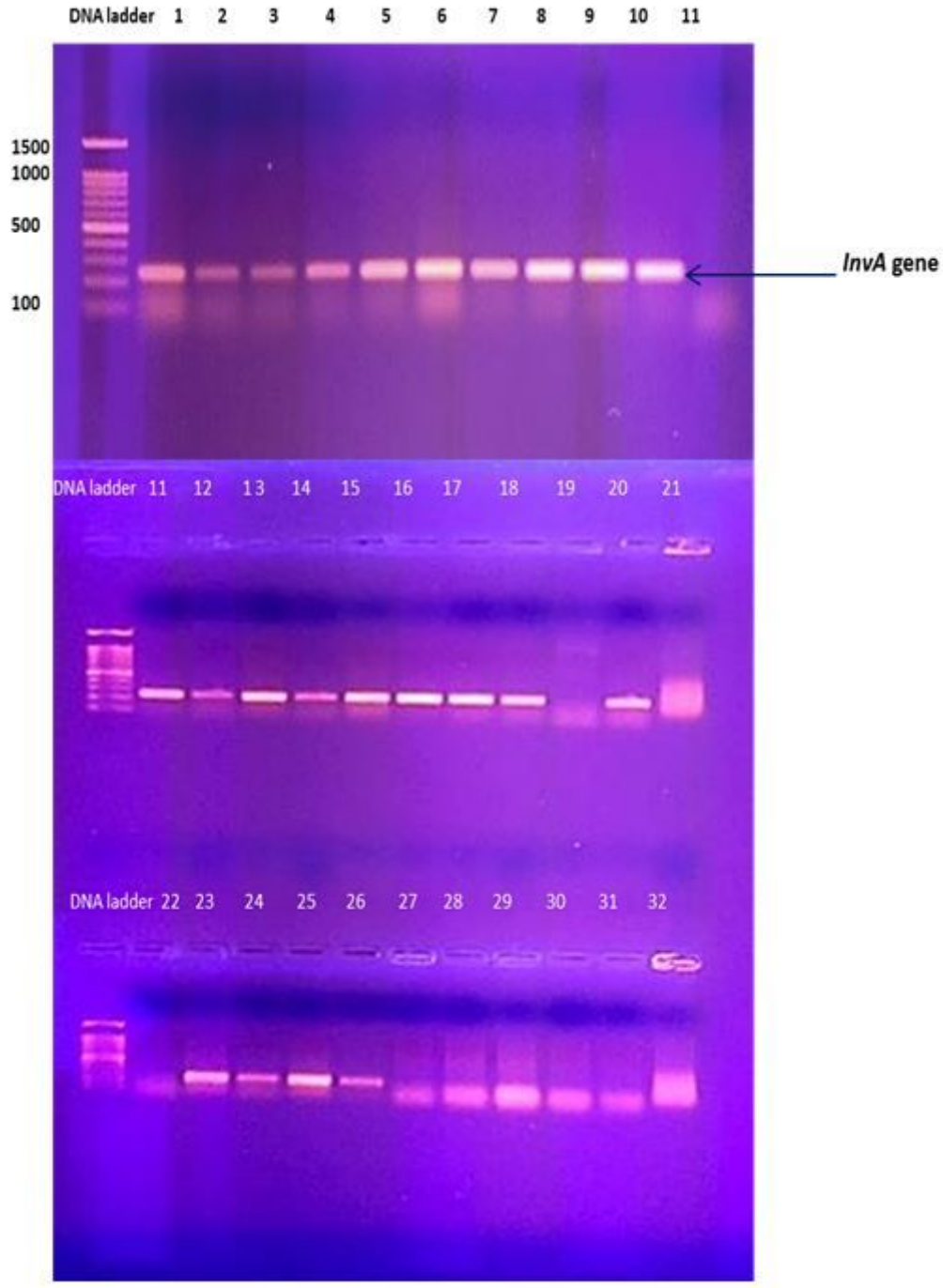

Figure 1

PCR products generated using genomic DNA invar gene primers Positive invar gene amplification for S. typhimurium ATCC 14028 and S. enteritis's ATCC 13076 (Lane 1 and 2) for 21 isolates (3–10), (11–18), (20) and (23–26). DNA band at 244 bp shows marker presence; visualized by gel electrophoresis using 1% agarose with an image analyzer (SYNGENE), DNA marker (1 kb ladder).

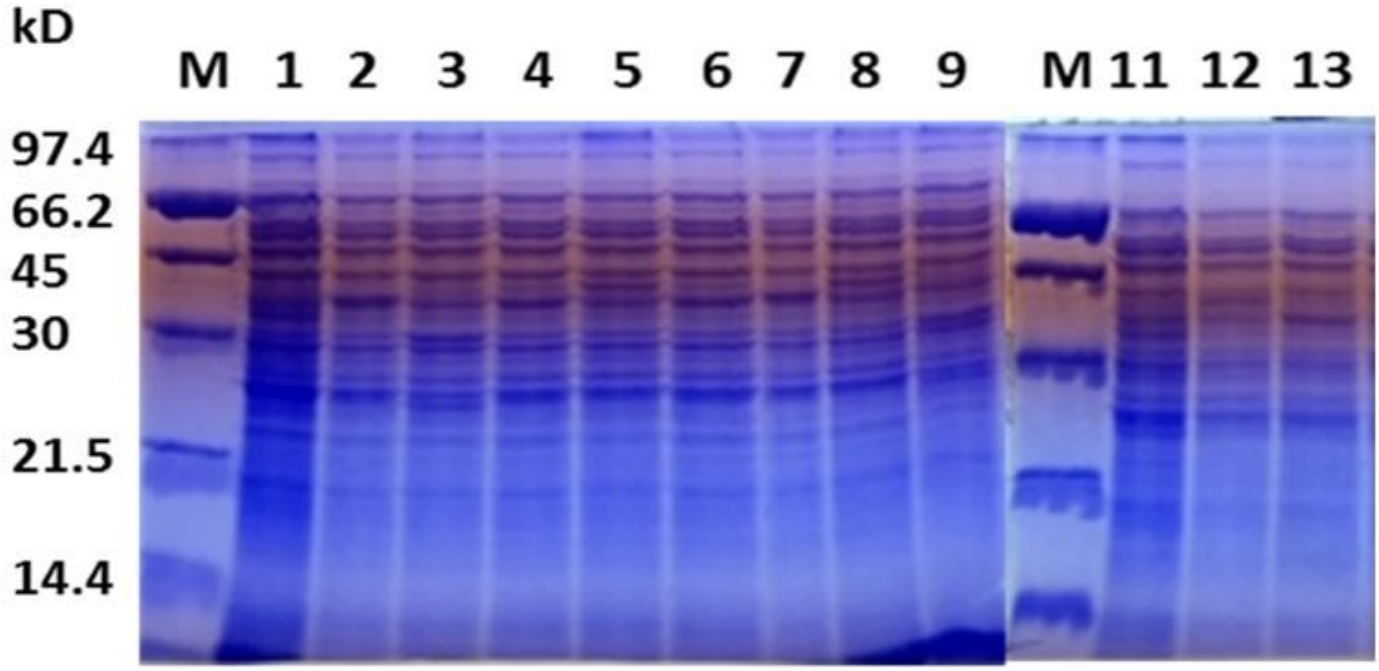

Figure 2

Total protein profiles of Salmonella typhimurium isolates by SDS/PAGE Lane M molecular weight standard. Lanes 1 Salmonella typhimurium ATCC 14028, Lanes 2–9 and 12–13: Salmonella typhimurium total protein and a positive anti-sera typhimurium.

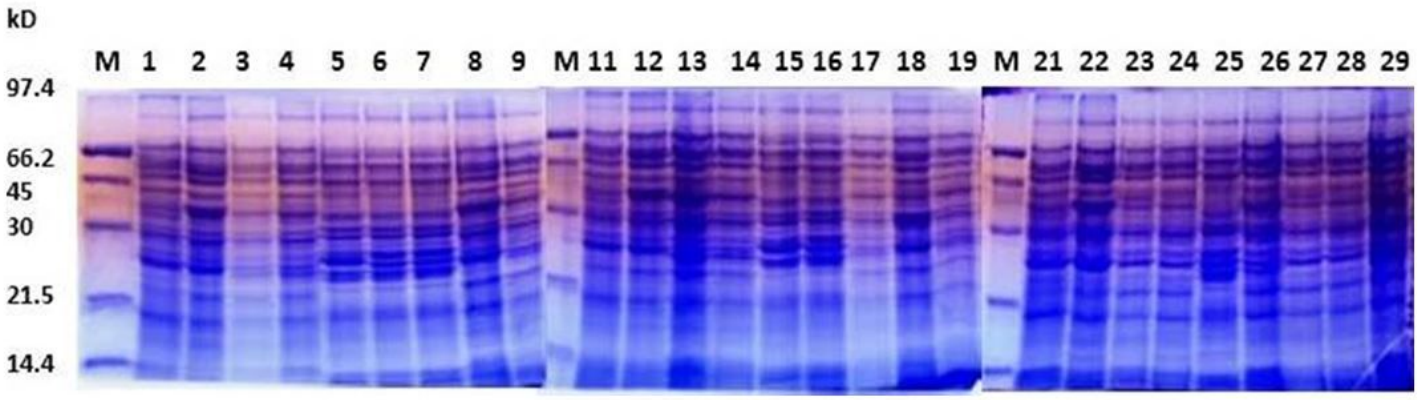

Figure 3

Total protein profiles of Salmonella isolates by SDS/PAGE Lane M: the molecular weight standard. Lanes 1, 11 and 21 Salmonella Typhimurium ATCC 14028, Lanes 2, 12and 22 Salmonella enteritis ATCC 13078. Lanes (3-9), (13-19) and (23-29), isolates were similar to standard strains.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table2a.JPG
- Table2b.JPG