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Molecular characterization through PCR based detection and confirmation of *Salmonella* spp. isolates by targeting *inv*A gene

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Abstract
The present study was designed to study the molecular characterization of the *Salmonella* spp. from pigs. A total of 175 fecal samples collected from different pig farms which are located in Bidar, Hassan and Tumakuru districts of Karnataka. Based on the high specificity of primers targeting *inv*ar gene, they were employed for the specific confirmation of the *Salmonella* spp. isolates to genus level. All the *Salmonella* spp. suspected cultures subjected to PCR amplification generated a product of approximate molecular size 284 bp. 100 bp DNA marker was used as a molecular weight marker. The band size detected in all the *Salmonella* spp. isolates and *S. typhimurium* reference strain was consistent as analyzed by agarose gel electrophoresis. Of the 24 isolates, all isolates were confirmed to be *Salmonella* spp. genus level by *inv*ar gene based PCR. The results revealed that all the 24 suspected isolates were confirmed to be *Salmonella* spp. and were harboring the *invA* virulence gene, indicating (100%) distribution of *inv*A.

Keywords: PCR, *invA,* virulence gene, forward primer, agarose gel electrophoresis

Introduction
Pig rearing is one of the traditional activities in India carried out by rural folk. Among various livestock activities, piggery is most efficient way of meat production utilizing kitchen waste, vegetable waste etc. Though initially local breeds have been raised, now-a-days exotic pig breeding is popular and pork from such animal is having wide acceptance. Further, pig farming requires small investment on building and equipment's.

The virulence of *Salmonella* spp.is linked to a combination of chromosomal and plasmid factors. Different genes such as *inv*A, *spv*, *fim* A and *stn* have been identified as major virulence genes responsible for Salmonellosis. *Salmonella* spp. pathogenicity islands (SPIs) are large gene cassettes within the *Salmonella* spp. chromosome that encode determinants responsible for establishing specific interactions with the host, and are required for bacterial virulence in a given animal like other pathogenicity islands. More than 20 SPIs have been described in *Salmonella* spp. (Sabbagh *et al*., 2010) [14]. The chromosomally located invasion gene *inv*A codes for a protein in the inner membrane of bacteria that is necessary for invasion of epithelial cells (Darwin and Miller, 1999) [6]. Whereas, an operon (*spv* RABCD), containing five genes, is present on plasmids commonly associated with some serotypes. One main function of the *spv* operon is to potentiate the systemic spread of the pathogen (Heithoff *et al*., 2008) [9]. The *spv* C is virulence-related gene on the plasmid required for survival within host cell (Chiu and Ou, 1996) [4]. Some studies have provided evidence that the virulence plasmid plays a significant role in human disease (Guiney *et al*., 1994) [8]. *Salmonella* spp. induced diarrhoea is a complex phenomenon on involving several pathogenic mechanisms, including production of enterotoxin. This enterotoxin production is mediated by the *stn* and plays a significant role in causing gastroenteritis by producing enterotoxin (Chopra *et al*., 1987) [5].

The subspecies of *Salmonella* spp. can be further divided into serotypes, also called serovares, differentiated from each other based on the presence of somatic (O) and flagellar (H) antigens. The number of serotypes that have been identified is continuously increasing, today adding up to more than 2500 (Grimont, 2007) [7]. The majority (1531) of these serotypes belong to *Salmonella enterica* subsp. *enterica* and were originally given names such as *Typhimurium*, Dublin, Infant is *etc*., while the serotypes belonging to other subspecies have been identified by numbers according to their antigenic formulae (Grimont, 2007) [7].

The purpose of the study will have a direct impact on the field application in the treatment of *Salmonella* spp. infection. Rapid detection of causative agent gene by PCR within 24 hrs will decrease the economic loss to the pig farming community.

Although bacteriological assays have historically been the method of choice for the recovery of *Salmonella* spp. from feaces and environmental samples, PCR has become an important technique for more-rapid detection of pathogens in feaces and environmental samples when an isolate is not required like bacteriological assays, PCR often requires enrichment of faecal samples to increase *Salmonella* spp. numbers and to aid in the dilution of compounds that may interfere with the PCR (Chiu and Ou, 1996) [4].

Materials and Methods

The material and methods used in the present study are presented in this chapter.

Standard / reference strain

The standard strain of *Salmonella* spp. (*S. typhimurium* No. Ref 0180 P) used study was obtained from 11 rue Emile 20LA B.P. 2332 38033 Grenoble Cedex 2 France. Microbiologics 200 Cooper Avenue North st. Cloud MN 56303, www.microbiologics.com, LIT: 2128, Rev F (Himedia laboratory Pvt. Ltd. Mumbai). Were this was inoculated into sterile nutrient broth and incubated at 37 °C for 24hrs. After incubation streaked onto selective media Hi Chrome *Salmonella* spp. agar were incubated at 24 hrs at 37 °C. After incubation pure colony picked up and inoculated into 20% nutrient-glycerol preservative were incubated at 37 °C for 24 hrs. After, incubation stored at -20 °C.

Isolation of DNA

The DNA of *Salmonella* spp. was extracted by boiling-snap chilling method. Approximately, colony of pure culture was taken from mac Conkey agar (isolate) plate, inoculated in nutrient broth, incubated at 37 °C for 12 hrs. After 24hrs, 500 µl incubated broth culture was taken in eppendrof tubes. Then samples were heated at 100 °C for 10 min. The boiled cell lysate was immediately cooled at -20 °C for 10 min and centrifuge at 14000 rpm for 2min. The supernatant DNA template for PCR (Karthik *et al*., 2016) [15].

Purity of the DNA samples by UV Spectrophotometry

The purity and concentration of the extracted genomic DNA was estimated by UV spectrophotometer. An aliquot of 20 µl of DNA sample was dissolved in 0.98 ml of sterile distilled water. The diluted DNA was transferred into 1ml micro cuvette and the optical density (OD) was read at 260nm and 280 nm in a UV spectrophotometer. Sterile DW was used as blank. The ratio of 260/280 OD was calculated. A ratio of 1.7 to 1.9 was considered pure.

Synthesis/ Procurement of PCR primers: All sets of primers used in this study were synthesized commercially bio nova supplies SIGMA-ALDRICH, Bengaluru (India), and they were reconstituted in NFW (Nuclease free water) as per the requirement and stored at -20 ºC.

Detection of virulence genes by Polymerase chain reaction (PCR)

All *Salmonella* spp. isolates were first screened for the presence or absence of virulence associated gene by using the PCR protocol standardized for detection of gene. The PCR was standardized for the detection of gene *inv*A used methodology as described by Chaudhary *et al.* (2015) [3], Kumar *et al*. (2008) [10] with suitable modification. Standardization of PCR was done by using standard strain of *S. typhimurium*.

Materials

1. 2X Master mix (Sigma).
2. Nuclease free water.
3. Extracted DNA.
4. Thin walled PCR tubes of 200 µl capacity (Bio-Red).
5. Primers.
6. Micropipettes and appropriate capacity tips.

Preparation of primers

Stock and working primer solutions were prepared according to the manufacturer's instructions.

Preparation of forward primer

[5′-GTGAAATTATCGCCACGTTCGGGCAA-3′]:
(Chaudhary *et al*., 2015) [3]

a) Primer containing tubes were centrifuged at high speed for 30 secs.
b) Sterilized nuclese free water of 334 µl was added to the tube containing oligo so as to obtain 100 µM of "stock solution".
c) Stock solution (10 µl) was further diluted 1:10 in nuclease free water (90 µl) to get 20 µM concentration "working solution".
d) 2 µl of "working solution" was used per 25 µl of PCR reaction mixture.

Preparation of reverse primer

[3′-TCATCGCACCGTCAAAGGAACC-5′]:
(Chaudhary *et al*., 2015) [3]

a) Primers containing tubes were centrifuged at high speed for 30 secs.
b) Sterilized nucleaase free water of 729 µl was added to the tube containing oligo so as to obtain 100 µM of "stock solution".
c) Stock solution (10 µl) was further diluted 1:10 in nuclease free water (90 µl) to get 20 µM concentration "working solution".
d) 2 µl of "working solution" was used per 25 µl of PCR reaction mixture.

Screening for invasion (*inv*A) gene

The isolates were screened for the presence of invA gene (284bp) by PCR method (Table-4) used Chaudhary *et al.* (2015) [3], Kumar *et al*. (2008) [10] with certain modification made as in this method preheated lid temperature 112 °C and annealing temperature 63 °C. The reaction was standardized in thin walled PCR tubes in 25 µl reaction volume with different concentration reactants under different annealing temperatures and cycling conditions. Finally, the reaction mixture was optimized to contain 12.5 µl 2X PCR master mix, 10 pmol of each forward and reverse primer, 7.5 µl nuclease free distilled water and 3 µl of concentration of DNA template. The reaction was performed UN the thermal cycler with pre-heated lid (Lid temp. 112 °C).

The reaction conditions employed were initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. A final extension of 5 min at 72 °C was employed. Quantity and concentration of various components used for PCR were as Table-4. Steps and conditions of thermal cycling for primer pair in PCR were as Table 5. On completion of the reaction the amplified product were analyzed on agarose gel electrophoresis through 1.5 per cent agarose gel. Visualized under UV light in Gel documentation instrument and results were noted.

Agarose gel electrophoresis
The PCR products were subjected to agarose gel electrophoresis using 1.5 per cent agarose gel.

Materials and Equipment's
a) Agarose (Hi media, Laboratories Ltd., Mumbai).
b) TAE buffer (Tris base, acetic acid, EDTA di-sodium), pH 8.5.

Stock solution 50X for 1000 ml
Tris Base - 242 gm
Glacial Acetic Acid - 57.1 gm
Disodium EDTA.2 H_2O - 37.2 gm

Working Solution 1X
Mix stock solution of TAE buffer 20 ml with 980 ml of triple distilled water to prepare the working solutions of 1X TAE buffer used for gel running and preparation of 1.5% agarose gel.

Table 4: Quantity and concentration of various components used in PCR

SL No	Components	Quantity Final	Concentration
1.	2X PCR master mix (Taq buffer, Taq polymerase, dNTPs)	12.50 µl	2X
2.	Nuclease free water	7.50 µl	---
3.	Forward primer (10 pmol/µl)	1.00 µl	10 pmol
4.	Reverse primer (10 pmol/µl)	1.00 µl	10 pmol
5.	DNA Template	3 µl	---
Total		25 µl	

Table 5: Steps and conditions of thermal cycling for primer pair in PCR

Primers	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
*inv*A (F)	94 °C	94 °C	63 °C	72 °C	72 °C
*inv*A (R)	3 min	30 sec	30 sec	30 sec	5min

Repeated for 35 cycles

I. Ethidium bromide
Ethidium bromide (10Mg) was procured from Biosciences, Merck specialties Pvt. Ltd., Bangaluru. One ml of DW was added and suspension was mixed properly to dissolve the dye. The stock solution was stored at room temperature and protected from light.

II. Gel loading dye (6X)
Gel loading dye (6X) was procured from Biosciences, Merck specialties Pvt. Ltd., Bangalore and stored at 4 °C.

III. 100 bp DNA ladder
Procured from Biosciences, Merck specialties Pvt. Ltd., Bangaluru.
a) Electrophoresis unit
b) UV-trans illuminator.
c) Horizontal electrophoresis apparatus with power pack (Banglore Genie, India).
d) Gel documentation unit (Bio-Rad, USA).
e) Micro-wave oven (LG, India).

Protocol
About 0.75 gm of analytical grade agarose was dissolved in 50 ml of 1X TAE buffer by heated in microwave oven to obtain a clear uniform suspension. Prior to casting the gel, the molten agarose was allowed to cool about 50 °C, after this add 3 µl of Ethidium bromide was added to a final concentration of 0.5 µg/ml and mixed thoroughly. Gel was cast on an appropriate gel casting tray fitted with acrylic comb allow 35-45 min for solidifying. The acrylic comb was carefully removed after the gel was set. The tray with the gel was then submerged in an electrophoresis tank containing 1X TAE buffer.
The DNA to be analyzed was mixed with $1/6^{th}$ volume of 6X loading dye and carefully loaded in to the wells using micropipette alongside 100 bp DNA molecular weight marker. The electrophoresis was carried out with 5V/cm until the tracking dye (Bromophenol blue) had just reach the anode end of the gel. The following electrophoresis, DNA bands were visualized and the images were captured using Trans illuminator unit (Gel documentation, Biorad).

Results
The present study involved PCR application of molecular characterization of isolated *Salmonella* spp. The result obtained during the programmer of research work was documented as follows.

Collection of samples
A total 175 samples were collected from different pig farms of Karnataka. The samples were processed in the Dept. of Veterinary Microbiology, Veterinary College and Bidar by standard protocols.

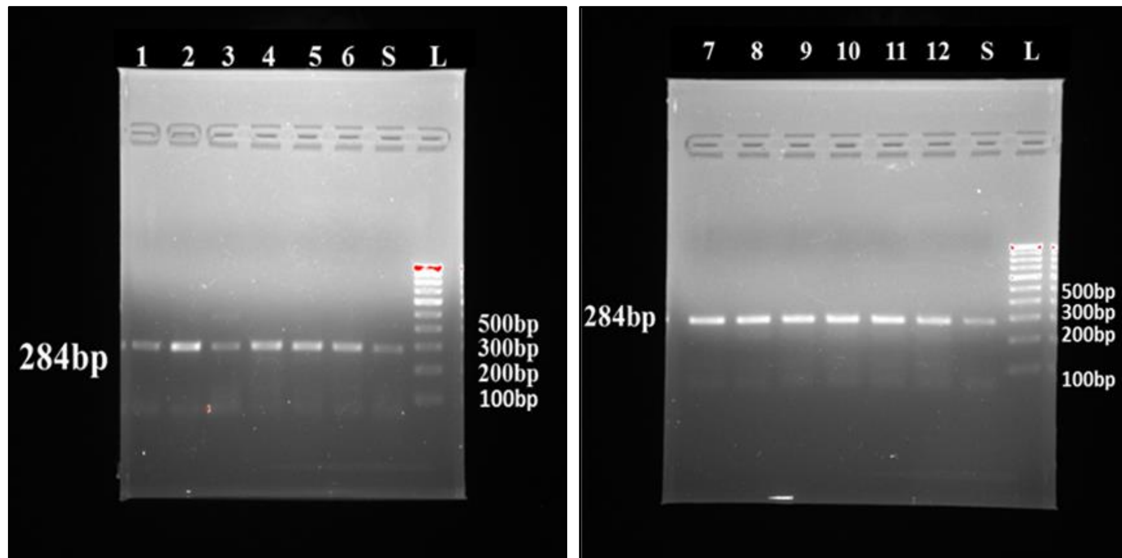
Isolation of *Salmonella* spp.
All the samples were subjected to bacteriological isolation, A total of 24 (13.71%) *Salmonella* spp. isolates were obtained in the present study.

Molecular characterization of *Salmonella* spp. isolates by PCR
Standardization of PCR for detection of *Salmonella* species
Initially, gradient PCR was set up for each primer to be used in the PCR. The annealing temperatures ranging from 55 °C to 65 °C were used in the gradient PCR and although all the primers amplified at 63 °C. In this study modifications are made as in this method preheated lid temperature 112 °C and annealing temperature 63 °C.The primers to be used were first evaluated for their specificity by PCR using known positive and negative controls. The PCR was successfully standardized and the desired amp icons were obtained. Positive reference cultures used yielded 284bp size amp icons specific for *Typhimurium* respectively, all the bands were well differentiated on agarose gel recorded in Gel documentation.

PCR based confirmation of *Salmonella* spp. to genus level

Based on the high specificity of primers targeting *inv*A gene, they were employed for the specific confirmation of the *Salmonella* spp. isolates to genus level. All the *Salmonella* spp. suspected cultures subjected to PCR amplification generated a product of approximate molecular size 284 bp. 100 bp DNA marker was used as a molecular weight marker. The band size detected in all the *Salmonella* spp. isolates and *S. typhimurium* reference strain was consistent as analysed by agarose gel electrophoresis. Of the 24 isolates, all isolates were confirmed to be *Salmonella* spp. genus level by *inv*A gene based PCR.

The results revealed that all the 24 suspected isolates were confirmed to be *Salmonella* spp. and were harboring the *inv*A virulence gene, indicating (100%) distribution of *invA*. All isolate subjected to PCR as follows (Plate 1).



Lane: 1-24 Positive samples,
S: standard strain,
L: DNA 100bp ladder

Plate 1: Agarose gel showing PCR amplification products of *inv*A gene (284 bp)

Discussion

The virulence of *Salmonella* spp.is linked to a combination of chromosomal and plasmid factors. Different genes such as *inv*A, *spv*, *fim* A and *stn* have been identified as major virulence genes responsible for Salmonellosis. *Salmonella* spp. pathogenicity islands (SPIs) are large gene cassettes within the *Salmonella* spp. chromosome that encode determinants responsible for establishing specific interactions with the host, and are required for bacterial virulence in a given animal like other pathogenicity islands. More than 20 SPIs have been described (Sabbagh e*t al*., 2010) [14]. The chromosomally located invasion gene *inv*A codes for a protein in the inner membrane of bacteria that is necessary for invasion of epithelial cells (Darwin and Miller, 1999 [6]). Whereas, an operon (*spv* RABCD), containing five genes, is present on plasmids commonly associated with some serotypes. One main function of the *spv* operon is to potentiate the systemic spread of the pathogen (Heithoff *et al*., 2008) [9]. The *spv* C is virulence-related gene on the plasmid required for survival within host cells (Chiu and Ou, 1996) [4].

Detection of virulence gene by PCR

Considering the difficulties in detection of virulence factors in routine identification of *Salmonella* spp. by conventional methods, number of virulence associated genes of *Salmonella* spp. needs to be identified to assess virulence potential of *Salmonella* spp. The polymerase chain reaction has tremendous potential for rapid, specific and reliable detection of food-borne pathogens as it has revolutionized field of molecular biology and therefore it is one of the most sought method in food microbiology in recent years. The PCR studies are being carried out to confirm the identity of isolate from natural sources. So in view of above, rapid and reliable diagnosis of Salmonellosis has been suggested to be ideally based on detection of virulence markers of *Salmonella* spp. by molecular technique (Rahman, 1999 [13], Bhatta, 2007) [2].

In this context the present study was undertaken with view to detection of virulence gene in *Salmonella* spp. isolates. Thus a total of 24 positive isolates of *Salmonella* spp. were subjected to PCR assay for the detection of virulence gene *inv*A.

In this study the PCR was standardized for the detection of gene *inv*A following the methodology described by Kumar *et al*. (2008) [10] with suitable modifications are made as pre heating lid temperature 112 °C and annealing temperature 63 °C. Standardization of PCR was done by using standard strain of *Salmonella* spp.

Detection of *inv*A virulence gene by PCR

In the present study, all the twenty four isolates of *Salmonella* spp. were subjected to PCR detection of the *inv*A virulence gene using method described by Kumar *et al*., (2008) [10] with suitable modifications. All the 24 isolates yielded desired amplified product of 284bp similar to that of reference strain of *Salmonella* spp. using the primer pair for *inv*A which is in accordance with Kumar *et al*. (2008) [10]. Chiu and Ou (1996) [4] found presence of *inv*A gene in all of 38 fecal isolates using enrichment broth culture-multiplex PCR combination assay employing different set of primers.

Abouzeed *et al*. (2000) [1] found *Salmonella* spp. isolates from beef cattle, broiler chicken and human Salmonellosis cases to contain *inv*A gene. Oliveira *et al*. (2003) [12] reported that all *Salmonella enteritidis* isolates of poultry, pigs, humans and

food origin contained *inv*A gene. Similarly Madadgar *et al*. (2008) [11] and Kumar *et al*. (2008) [10] detected *inv*A gene in all (60 and 50, respectively) strains of *Salmonella.* Findings in the present study are in accordance with earlier workers reports.

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