Detection of *INVA* Gene and Cytotoxin of *Salmonella entertidis* in Food Samples Using Molecular Methods

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Authors' contributions

Article Information

Original Research Article

ABSTRACT

Salmonella entertidis is a foodborne pathogen that causes various diseases in human beings worldwide. The toxin of *Salmonella* can cause infectious diseases. In this research project, *Salmonella* was detected through various microbial, biochemical and molecular tests in diverse food samples collected from highly populated, moderately populated and less populated areas of Lahore, Pakistan. Enriched cultures of all food samples such as apples, tomatoes, yogurt and mayonnaise was streaked on violet-red bile glucose agar, Simmon's citrate agar and eosin-methylene blue agar (EMB). *Salmonella* isolates were screened for the presence of toxin encoding gene through PCR. 27% apples, 19% tomatoes, 5% mayonnaise and 7% yogurt were found to be

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positive for *INVA* genes (invasion protein genes). In medical and pharmaceutical point of views the INVA gene can also help to develop specific medicines against *salmonella*. The cytotoxin that is protein in nature was confirmed by SDS PAGE in mayonnaise samples. This study illustrates that foods of highly populated areas are reservoir for *Salmonella entertidis* in Pakistan. There is need to develop specific drugs, precautionary measures to control *salmonella* and its disease.

1. INTRODUCTION

Salmonella is a most renowned entero-pathogen associated with serious illness in humans and animals including *entertidis* and diarrhea [1]. It has received attention for many years due to its large number of outbreaks reported in different regions of the world [2]. *Salmonella enetrica* has approximately 2500 serotypes and most of the serotypes are infectious and cause many diseases in mammalian species. *Salmonella entertidis* causes salmonellosis which is a gastrointestinal infection that results in fever, abdominal cramps, diarrhea after 12 to 72 h of post-infection [3]. Poultry products have been reported as the important cause of salmonellosis because this bacterial serotype is found in the intestinal tracts of chickens. Owing to large amount of the consumption of broiler chickens, eggs and poultry products, outbreaks of salmonellosis have been increased overtime. According to the survey, it has been estimated that there exit about 13 million cases of salmonellosis across the Asian countries due to the consumption of poultry products [4]. In the same way, dairy products made from improper pasteurized milk are reservoir of *Salmonella* [5].

There are many virulence factors responsible for the production of enterotoxins and ultimately causes diseases. According to the research, there are around 200 virulence factors present in Salmonella species [6]. Among these the most frequently involved genes are *invA* (invasion in the epithelial cells), *fimA* (major fimbrial subunit encoding gene), *stn* (enterotoxin gene) and *hilA* (invasion gene transcriptional activator) [7]. When it enters the body of an organism, it begins to divide immediately as a consequence gastroenteritis occurs that lasts for a week or more. *Salmonella* species produce toxins that is termed as cytotoxin which leads to the inhibition of protein synthesis responsible of intestinal mucosal damage, enteric symptoms and inflammatory diarrhea that ultimately cause urinary tract infection, osteomyelitis and septicemia [8]. The purpose of this study was to investigate the prevalence of *Salmonella*

entertidis in diverse food samples like fruits (apples), vegetables (tomatoes), dairy (yogurt) and poultry (mayonnaise) products collected from different areas of varying population. *Salmonella* was isolated by different microbial methods and tested through biochemical methods. Finally, toxin producing gene (*invA*) was identified by polymerase chain reaction and toxin was analyzed by using SDS-PAGE

2. MATERIALS AND METHODS

The research was carried out by collecting samples of varying quality from highly populated, moderately populated and less populated areas of Lahore, Pakistan i.e., Paragon city (PC), Lahore Press Club (LPC) and Harbanspura (HP).

2.1 Sample Collection

A total of 64 food samples (tomatoes, apples, yogurt and mayonnaise) of varying quality were collected from above mentioned localities. The samples were taken in sterilized tubes and covered with aluminum foil then immediately transferred to lab. Clear extracts of apples and tomatoes were obtained after blending and filtration. Yogurt and mayonnaise samples were mildly blended to get homogenized mass and stored at 4°C.

2.2 Pre- enrichment

All food samples were subjected for pre-enrichment in peptone buffered water (pH 7.0) with ratio of 9: 1 (media: sample) and allowed to incubate overnight at 37°C in shaking incubator.

2.3 Isolation and Screening of *Salmonella entertidis*

The samples were streaked on different selective media i.e. Violet red bile glucose agar, Simmon's Citrate agar and Eosin methylene blue agar and incubated overnight at 37°C. The colonies with colorless in case of Violet red bile glucose agar [9], bluish in case of Simmon's Citrate agar [10]

and colorless on Eosin methylene blue agar [11] were picked and tested for the presence of the desired serotype using TSI agar slants [12], indole test [13], methyl red test [14], and catalase test [15]. The samples showing positive results were picked and analyzed for the detection of gene encoding *invA* toxin.

2.4 Detection of INVA Gene of *Salmonella entertidis*

2.4.1 Culturing of *Salmonella entertidis*

The colonies of *Salmonella* isolated by selective media, were picked in laminar flow hood with the help of sterile loop. These colonies were allowed to grow in 15 ml of autoclaved LB media (pH 7.4) in sterile falcon tubes in shaking incubator at 220 rpm at $37^{o}C$ for 24 hrs.

2.4.2 PCR for detection of *Salmonella entertidis*

Bacterial DNA was isolated by phenol-chloroform method [9]. The presence of DNA was detected on 1% agarose gel. The isolated DNA was used for the detection of *invA* gene of *Salmonella entertidis*. Reaction mixture was prepared by adding 2.5 µl of PCR buffer, 1 µl of MgCl2, 1 µl of forward primer (5' ACAGTGCTCGTTTACGACCTGAAT 3'), 1 µl of reverse primer (5' AGACGACTGGTACTGATCGATAAT 3'), 0.5 µl of taq polymerase, 2 µl of template DNA and 15 µl of autoclaved water in autoclaved PCR tubes. PCR reaction was carried out in BioRad thermal cycler with the profile of initial denaturation at $94^{o}C$ for 10mins, denaturation at $94^{o}C$ for 30 sec, annealing at $59^{o}C$ for 30 sec, extension at $72^{o}C$ for 30 sec and final extension at $72^{o}C$ for 10mins. After completion of 30 cycles of PCR, the product was run on 1% agarose gel, stained with ethidium bromide (0.5 µg/ml) and photographed using a gel documentation system.

2.5 Detection of Salmonella Toxin

2.5.1 Extraction of toxin

A 4 days old bacterial culture was centrifuged at 10,000 rpm for 8mins at $4^{o}C$. After centrifugation, the pallet was suspended in about 5 ml of 5x PBS. Centrifugation was again carried out and pallet was re-suspended in about 3ml of 5x PBS. Suspended pallet was sonicated twice for 4 mins and again centrifuged at 12,500 rpm for 20 mins at $4^{o}C$ and supernatant was used for detection of

toxin. The supernatant was used for the detection of cytotoxin on SDS-PAGE using 15% polyacrylamide gel.

3. RESULTS

3.1 Microbial and Biochemical Tests

Microbial methods indicated the presence of *Salmonella* strains in 43 out of 64 food samples. The presence of *Salmonella* was observed by the development of colorless on Violet red bile glucose agar (Fig. 1A), blue coloration on Simmons's citrate agar (Fig. 1B) and colorless on EMB (Fig. 1C).

3.1.1 Biochemical tests

All the *Salmonella* positive colonies from media were picked and inoculated in TSI slant, trypton broth, methyl red broth and normal saline in which the presence of *Salmonella* was confirmed by observing the respective change in color. (Figs. 2, 3, 4, 5).

3.1.2 Polymerase chain reaction

PCR was done to detect the presence of *invA* genes; out of 64 samples of *Salmonella* 47 samples confirmed the presence of *invA* genes.

3.1.3 SDS page of *Salmonella* toxin

SDS- PAGE was performed of all samples that were tested for the presence of cytotoxin of *Salmonella entertidis*. Out of 64 food samples, 57 food samples showed positive result.

4. DISCUSSION

Salmonella entertidis is most reported foodborne pathogen that contaminates large amount of food each year and causes gastrointestinal disease (salmonellosis) in human beings. If not treated with antibiotics, serious conditions arises that cause death [16]. In daily routine various biochemical and microbial tests are done to identify the strain but these methods are time consuming and less reliable. On the other hand PCR technique provides more reliable epidemiological data to trace the pathogenic strain [17]. In this research the bacterial strain was first identified by biochemical assays. It was further confirmed by microbial techniques. Then results were further confirmed by PCR and bacterial cytotoxin was identified by SDS PAGE. The differentiable PCR product was easily

visualized along with negative control and high quality primers. In this study the differential feature of *Salmonella entertidis* was utilized in order to identify in food samples. All the food samples were collected from densely populated areas. It is glucose fermenting bacteria that ferments glucose and appears as colorless colonies on Violet Red bile agar at 25°C after overnight incubation. Thus this media is selected that readily distinguish glucose fermenting bacteria with those that do not ferment glucose. This bacteria releases cytotoxins that help in pathogenicity of the organism. In this study the cytotoxin was isolated by SDS PAGE.

In case of biochemical assays, Simmon's citrate agar gives most differential results. *Salmonella entertidis* is citrate positive strain and can be differentiated from other Salmonella sub-genus that are citrate negative (*Salmonella typhi*) [18]. This is selected media for *Salmonella entertidis* that changes the color of media from green to royal blue due to production of citrate. A variety of microbial and biochemical tests were used to identify this pathogenic strain [19-26]. In PCR assay *invA* gene was found in 47 samples.

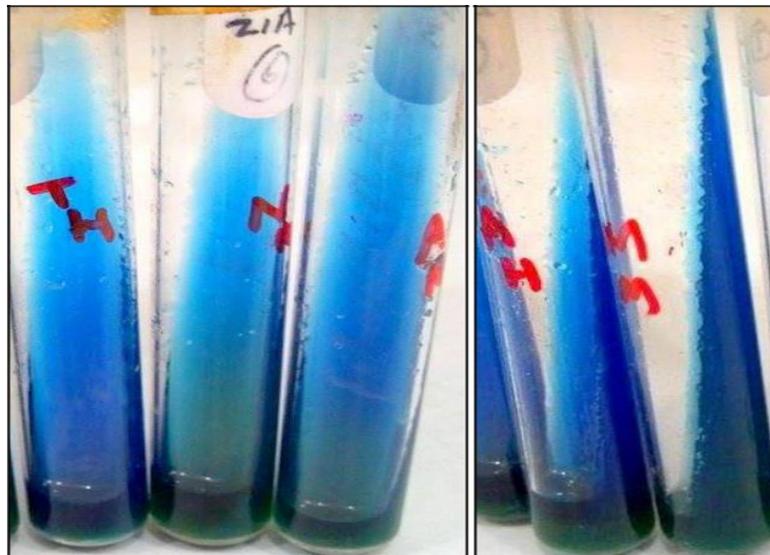**Fig. 1A. Growth of *S. entertidis* on violet red bile agar – colorless colonies represents the presence of *Salmonella***

Fig. 1B. Growth of *S. entertidis* on violet red bile agar – blue color indicates the presence of *Salmonella*
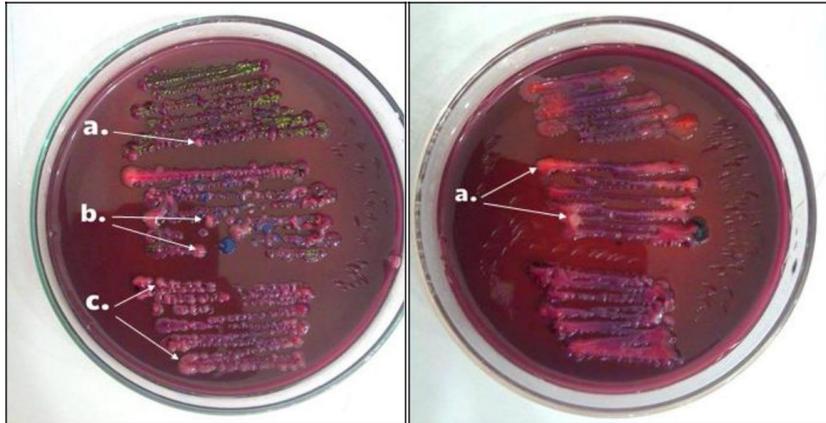
Fig. 1C. Growth of *S. entertidis* on EMB agar – colorless colonies represents the presence of *Salmonella*

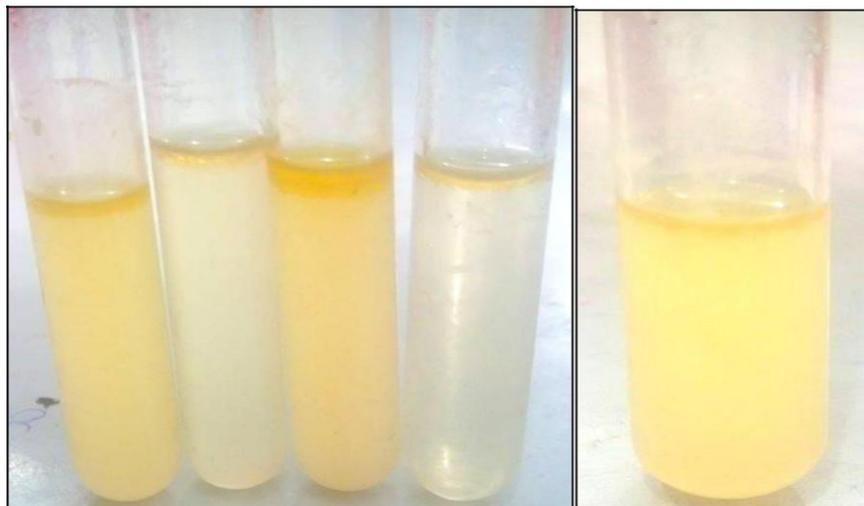**Fig. 2. TSI agar test – Red coloration indicating the presence of *Salmonella enteridis***

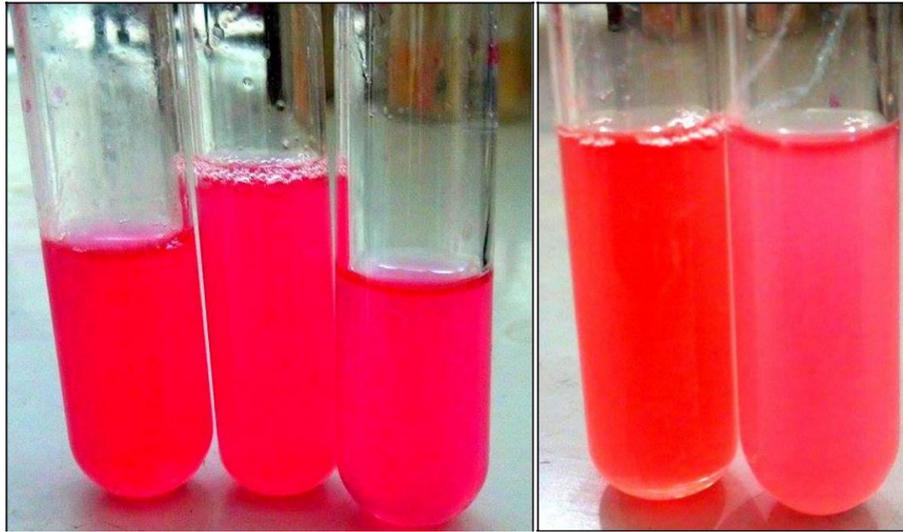**Fig. 3. Indole test – Colorless form indicating the presence of *Salmonella***

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Fig. 4. Methyl red test –Red coloration showed positive results for *Salmonella*

5(a)

5(b)

5(c)

Fig. 5. Catalase test – Slide a, b and c indicating no bubbling showing negative results
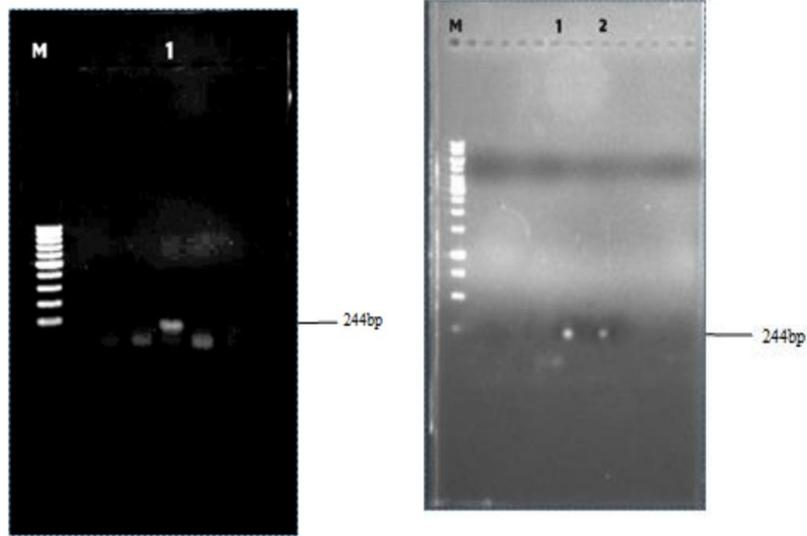
Fig. 6. Electrophoresis analysis of food samples of highly populates area- Lane M is 1kb ladder, Lane 1, 1and 2 are INVA gene
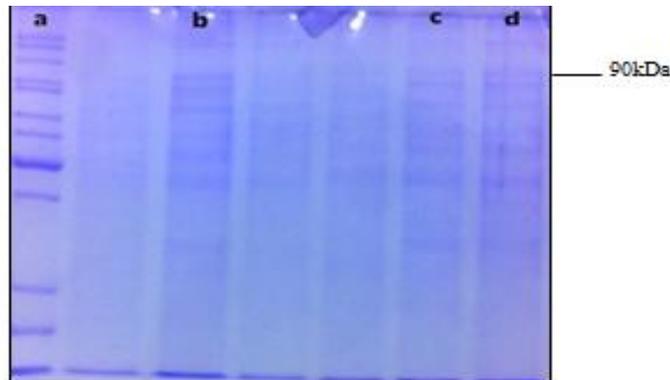

Fig. 7. SDS Polyacrylamide gel electrophoresis - Lane a: Unstained protein ladder, Lane b, c showing the presence of toxin of Salmonella entertidis

5. CONCLUSION

The presence of *Salmonella entertidis* in food is of great concern. It is a causative agent of various kind of gastrointestinal diseases. Thus from this study, the detailed analyses were made using different type of detection methods. Their detailed limitations and advantages were also analyzed. Finally, it is concluded that molecular based methods like PCR is a reliable way to detect this bacterial strain in food samples.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/66059