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*Original paper*

# Prevalence of Salmonella spp. in chicken meat from Quetta retail outlets and typing through multiplex PCR

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## Abstract

A study was conducted to investigate the prevalence of Salmonella spp. in processed raw frozen and fresh poultry meat sold in retail markets and shops of Quetta, Pakistan. A total of 200 samples (100 each of fresh and frozen meat samples) were randomly collected from retailers during the period of March to September 2016. Out of 200 samples tested, 66 (33%) were found to be contaminated with Salmonella spp. The contamination rate of frozen samples was 30% as compared to 36% of fresh meat samples. Out of 66 Salmonella positive samples, the most prevalent sero-groups identified were S. enteritidis (43.9%) followed by S. typhimurium (30.3%), S. gallinarum (16.6%), S. pullorum (7.6%) and S. typhi (1.5%). Our findings highlight the magnitude of Salmonella contamination in chicken meat sold in the city of Quetta and demonstrate the efficacy of biochemical characterization and typing through multiplex PCR. The results indicate an urgent need for applying proper food hygiene practices in the Quetta region to reduce the incidence of foodborne diseases.

**Keywords** Chicken meat, contamination, multiplex PCR, *Salmonella*, Salmonellosis, serotyping

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## Introduction

Foodborne illnesses are a major public health problem throughout the world (HAVELAAR & al. [1]; KIRK & al. [2]; HOFFMANN & al. [3]; YANG & al. [4]). *Salmonella* spp. are a major foodborne pathogen causing illnesses and economic costs worldwide (AOUST & al. [5]; AHMED & al. [6]). The microorganism has been isolated from a variety of foods in nearly all countries in which it has been examined (RAJASHEKARA & al. [7]). Transmission of *Salmonella* to humans is usually by consumption of undercooked meat, poultry and dairy products and other cross-contaminated foods (SUMNER & al. [8]; CALLAWAY & al. [9]; FOLEY & al. [10]). In recent decades, poultry meat has emerged as a key source of proteins in daily rationing. It also leads to foodborne infections because of poor hygiene conditions during processing and has become one of the most investigated and understood sources of *Salmonella* contamination (MEAD [11]; GUNASEGARAN & al. [12]; CHAI & al. [13]). In developing countries, meat products have gained popularity because they represent quick easily prepared meals and allow the processors to convert the various types of meat into unified products. On the other hand, meat products are liable to harbour different types of microorganisms through a long chain of handling, processing, distribution and storage as well as preparation (AHMED [14]).

*Salmonella* is a Gram negative, non-spore-forming, usually motile, facultative anaerobic bacilli belong to the family *Enterobacteriaceae*. *Salmonella* spp. comprises thousands of serotypes and this genetic diversity gives them the ability to have a wide host range including mammals and birds as well as cold-blooded animals in addition to humans. The symptoms of Salmonellosis range from mild to severe and can be fatal if not treated successfully. Of further concern is that *Salmonella* serotypes are becoming resistant to commonly used and last-line antibiotics (ALCAINE & al. [15]; FOLEY & LYNNE [16]; LOU & al. [17]). It is therefore essential to have reliable, rapid and sensitive methods for detecting and classifying *Salmonella* spp. in foods and environmental sources and on surfaces.

Conventional culture techniques have been considered as the "Gold Standard" for isolation and documentation of foodborne bacterial pathogens, including *Salmonella* spp. (JASSON & al. [18]; BELL & al. [19]). They entail several steps including nonselective enrichment, selective enrichment, differential/selective plating and they are also used for morphological, biochemical and serological confirmation. The culture technique is rather sensitive and quite inexpensive, but also labour-intensive and laborious because it takes 3 working days at least for producing a negative result and around 5-10 working days for establishing positive results. Also, environmental factors are associated with variation in gene expression of microorganisms that might affect the results of biochemical tests. Furthermore, feasible but non-cultivable cells are not detected through conventional culture procedures (MALORNY & al. [20]). More rapid techniques have been developed for recognising *Salmonella* in foods such as electrical methods, nucleic acid probes and immunoassays (JENÍKOVÁ & al. [21]), but there are still problems with their sensitivity and specificity. PCR is a rapid technique that is highly sensitive and specific for detecting and identifying specific disease-causing bacteria from different food materials (HILL [22]; McKILLIP & DRAKE [23]) and has been successfully used for *Salmonella* spp. (MALKAWI [24]; DESAI & al. [25]; EL-TAWWAB & al. [26]; OSCAR [27]; RODRIGUEZ-LAZARO & al. [28]; DINH THANH & al. [29]; HYEON & DENG [30]).

The objective of this study was to analyse poultry meat from retail outlets in the city of Quetta, Pakistan for the prevalence of *Salmonella* spp. using conventional culture and biochemical methods as well as multiplex PCR.

## Materials and Methods

**Sample collection**

A total of 200 poultry (chicken) meat samples (100 each of processed/frozen and fresh meat) were randomly collected from retail markets and shops in Quetta, Pakistan during the period of March to August 2016. Samples were stored in sterilised plastic containers in an ice box and brought to the CASVAB Bacteriology laboratory, University of Balochistan (Quetta, Pakistan) for immediate processing.

**Isolation and identification of *Salmonella***

Approximately 25 g of meat was excised from each collected sample, minced and placed in Buffered Peptone Water (BPW-iso, Oxoid, UK) (225 mL) as pre-enrichment media, and incubated at 37 °C for 18 ± 2 hours. Aliquots from the pre-enrichment were inoculated into selective enrichment liquid media at a ratio of 1:10 in Selenite-Cysteine broth and incubated at 37 °C for 24 ± 2 hours. At the end of incubation a loopful of broth was streaked on to plates of *Salmonella-Shigella* agar (SS agar) and Xylose-Lysine-Deoxycholate agar (XLD agar, Oxoid, UK). The temperature and the period of incubation were standardised at 37 °C for 24 ± 2 hours, respectively. Suspected colonies of *Salmonella* (Fig. 1) from each plate were collected for presumptive identification by their morphological characteristics and biochemical tests. The primary tests included Gram's stain, catalase, oxidase, motility, Triple Sugar Iron agar (TSI agar), indole, methyl red, Voges-Proskauer, citrate utilisation test and sugar fermentation tests (Fig. 2).

**DNA extraction**

DNA extractions of enrichment broths were performed by the phenol-chloroform method as described by FADL & al. [31]. Briefly, aliquots (1 mL) were centrifuged (2,000

x g, 4 minutes) and the pellet was resuspended in 474 µL of TE buffer (10 mM Tris-HCL pH 8, 1 mM $Na_2EDTA$), 25 µL 10% SDS and 1.25 µL proteinase K (20 mg/mL). After incubation at 55 ºC for 30 minutes, 500 µL of phenol-chloroform pH 8 (1:1) was added, then the samples were mixed vigorously and centrifuged (10,000 x g, 4 minutes). The aqueous phase was transferred to a fresh microtube and DNA was precipitated with 3 M sodium acetate and ice-cold isopropanol for 30 minutes. Samples were centrifuged (16,000 x g, 10 minutes) and the pellet was washed with 80% ethanol. The final pellet was resuspended in 50 µL of TE buffer and the extracted DNA was quantified on a Shimadzu UV/VIS photometer before storage at 4ºC until PCR was performed.

**PCR conditions**

PCR was performed using the same conditions as those described previously for amplification of the following genes: *invA* (*Salmonella* spp.) (RAHN & al. [32]; OLIVEIRA & al. [33]), *fliC* (*S. typhimurium*) (SOUMET & al. [34]), *sefA* (*S. enteritidis*) (DORAN & al. [35]), *fliC* (*S. typhi*) (SONG & al. [36]; FRANKEL [37]), *rfbS* (*S. pullorum* and *S. gallinarum*) (LUK & al. [38]) (Table 1).

**Electrophoresis of PCR products**

Amplified DNA products from *Salmonella*-specific PCR were analysed by electrophoresis on agarose gels (2% w/v). PCR product (8 µL) was mixed with 6x loading dye (3 µL) and loaded on to the gel. A 1000 bp DNA ladder was used as a size marker for the PCR products (Fig. 3 and Fig. 4). Gels were run at 120 V, stained with ethidium bromide and visualized by UV illumination.

**Characterisation using Remel RapID ONE kits**

RapID ONE kits were also used to further identify *S. gallinarum* and *S. pullorum* species after PCR confirmation of the *rfbS* gene (Fig. 5).

**Fig. 1.** Typical *Salmonella* colonies on *Salmonella-Shigella* agar and Xylose-Lysine-Deoxycholate agar.


**Fig. 2.** Example of biochemical tests for the preliminary identification of *Salmonella* spp.

**Table 1.** PCR primers used in this study

| Gene | PCR primer 5'-3' | Product size | Target organism | Reference |
|---|---|---|---|---|
| *invA* (1) | F-GTGAAATTATCGCCACGTTCGGGCAA<br>R-TCATCGCACCGTCAAAGGAACC | 284 bp | *Salmonella* spp. | RAHN & al. [32]<br>OLIVEIRA & al. [33] |
| *sefA* (2) | F-GATACTGCTGAACGTAGAAGG<br>R- GCGTAAATCAGCATCTGCAGTAGC | 488 bp | *S. enteritidis* | DORAN & al. [35] |
| *fliC* (3) | F- ACTGCTAAAACCACTACT<br>R- TTAACGCAGTAAAGACAG | 495 bp | *S. typhi* | SONG & al. [36]<br>FRANKEL [37] |
| *fliC* (4) | F-CGGTGTTGCCCAGGTTGGTAAT<br>R-ACTGGTAAAGATGGCT | 620 bp | *S. typhimurium* | SOUMET & al. [34] |
| *rfbS* (5) | F-TCA CGA CTT ACATCC TAC<br>R-CTG CTATAT CAG CAC AAC | 720 bp | *S. pullorum* and *S. galinarum* | LUK & al. [38] |

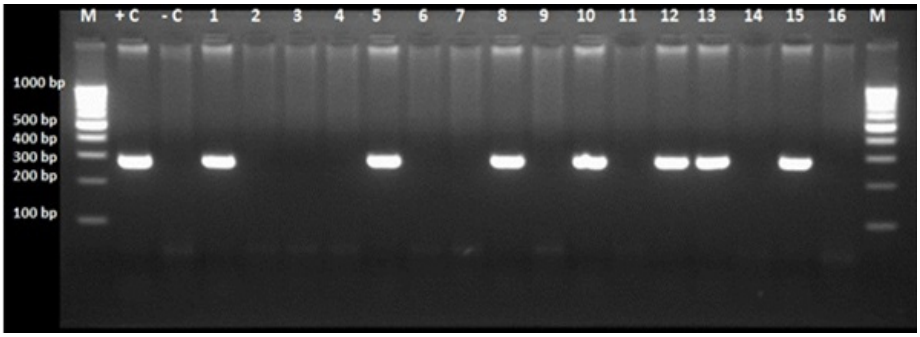


**Fig. 3.** Example of agarose gel (2%) electrophoresis showing PCR amplification of fragments of *Salmonella* spp. Lanes 1, 5, 8, 10, 12, 13 and 15 contain positive amplifications of *Salmonella* spp. (*invA*, 284 bp), +C: positive control, -C: negative control, M: bp markers.

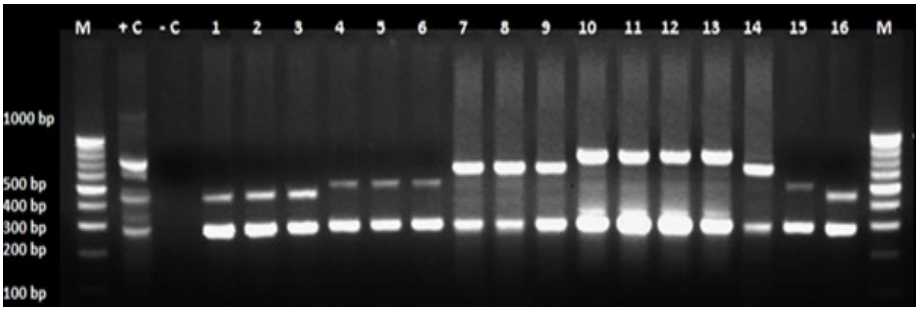


**Fig. 4.** Example of agarose gel (2%) electrophoresis showing PCR amplification of fragments of *Salmonella* spp. pathotypes. Lanes 1, 2, 3 and 16 contain positive amplifications of *S. enteritidis* (488 bp), Lanes 4, 5, 6 and 15 contain positive amplifications of *S. typhi* (495 bp), Lanes 7, 8 and 9 contain positive amplifications of *S. typhimurium* (620 bp), Lanes 10, 11, 12 and 13 contain positive amplifications of *S. gallinarum*/ *S. pullorum* (720 bp). +C: positive control, -C: negative control, M: bp markers.

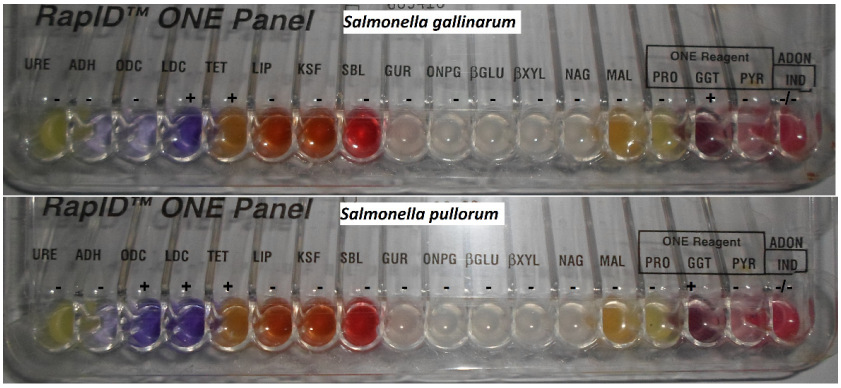


**Fig. 5.** Example of RapID ONE test kit results for identification of *Salmonella* spp.

**Table 2.** Detection of *Salmonella* spp. in processed raw frozen and fresh chicken meat samples

| No. of samples | *Salmonella* detected | *S. typhi* | *S. enteritidis* | *S. typhimurium* | *S. gallinarum* | *S. pullorum* |
|---|---|---|---|---|---|---|
| Frozen 100 | 30 samples | 1 (3.3%) | 13 (43.3%) | 9 (30.0%) | 4 (13.3%) | 3 (10.0%) |
| Fresh 100 | 36 samples | 0 (0%) | 16 (44.4%) | 11 (30.6%) | 7 (19.4%) | 2 (5.6%) |
| Overall 200 | 66 samples | 1 (1.5%) | 29 (43.9%) | 20 (30.3%) | 11 (16.7%) | 5 (7.6%) |

## Results and Discussion

A total of 200 poultry (chicken) meat samples (100 each of processed frozen and fresh poultry meat) were collected randomly from meat retail markets and butcher shops and tested for the prevalence of *Salmonella* spp. Samples were first analysed through presumptive identification methods including selective enrichment, selective/differential plating, microscopy and biochemical tests. For biochemical identification, the tests used were ONPG, oxidase, citrate utilisation, reaction on TSI, indole, MR/VP, urease, gelatinase, nitrate reduction, LDC, ODC and sugar fermentation tests (dulcitol, mannitol, rhamnose, sorbitol, glucose and arabinose) (Fig. 1 and Fig. 2). Presumptively identified samples were then subjected to PCR confirmation and typing through use of species-specific primers (Fig. 3 and Fig. 4). Both presumptive and PCR identification methods were found to be equally effective. For the typing of *S. pullorum* and *S. gallinarum*, *rfbS* gene-specific primers were used, which is common for both species. After positive amplification of the 720 bp fragment, the samples were further subjected to the RapID ONE identification system to differentiate between *S. pullorum* and *S. gallinarum* (Fig. 5).

The results showed that *Salmonella* prevalence was 30% in processed frozen poultry meat samples, of which *S. typhi* was 3.3%, *S. enteritidis* 43.3%, *S. typhimurium* 30.0%, *S. gallinarum* 13.3% and *S. pullorum* 10.0%. The prevalence of *Salmonella* species in fresh poultry meat samples was 36% of which *S. typhi* was 0%, *S. enteritidis* 44.4%, *S. typhimurium* 30.6%, *S. gallinarum* 19.4% and *S. pullorum* 5.6% (Table 2). The overall prevalence of *Salmonella* species in both processed and fresh samples was 33% of which *S. typhi* was 1.5%, *S. enteritidis* 43.9%, *S. typhimurium* 30.3%, *S. gallinarum* 16.7% and *S. pullorum* 7.6% (Table 2).

*Salmonella* is a leading pathogen producing food-borne outbreaks all over the world (AKTAS & al. [39]). Poultry are one of the foremost reservoirs of *Salmonella* that can be transmitted to humans through the food-chain. The most common serotypes isolated from humans are *S. typhimurium* and *S. enteritidis*. Traditional approaches for detecting *Salmonella* are based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests that can take up to 10 days to confirm the results (STONE & al. [40]). When a foodborne outbreak is suspected, it is important that the source of the pathogen is identified as soon as possible so that the public can recover confidence in the food supply

(BHAGWAT & LAUER [41]). The present study was therefore conducted to investigate the prevalence of *Salmonella* species in poultry meat samples collected from retail outlets in Quetta, Pakistan.

Detection of *Salmonella* spp. in food sources has been reported by studies all over the world. In Pakistan, SHAH & KOREJO [42] reported a 48.8% prevalence of *Salmonella* in chicken meat in Karachi of which *S. typhi* was 20.5%, *S. enteritidis* 48.7%, *S. typhimurium* 10.3% and *S. pullorum* 20.5%. Another study by SOOMRO & al. [43] in Hyderabad reported 38.0% of *Salmonella* in poultry meat of which *S. typhi* was 28.9%, *S. enteritidis* 42.1%, *S. typhimurium* 10.5% and *S. pullorum* 18.4%. Similar results for *Salmonella* contamination of food in Faisalabad have been reported (AKHTAR & al. [44]).

The relatively high prevalence (43.9%) of *S. enteritidis* observed in our present study is comparable to the situation described in most developing countries (BAILEY & al. [45]; CARDINALE & al. [46]). It appears that the presence of this pathogen in the intestinal tract of broilers can contaminate carcasses during slaughter and processing (ORJI & al. [47]). Similarly, the factors associated with *Salmonella* contamination of beef meat in Pakistan have been reported in detail previously (NAUMAN & al. [48]). The presence of *S. typhi* and *S. typhimurium* in poultry is of considerable importance from the standpoint of public health, whilst *S. pullorum* isolated in the present study indicates faecal contamination of carcasses (ORJI & al. [47]). The incidence of *Salmonella* in chicken meat may be a result of cross-contamination from intestines during processing and cutting or from cages, floors and workers during retailing or marketing. A 13% prevalence of *Salmonella* isolates from imported chicken carcasses in Bhutan was reported (ELLEBROEK & al. [49]), whilst there was a 25% prevalence of *Salmonella* in different types of meat including chicken in Thailand (MINAMI & al. [50]). These reports are all consistent with *Salmonella* being more prevalent in chicken or poultry than in other meats (ÁLVAREZ-FERNÁNDEZ & al. [51]). A 4.2% and 11.0% prevalence of *Salmonella* contamination in retail chicken meat was reported in similar studies from Washington, USA (ZHAO & al. [52]; MAZENGIA & al. [53]) and 4.0% and 5.1% from the UK and the Republic of Ireland, respectively (MELDRUM & WILSON [54]; MADDEN & al. [55]), whilst 29.3% was reported in Turkey (ARSLAN & EYI [56]), 34.3% in Guatemala (JARQUIN & al. [57]), 37.0% in Colombia (DONADO-GODOY & al. [58]) and 43.3% in China (YANG & al. [59]). Hence, the general trend is for a lower prevalence of *Salmonella* contamination in retail poultry from more developed countries and regions.

## Conclusions

This study clearly demonstrates the extent of *Salmonella* contamination in chicken meat available from retail outlets of Quetta, Pakistan. It is important that there is satisfactory consumer protection against *Salmonella*. To prevent zoonotic *Salmonella* serovars from entering the food chain, bacteriological monitoring of broiler groups and separation of infected groups from food production combined with an overview of good manufacturing practices and hygiene control must be applied. The high potential for spreading contamination of *Salmonella* in the poultry industry means that specific epidemiological studies at various levels of production are required on an ongoing and long-term basis. Another measure to be taken in the consumer part of the food chain is to apply sufficient heating treatments of chicken meat before consumption and to avoid too many manipulations of the meat before cooking.

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