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Original Article

Isolation, phenotypic characterization and genotypic identification of *Salmonella* species isolated from food and water samples in Karachi, Pakistan.

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Abstract

Background: Foodborne illness is a global health problem, and *Salmonella* is one of the leading bacterial pathogens to cause salmonellosis and typhoid fever worldwide. These infections remain an obstinate risk to human and animal health despite substantial innovations and cleanliness practices. The high incidence of these infections is resulting not only from infected eggs and chicken but also from different food commodities. This research was designed in the direction of the certainties mentioned above and the high occurrence rate of extensively drug-resistant (XDR) infections in Pakistan.

Methodology: The study focused on the isolation and identification of *Salmonella* species from food and water samples collected from different regions of Karachi, Pakistan. The standard protocol of enrichment, culturing, and biochemical confirmation from Biological Analytical Manual (BAM) was used to characterize *Salmonella*'s phenotypic isolation and characterization. These isolates were then subjected to Polymerase chain reaction (PCR) and resolved on 1.5% agarose via electrophoresis.

Results: From the total of 1010 samples, 10 genotypically confirmed *Salmonella* isolates were obtained by detecting the *invA* gene by using PCR. Food items of different kinds exhibited 80% of the total positive isolates, while 20% is from the water sample.

Conclusion: The study exhibited the highest prevalence of *Salmonella* in chicken meat, which may indicate insufficient hygiene and sanitation practices during slaughtering and the supply chain. Incidence of *Salmonella* was also found in water and spices, signifying the improper sanitary systems and unhygienic handling of food items in many localities of the city, affecting human health.

Keywords

Salmonella, Prevalence, Polymerase Chain Reaction, Electrophoresis, Typhoid Fever.

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Introduction

Foodborne illness is a global health problem, and *Salmonella* is one of the leading bacterial pathogens to cause foodborne disease worldwide[1]. *Salmonella* is the genus of gram negative rods belonging to the Enterobacteriaceae family, causing salmonellosis and typhoid fever after ingesting contaminated food. It is comprised of more than 3000 serovars which are categorized based on host specificity and invasiveness into three groups[2]. Group 1 is incredibly host adaptive and invasive such as *Salmonella enterica* subsp. *enterica* ser. *Typhi;* Group 2 is invasive but non-host-adaptive such as *Salmonella enterica* subsp. *enterica* ser. *Enteritidis* and *Salmonella enterica* ser. *Typhimurium;* while Group 3 consists of non-host-adaptive but highly intrusive serotypes[3-6].

Salmonellosis and other infections caused by *Salmonella* remains an obstinate risk to human and animal despite substantial innovations and cleanliness practices in plant and food manufacturing and production. High incidence of these infections resulting from infected eggs, meat, and various food commodities. These can be a source of illness as a consequence of insufficient cooking and cross-contamination of working areas[2,7]. Disease caused by *Salmonella* is generally self-limiting but may cause severe complications in a vulnerable group such as infants and older adults, depending on its serotype and host specificity[2,8-10].

According to World Health Organization (WHO), 3/4 of all broiler chicken meat was infected with *Salmonella* spp[11]. In recent years, outbreaks of salmonellosis involving beef, spicy sprout, black and red pepper, etc., have been reported[12]. Vegetables, raw meat, and dairy items are the actual vehicles for transmitting foodborne pathogens, signifying a risk to the human population. The highest frequency of *Salmonella* was observed in pork and chicken (42.4% and 41.2%, respectively), followed by beef (31.5%), cucumber, and lettuce (3.8%) in Shanghai[1]. The most common serotypes isolate Enteritidis and Typhimurium. Ready-to-eat products and chicken meat are considered as a critical cause of *Salmonella* infection worldwide[13,14].

Besides, contamination of chicken meat with multi-drug resistant bacteria can create a severe problem in public health[15]. *S.* Typhimurium and *S.* Enteritidis isolated from chicken meat, collected from the retail chicken shop in China, were multidrug-resistant and carried numerous genes for virulence, a potential hazard to health well-being[16,17]. *S. enterica* ser. Typhimurium and Enteritidis signify the leading serovars associated with salmonellosis in humans. Contamination of meat and its products with these two serotypes is the primary source of infection[18].

Non-typhoidal *Salmonella* is a zoonotic pathogen, largely impacting human health [19]. According to World Health Organization, *Salmonella* graded first in causing food borne illnesses amongst 22 bacterial, protozoal, and viral agents in 2015[20]. Routes of transmission for non-typhoidal salmonellosis include eggs and poultry, milk and its products, and contact with pets. Person-to-person contact may also result in its transmission[21]. Consumption of contaminated tomatoes, sprouts, fruits, spinach, and peanuts is also the primary source of infection spread[22-26]. Drinking water is a major source of dissemination of *Salmonella* Typhi. Besides, food washed with contaminated water is also contributing in the spread of different *Salmonella* species[27,28].

Antimicrobial resistance is a developing issue these days leading to the failure of present antibiotics, which may prolong nosocomial and community-acquired infections. *Salmonella*'s Antimicrobial resistance genes are usually present on the mobile elements as plasmids, provides elasticity to host bacterium, and helps them distribute these genes across the diverse population[2, 29].

Pakistan is viewed as an epidemic of XDR *Salmonella* infection ascending from Hyderabad and some other cities since November 2016[30]. A total of 5423 out of the 8630 laboratories investigated cases from ten different hospitals of Karachi were categorized as XDR Typhoid from Jan 2017 to Jun 2019[31]. The implementation of hygienic practices must be forced on the workers in slaughtering places, and

the public needs to be educated for clean and safe food from the markets [32]. This study was designed due to the high occurrence rate of food poisoning and the XDR infections caused by *Salmonella* in Pakistan. In this research, the presence of *Salmonella* spp. was evaluated in water and different food products in Karachi city, Pakistan.

Methodology

Sample Collection & Preparation

Different categories of export quality food products and samples of water and food items collected locally from different areas in Karachi in both raw and cooked form. A total of 1010 samples were collected, homogenized, and processed for *Salmonella* isolation and identification, based on the BAM with some modifications.

Salmonella Detection by Conventional Culture-Based Method

The samples were subjected to non-selective pre-enrichment by homogenizing 25 g of the sample (for liquids; 25 ml) with 225 ml of sterile Lactose broth (LB). This homogenized mixture was incubated for 24 ± 2 h at 35ºC after kept for 1 h at room temperature. For spices, Trypticase Soy Broth (TSB) was used with 0.5% K_2SO_3 instead of LB due to high microbial load. A total of 0.1 ml of the overnight incubated lactose broth was added to 10 ml Rappaport-Vassiliadis (RV) medium and 1 ml to Tetrathionate (TT) broth, respectively for selective enrichment of *Salmonella* species, and incubated for another 24±2h at their appropriate incubation temperatures in circulating, thermostatically-controlled water bath.

A loopful of the inoculum from both of the enrichment broths was streaked on hektoen enteric agar (OXIDE), bismuth sulfite agar (OXIDE), and xylose lysine deoxycholate agar (OXIDE) separately, and incubated for 24 ± 2 h at 35ºC. Gram's staining reaction confirmed the presumptive *Salmonella* isolates, as well as by biochemical identification on triple sugar iron slants and lysine iron agar slants and hydrogen sulphide production[33].

Genotypic Detection of *Salmonella* by Polymerase Chain Reaction

a. DNA Extraction of *Salmonella*

Bacterial isolates were streaked on Trypticase soya agar to get pure isolated colonies. Suspensions were prepared with overnight *Salmonella* cultures in microcentrifuge tubes containing 600 µl distilled water (D/W) and vortexed. These tubes were then positioned in the water bath at 60 ºC for 10 minutes for cell lysis. DNA was then extracted and used for amplification with *Salmonella* specific primers.

b. Primers Set and PCR Amplification

The *invA* gene (389 bp) was amplified to detect *Salmonella* spp. by using specific primers. The sequence of the primers used were Salm3 (5'-GCTGCGCGCGAACGGCGAAG-3') and Salm4 (5'-TCCCGGCAGAGTTCCCATT-3')[33]. The PCR mix was prepared by GoTag® Green Master Mix (Promega), Forward Primers, Reverse Primers, and Nuclease-Free Water. A total of 23 µl of the mixture was added to each PCR tube. Bacterial lysate (2.5 µl) was then added to make the volume up to 25.5 µl. *S.* Typhimurium ATCC 14028 was used as a reference strain. PCR was run by using Thermal Cycler (Bio-Rad) with the following condition: preliminary denaturation at 95ºC (5 min), followed by 35 cycles of denaturation at 95ºC (90 sec), annealing at 62ºC (60 sec) and elongation at 72ºC (90 sec). Termination of the cycle was done with the final extension at 72ºC (7 min)[34].

c. Preparation of Agarose Gel and Resolution by Electrophoresis

1.5% agarose gel was prepared by dissolving 1.5g agarose powder in 100 ml Tris-Borate EDTA (TBE) buffer by heating. After reaching room temperature, 4 µl of Ethidium bromide was added and immediately poured into the gel cast. After placing the gel in an electrophoretic tank, TBE buffer was added. A total volume of 12 µl for each PCR-amplified product was loaded in each well, and 9 µl of the DNA ladder (100 bp) was loaded as a marker. The electrophoresis was done at 120 V for 1.5 to 2 hours. The gel was exposed to UV transillumination to visualize the DNA bands using a Bio-Rad Gel doc system.

Data Collection and Analysis

Data was collected by calculating the frequencies and percentages based on colony morphology on selective media, change in color during biochemical reactions, production of hydrogen sulphide gas, and observing the shape of cells and gram staining reaction in microscopy. Genotypically, the assessment was done on the basis of the presence of the *invA* gene.

Results

Phenotypic identification of *Salmonella*

After pre-enrichment in LB and selective enrichment in RV and TT broth, streaking was done on HE, BS and XLD agar for the assessment of the colony morphology of *Salmonella* (Figure 1-3), which was then confirmed by gram staining and biochemical reactions (Figure 4 & 5). Twenty-one out of 1010 samples were positive for *Salmonella* spp. by this conventional culture-based method of *Salmonella* identification.

Table 1: Presumptive *Salmonella* isolates with their respective source (Based on the culture-based method).

Isolates	Sample	Isolates	Sample
S1	Raw chicken	**S12**	Raw chicken
S2	Sweet	**S13**	Spinach
S3	Raw chicken	**S14**	Raw chicken
S4	Raw chicken	**S15**	Raw chicken
S5	Vermicelli	**S16**	Lyari water
S6	Rose water	**S17**	Green City water
S7	Garlic paste	**S18**	Raw chicken
S8	Red chilli powder	**S19**	Ready to cook spice
S9	Gharo Water	**S20**	Chutney
S10	Quaidabad water	**S21**	Aalu palak curry
S11	Coriander leaves		

Table 2: Colony morphology of *Salmonella* on different Selective media

Isolates		Colony Morphology	n(%)
HE Agar	S17	green colony	1(4.8)
	S1-16, 18-21	black centered light green colony	20(95.2)
XLD Agar	S1, S4, S10, S12, S13, S16, S19, S20	pink translucent colony + media turns pink	8(38.1)
	S2, S3, S5-9, S11, S14, S15, S17, S18, S21	black centered pink translucent colony + media turns pink	13(61.9)
BS Agar	S3, S8, S14,	black colony surrounded by grey outline	3(14.3)
	S5-7, S9-11, S13, S15, S18-21	black colony surrounded by grey outline + metallic sheen	12(57.1)
	S1, S2, S16, S17	light grey colony	4(19)
	S4, S12	No Growth	2(9.5)

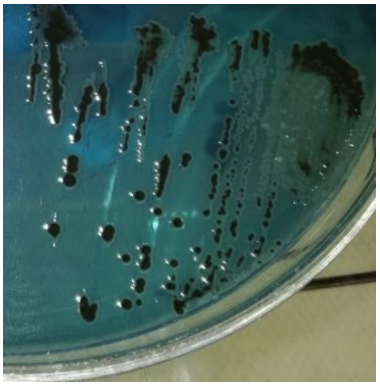


Figure 1: Enlarged Separated Colonies of *Salmonella* on HE Agar

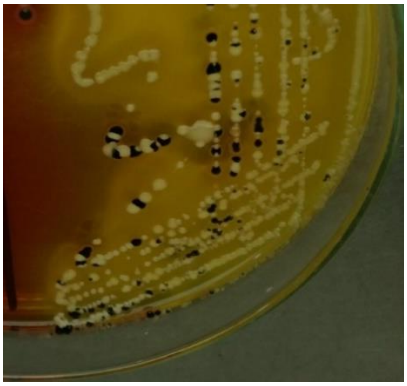


Figure 2: Enlarged Separated Colonies of *Salmonella* on XLD Agar

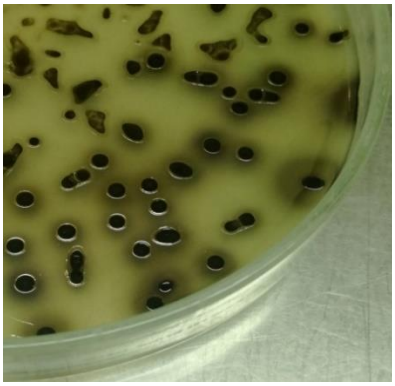


Figure 3: Enlarged Separated Colonies of *Salmonella* on BS Agar

Table 3: Biochemical identification of *Salmonella*

Isolates	**Reaction on TSI (slant/butt)**	**n(%)**
S1-3, S6, S7, S10, S12, S14-21	R/B	15(71.4)
S9, S13	R/B Y	2(9.5)
S4, S5, S8	Y/B	3(14.3)
S11	Y/B Y	1(4.8)
Isolates	**Reaction on LIA (slant/butt)**	
S4-9, S11, S18-21	P/P+ black streak	11(52.4)
S1-3, S12-17	P/Y	9(42.9)
S10	Y/Y	1(4.8)
Isolates	**H2S Production**	
S1-5, S10, S12-17, S20	-	13(61.9)
S6-9, S11, S18, S19, S21	+	8(38.1)

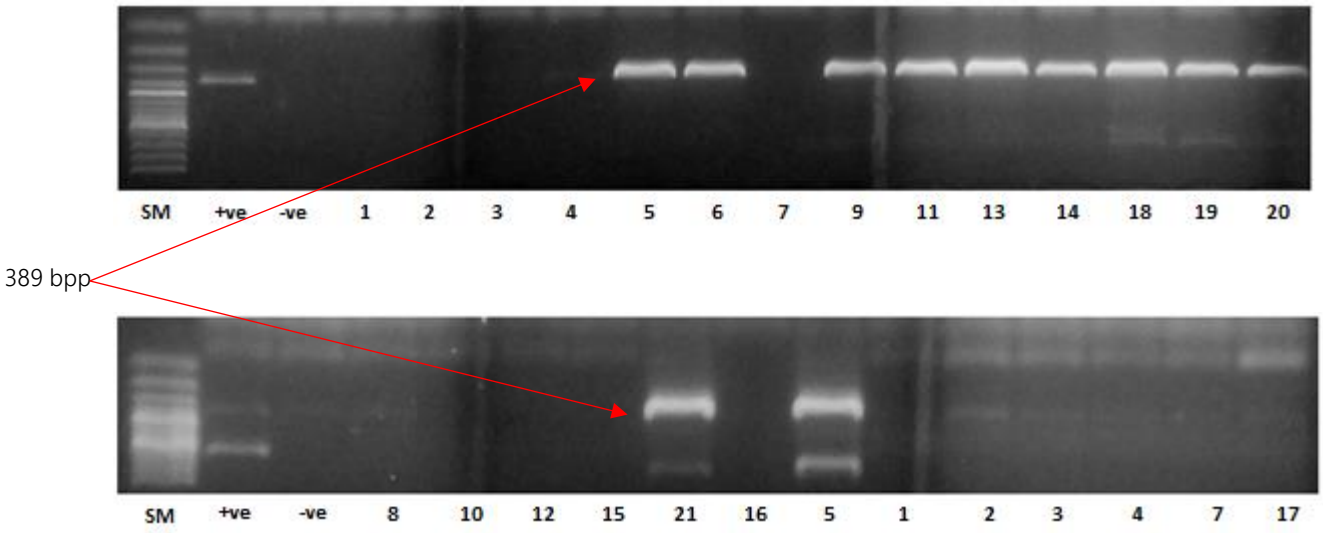
R (red); B (black); Y (yellow)
P (purple); Y (yellow)

Figure 4: Triple Sugar Iron agar with Red Slant, Black Junction & Yellow Butt showing Positive *Salmonella* reaction

Figure 5: Lysine Iron Agar with Purple Slant and Purple Butt, showing Positive *Salmonella* reaction

Genotypic Identification of the *Salmonella* Isolates

Salmonella isolates were tested genotypically by Polymerase Chain Reaction. The *invA* gene was amplified by PCR for the detection of *Salmonella* spp. and was resolved on 1.5% agarose Tris-EDTA gel by electrophoresis. Bands that coincide with the positive control band of *Salmonella* Typhimurium ATCC 14028 were considered positive for *Salmonella* (Figure 6).



(Lane 1: DNA Ladder 100 bp; Lane 2: Positive control (*S.* Typhimurium ATCC 14028); Lane 3: Negative control; Other Lanes: Samples run)

Figure 6: A representative agarose gel electrophoresis image of Polymerase Chain Reaction for the detection of *Salmonella*. Bands coincide with the Positive control (*Salmonella* Typhimurium ATCC 14028), representing the amplified product of 389 bp for the *invA* gene.

The total occurrence of *Salmonella* was 0.99% (10/1010), from which 80% were isolated from different food items (Green veges 25%, Processed food 25%, Raw chicken 25%, Ready to eat food 12.5% & Spices 12.5%), while only 20% were from water samples.

Discussion

The current study is focusing on the isolation and characterization of *Salmonella* from the water and food items collected from different areas in Karachi city, Pakistan. In the present research, 21 isolates were recognized as *Salmonella* from the total 1010 examined samples using a conventional culture-based identification method. Samples were of export as well as local quality, having raw and cooked both types. The detection of *Salmonella* was done on the basis of their morphological characteristics and biochemical reactions. Identification based on genotype was also performed. The *invA* gene was exponentially amplified using PCR and resolved on agarose gel by electrophoresis[34]. As studied by O'Regan *et al.* (2008), this gene has been considered as a golden marker for the genetic identification of *Salmonella*[35]. Results of PCR showed somehow different results as compared to phenotypic identification. From 21 phenotypically positive isolates, only 10 exhibited the presence of the *invA* gene.

These variable results of two different testing methods provide a good comparison of phenotypic and genotypic identification systems. PCR gives more accurate results quickly compared to the 5-day phenotypic identification-based results, including colony morphology, gram staining, and biochemical confirmation. The total occurrence of *Salmonella* was 0.99% (10/1010), from which 80% were isolated from different food items (Green veges 25%, Processed food 25%, Raw chicken 25%, Ready to eat food 12.5% & Spices 12.5%), while only 20% were from water sample.

Wang et al. (2020) also calculated the prevalence of different *Salmonella* serovars in both sick and healthy chickens. On serotyping and PCR based tests, 84.7% were revealed for *S.* Pullorum, *S.* Gallinarum and *S.* Enteritidis. *Salmonella* ser. Pullorum was showed to be the most prevalent in both sick and healthy chicken. From these three isolates, last one was found only in healthy chickens[36]. El-Sharkawy et al. (2017) also designed the prevalence of *S. enterica* in broiler chicken. Sixty-seven isolates of *Salmonella enterica* were obtained, which showed the presence of *S.* Typhimurium and *S.* Enteritidis[2]. The prevalence of *Salmonella* was also found high in different spices.

This study provides a good comparison between two different identification systems; i.e., phenotypic and genotypic methods, though it was limited till genotype level rather than to identify different serovars of *Salmonella*.

Conclusion

From this study, it is concluded that *Salmonella* is most prevalent in chicken meat, which may indicate the unhygienic slaughtering process or the insufficient general hygiene and cleaning. The study showed that the improvement in sanitation systems and the implementation of hygienic practices could lessen the contamination of *Salmonella* and eventually the incidence of human diseases.

Conflicts of Interest

The authors have declared that no competing interests exist.

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