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Detection of *invA* Gene in Isolated Salmonella from Marketed Poultry Meat by PCR Assay

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Abstract

Background: The aims of the current study were to detect the *invA* gene from chicken samples meant for human consumption from N.E India.

Materials and method: After *Salmonella* sp. identification with culture method, PCR assay were developed for detection of pathogenic genes and antibiotic resistance genes of *Salmonella* sp.

Results: *Salmonella* was detected in 80 samples of poultry carcasses from main poultry markets in Silchar, Assam, N.E India. A total of 40 *Salmonella* isolates were found in chicken samples (43%) and the isolates had a growth on brilliant green agar and De-oxycholate citrate agar medium, were oxidase negative and catalase positive and exhibited no changes in the colour of the medium with 100% motility. All the strains were subjected to Salmonella-specific gene (*inv*A) and were confirmed as *Salmonella* positive by the predicted product of 284-bp DNA fragment. Salmonella isolates recovered from poultry samples were tested for antibiotic susceptibility against 5 selected antibiotics of which ciprofloxacin was observed to be highly susceptible (77.5%).

Conclusion: Our results recommended the use of PCR for detection of pathogenic genes of bacteria as a safe, rapid, and accurate method in laboratories. High levels of Salmonellosis infections in poultry farms has raised an eye amongst the poultry management personnel's to consider various effective control programs to prevent the economic loss resulting from mortality and spreading of infection.

Keywords: Poultry meat; Salmonella; PCR; *Inv*A gene

Introduction

Poultry provides an immense supply of food for the entire world's population where poultry meat and eggs in majority are all time preferred to other kinds of animal food products for a variety of reasons, but most of these have been implicated as a major source of *Salmonella* infection in human [1]. Today, reports on mishandling of poultry products and raw poultry carcasses, uncooked poultry meat are one of the frequent causes of human infection caused by *Salmonella* species [2]. Also, a number of *Salmonella* serotypes can be transferred to poultry from sources, such as feedstuffs, breeding flocks, rodents, and wild birds, etc. of which may act as an important hindering factor for the economy and growth of a country. Thus in the present time, several new techniques in this regard have been developed for the rapid detection of *Salmonella* serovars such as selective culture medium and enzyme-linked immunosorbent assay [3]. However, today the Polymerase Chain Reaction (PCR) method has emerged as a powerful, rapid and a reliable tool for detection and identification of food- borne pathogens such as *Salmonella*. Malorny [4] where several chromosomal genes including *invA* are target genes for PCR amplification of *Salmonella* species [5]. The *invA*, gene of *Salmonella* contains those sequences that are unique to this genus and has been proved as a suitable PCR target with potential diagnostic applications [6]. Thus, keeping the above in view, the present investigation highlights the screening of poultry meat samples for *Salmonella* sp. and their confirmation by *invA* specific PCR methods.

Materials and Methods

Clinical isolates and phenotypic identification

A total of 80 poultry samples were collected from various parts of Silchar, Cachar District, Assam, comprising of meat, liver, intestine scrapings and faecal materials. The samples mostly belonged to highly crowded areas, slaughter houses, swabs from butchers knife used for cutting chicken. Samples were aseptically enriched in selenite F broth (Himedia) and incubated at 37°C for 24 hours. A loopful from the broth was then streaked on brilliant green agar (Himedia) and De-oxycholate citrate agar (Himedia) for further incubation at 37°C for 24 hours. The distinct isolated colonies were identified on the basis of their morphological and biochemical characters. The shape and colours of the colonies were examined under the microscope after Gram staining. Isolates were biochemically confirmed for the activities of oxidase, catalase, methyl red, voges-prauskauer test, urease, motility, indole production, citrate utilization, TSI and sugar fermentation tests through a series of conventional biochemical tests. Colonies that depicted biochemical reactions like *Salmonella* were transferred to nutrient agar slant (Himedia) and incubated at 37°C for 24 hrs.

Antibiotic susceptibility test

Antibiotic susceptibility of the isolates was assayed according to Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA) [7]. The isolates were freshly inoculated on saline water for detection of turbidity via comparison with 0.5 Mc. Farland solution (1.5 x 108 CFU/ml). The antibiotic disks used were of HI-MEDIA Laboratories, Mumbai, India, consisting of the following: Kanamycin (30 µg), Ciprofloxacin (5 µg), Ampicillin (25 µg), Norfloxacin (10 µg) and Nalidixic acid (30 µg). Positive tests were indicated by zones of

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inhibition which were measured by using the zone size interpretative tables provided by the manufacturer of the discs.

Genotypic screening of *invA* gene from isolated strain by PCR assay

DNA of *Salmonella* isolates was extracted and purified using Genomic DNA Mini Kit (cultured cell).

Primers set and PCR amplification program: The primers (Forward and Reverse) used in PCR were specific for *invA* gene which was chosen according to Rahn [6]. The sequence of primer used in this study is presented in Table 1.

Electrophoresis of PCR products

PCR for amplification of *invA* gene was performed using 12.5 µl of Tag Green Master Mix 2x DNA polymerase [8], specific primers for *invA* gene (1 µl forward and 1 µl reverse), 2 µl of DNA extract as a template, 8.5 µl of nuclease free water to make the final reaction volume up to 25 µl. The PCR conditions started with thermocycler (Eppendorf) program that is initial denaturation at 95°C for 2 minutes, 34 cycles of denaturation at 95°C for 15 seconds, 45°C for 1 minute, and 72°C for 45 seconds and final extension at 72°C for 7 minutes.

Agarose gel electrophoresis

Five microliters of the reaction mixture and 1 µl marker DNA (100 bp DNA ladder mix; MBI Fermentas, USA) mixed separately with 1 µl of 6X gel loading dye (MBI Fermentas, USA) and were analyzed by submarine gel electrophoresis in 1.2% agarose (Hi-Media, Mumbai, India) at 60 V for 1 hour and 20 min. or until the second dye marker had run 3/4th of the gel. The reaction products were visualized with UV light after staining with ethidium bromide. The identities of the amplicons were confirmed by comparison of the amplicon sizes with the predicted sizes and photographed [8].

Results

A total of 80 chicken samples were procured for this study, of which 40 isolates of *Salmonella* sp. were recovered. Table 2 represents the total number of isolates and their percentage.

All the 40 isolates had a growth on brilliant green agar and De-oxycholate citrate agar medium, were oxidase negative and catalase positive and exhibited no changes in the colour of the medium. 100% motility was observed in all the isolates; indole and methyl-red tests were observed as positive and voges-Proskauer test as negative; all of them represented citrate utilization, urease test as negative, were non-lactose fermenters on MacConkey agar medium and fermented glucose, fructose and maltose. TSI was observed as $K/A^{G+H2S}+$. All the 40 isolates were identified as *Salmonella* organisms.

Forty *Salmonella* isolates recovered from poultry samples were tested for antibiotic susceptibility against 5 selected antibiotics. Percentages of *Salmonella* isolates susceptible to antibiotics are represented in Table 3.

Forty *Salmonella* strains were isolated from poultry specimens, by culturing in selenite F and then transferring to Salmonella-Shigella agar, when subjected to Salmonella specific-PCR using primers *invA* F and *invA* R where all isolates including positive control and a single 284 bp amplified DNA fragment, on 1.2% agarose gel (Figure 1).

Discussion

The present study evaluated the microbial quality of poultry meat sold at various retail markets of Silchar, were heavily contaminated with *Salmonella* sp. (43%) [9-11]. Presence of bacteria in meat is of great public health concern [12] indicating a potential breakdown of hygiene at various stages of the food processing and distribution chain and/or a lack of refrigeration of meat. As observed during the course of this study, the methods of slaughtering of animal are responsible for this microbial contamination. Traditional method of butchering using knives and cutting lines appears more capable of minimizing faecal contamination then modern mechanized system which are manned by a team of operators. This was inferred because poultry samples collected from the local abattoir were less contaminated, than those collected from the areas where modern equipment is used [13].

Biochemical reactions are very important for speciating the isolates, while isolates could get a correct identification only when based on genetic methods. In the present study, all the 40 (43%)

Name of primer	Target gene	Primer sequence (5'-3')	Amplified product size	Reference
InvA F	invA	GTGAAATTATCGCCACGTTCGGGCAA	284 bp	Rahn et al. [15]
InvA R		TCATCGCACCGTCAAAGGAACC		

Table 1: Sequences of primers for *invA* gene.

SL No.	Samples from different parts of poultry	Samples procured	Total no. of isolates isolated	Percentage of *Salmonella sp*
	Meat	29	15	52%
	Liver	23	12	52%
	Scrapping	11	5	45%
	Fecal	17	8	47%
	Total	80	40	43%

Table 2: Percentage of *Salmonella* sp. contamination in poultry chicken samples from the different areas of Silchar.

Antibiotics	Total susceptible isolates	% of susceptible isolates
Ampicillin	25	62.5%
Ciprofloxacin	31	77.5%
Kanamycin	30	75%
Norfloxacin	23	57.5%
Nalidixic acid	26	65%

Table 3: Percentages of *Salmonella* isolates susceptible to antibiotics from poultry samples.

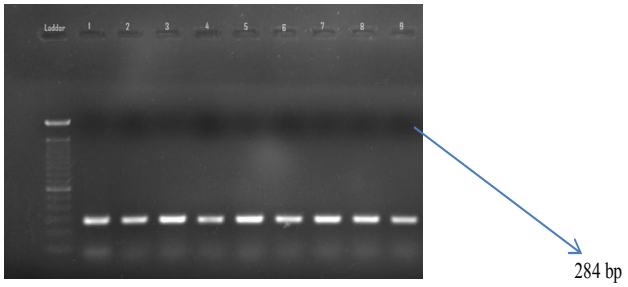

Figure 1: Gel image showing amplification of *inv*A gene. Ladder used is 100 bp ladder. Lanes 1,2,3,4,5,6,7,8 and 9 exhibits the amplified *inv*A gene (284 bp).

isolates exhibited typical biochemical characteristics of *Salmonella* on the basis of IMViC reaction, gas production and sugar fermentation as per standard techniques. All the isolates tested negative oxidase, positive catalase, positive indole in typtone broth, positive methyl red, negative Voges-Proskauer, urease negative and citrate positive tests and fermented glucose, fructose and maltose.

Today, the frequencies of bacterial strains resistant to antimicrobial agents have increased dramatically in the environment as a consequence of the wide spread use of drugs [14]. A significant public health concern and the possibility of transfer of resistant genes between bacteria in the natural habitats have attracted attention. In the present study, antimicrobial susceptibility pattern against 5 antibiotics was carried out for 40 *Salmonella* isolates of which ciprofloxacin was 77.5% susceptible, whereas kanamycin exhibited (75%) susceptibility, nalidixic acid (65%), ampicillin (62.5%) and norfloxacin (52.5%). It was observed that norfloxacin which was routinely used in present prognosis was the least susceptible antimicrobial agent found in the present study. Also, this is the first report on the prevalence of *Salmonella*, determining its antimicrobial-resistance pattern along with detection of *inv*A gene of *Salmonella* from poultry farms and slaughter houses of this geographical area. In the current study, the overall prevalence of *Salmonella* and fecal shedding of *Salmonella* was lower in organized farms than the slaughter houses. Despite the use of selective broth, we recovered higher proportions of *Salmonella* (43%) from the both clinical as well as post-mortem samples.

The present study supports the ability of these specific primer sets to confirm the isolates as *Salmonella*. All PCR products of isolates included positive control, screened by PCR, resulted in 284 bp amplified fragment. No amplified DNA fragments were obtained from non-Salmonella species. The ability of *Salmonella* specific primers to detect *Salmonella* species rapidly and accurately is primarily due to the primer sequences that are selected from the gene *inv*A [15]. The *inv*A gene codes for protein in inner membrane of bacteria, which is necessary for invasion to epithelial cells [16]. The PCR assay carried out in this study for the detection of the *inv*A genes in 40 *Salmonella* isolates from poultry meat samples was present in 22 isolates (55%). These findings are in agreement with the earlier reports [17-20]. Furthermore it has been observed that this gene is involved in the invasion of the cells of the intestinal epithelium and is present in pathogenic *Salmonella*. Therefore for salmonellosis to occur it is important that a gene responsible for invasion must be present. This gene is essential for full virulence in *Salmonella* and is thought to trigger the internalization required for invasion of deeper tissue [21].

Conclusion

High levels of Salmonellosis infections in poultry farms has raised an eye amongst the poultry management personnel's to consider various effective control programs to prevent the economic loss resulting from mortality and spreading of infection. In the present work, PCR based methods with genus-specific primers belonging to *inv*A, proved to be quick, specific and sensitive and certain in identification and confirmation of *Salmonella* isolates.

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