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PCR based detection of *Salmonella* from fresh and processed chicken meat from Quetta, Pakistan

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Abstract

Salmonella is the most common cause of food borne infections worldwide. Approximately causing 16 million cases of typhoid fever, 1.3 million fatalities all around the world each year. The primary vehicle of transmission of the pathogen is Chicken meat which is largely consumed throughout the country. Due to lack of good hygiene and proper handling, pathogen is easily transmitted from meat to consumers causing diseases. The present study aims to investigate the prevalence of Salmonella in fresh and processed chicken meat through Polymerase Chain Reaction using Inv A gene as a genetic target. Total of 150 samples of raw chicken meat were collected in which 100 of freshly slaughtered samples were collected in pre-sterilized plastic bags and 50 of processed chicken samples were obtained in their packaging, from different shops and markets of Quetta. Out of 150 samples 37 (24.6%) were found *Salmonella* positive and 113 (75.4%) were negative. In raw chicken and processed chicken the contamination rate was found 26% and 22% respectively. The study revealed that many shops may not practices good hygiene which is making *Salmonella* a potential threat to consumer's health. To control the food-borne illnesses and to keep the microbial load of raw and processed meat in check, the food safety requirements should be followed strictly in accordance with HACCP (Hazard analysis critical control point)..

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Introduction

Food-borne diseases and food poisoning are most significantly affecting public health worldwide. It is estimated that one in three persons suffers from food-borne illnesses, 1.8 million deaths occur from severe food borne diarrhea, each year (Bhunia, 2008). Among food-borne pathogens, *Salmonella* is known to be the major cause of diseases in humans, causing 16 million cases of typhoid fever, 1.3 million fatalities all around the world each year (Barura *et al.*, 2013). *Salmonella* outbreaks have been related to various foods. Epidemiological studies report that poultry meat is still a primary cause of food poisoning (Ishola and Taiwo, 2014).

Poultry are the most important reservoir for *Salmonella*, with prevalence in chicken ranging from 20-70% in most countries (Dumen *et al.*, 2015). In last few decades, Pakistan has made great strides in the industry of poultry by producing 0.652 million tons of meat per year, constituting the 20 to 25% of the total meat production in the country (Somroo *et al.*, 2010). Raw meat may harbor many important pathogenic microbes including *Salmonella*, making the meat a significant risk for human health. Particularly chicken that is largely consumed throughout the world. Chicken consumption is mainly influenced by its nutritional content and its accessible price, implicated in many outbreaks of human Salmonellas (Bhunia, 2008; Ahmad *et al.*, 2013). Commercial poultry is one the fastest growing sectors that is advancing to reduce the prevalence of *Salmonella* contamination in Processes poultry (Foley *et al.*, 2011). Several studies have been conducted on the prevalence of *Salmonella* in processed poultry. Yet there is still less information given regarding the prevalence in processed chicken meat.

The contamination of *salmonella* was studied in processed cooked and uncooked by Dominguez and Schaffner (2009); Foley *et al* (2011); Moschonas *et al.* (2012) who found them associated with Salmonellas outbreaks because of not properly being cooked before consumption which makes it potentially dangerous for health.

Foley *et al* (2011) reported that fresh and processed poultry account for ~29% of all *Salmonella* infections in humans. *Samonella* is characterized by its wide host range that comprises most animal species including mammals, birds and cold-blooded animals in addition to humans, therefore, it has been isolated from a range of foods in almost every country (Somroo *et al.*, 2010), and it can be transmitted through the food chain, from feed to poultry meat and then to human causing localized or systematic infection, chronic asymptomatic carrier state and zoonotic disease such as Salmonellas (Shahzad *et al.*, 2012; Nader *et al.*, 2015).

Most of the *Salmonella* infections cause self-limiting diarrhea that do not need to be treated with antimicrobial drugs. However, in case of complications the first choice of drugs is fluoroquinolones and cephalosporin (Ziech *et al.*, 2015). Resistant strains have been reported against multidrugs such as ampiciline, gentamycin, trimthoprime-sulphame thaxazole (Adeyunji and Ishola, 2014). It is important to know the behavior of strains against antimicrobial agents (Ziech *et al.*, 2015).

In present days there is a great demand for the rapid detection of *Salmonella*. Among many techniques Polymerase Chain Reaction is a simple, rapid, very specific and inexpensive (Dumen *et al.*, 2015), compared to the conventional method involving the steps such as primary enrichment, selective enrichment, selective plating and biochemical confirmation which is very time consuming that takes 5 to 7 days. Although the culture base method is still gold standard technique has an advantage of detecting the viable bacterial cells offering an epidemiological advantage over the PCR that can detect even the dead cells (Koyumcu *et al.*, 2010). The present study aims to investigate the prevalence of Salmonella in fresh and processed chicken meat.

Materials and method

Sample collection

Total of 150 samples of raw chicken meat were collected in which 100 of freshly slaughtered samples were collected in pre-sterilized plastic bags and 50 of

processed chicken samples were obtained in their packaging, from different shops and markets of Quetta. Samples were brought to the post graduate laboratory of CASVAB, University of Balochistan, Quetta for further processing.

Preparation of samples

Frozen samples were thawed by overnight refrigeration before fur there processing (Hassaneien *et al*., 2011).

Isolation of Salmonella

For the analysis of samples, the technique recommended by the International Organization for Standardization (ISO) 6579 (2002) was adapted with some modifications. Briefly, 25g of each sample was homogenized in 225ml of buffered peptone water (BPW) (Oxoid, England), crushed in the stomacher bags and was incubated at 37°C for 18 to 20 hours. After incubation1ml pre-enrichment broth was added to 10ml of Rappaport-Vassiliadis (RV) broth (Oxoid, England) and was incubated at 42°C for 18 to 24h. Each selective enrichment broths were streaked onto xylose lysine deoxycholate (XLD) agar and salmonella shigella (SS) agar. A 10µl loop full spread on XLD agar, and SS plates and were incubated at 37 °C over night (18-24 hours). Suspected colonies of *Salmonella* from XLD and SS were then streaked onto nutrient agar and incubated for 24 hours at 37°C for biochemical conformational serotyping.

Identification of Salmonella

The initial identification step was done using Gram stain smears and Ready to use kit“ Rapid ID One system” was used for the confirmation of Salmonella following the manufacturer’s protocol.

Antibiotic Susceptibility Test

Antibiotic susceptibility test for isolates was evaluated using the Kirby-Bauer disc diffusion method. Each isolate was inoculated in brain heart infusion broth (BHI) separately and incubated for 24 hours at 37°C. The broth were streaked using sterile cotton swabs on Mueller-Hinton agar plates, Plates were kept at room temperature for 5 min, and then discs with antimicrobial drugs were placed on the plates and incubated for 24 hours at 37°C. The antibiotics discs (Oxoid, UK) used were ampicilline (10µg), gentamicin (10µg), kanamycin (30µg), to bramycin (10µg), amikacin (30µg), nalidixicacid (30µg), of loxacin (5µg), levofloxacine (5µg), chloramphenicol (30µg), tetracycline (30µg), oxytetracycline (3 µg), sulphamethox/trimethoprim (25µg).

DNA extraction for PCR

DNA was extracted through (cetyletrimethyle ammonium bromide) CTAB method as earlier described by Minas *et al*., 2011. Briefly, 1 ml broth of each culture was centrifuged at 6000 rpm, and pellet was dissolved in 400µl TE buffer, 70µl OF 10% SDS and 50µl proteinase K (10mg/ml) and kept in water bath at 60°C. Thawing and freezing was done by adding 100µl 5m Nacl and 100µl of 10% CTAB. 700µl phenol/chloroform/iso-amyle alcohol with a concentration of (25:24:1) was added to the tubes and were centrifuged on 12000 rpm. The upper most layers from the tubes was separated and dissolved in pre-cooled isopropanolol and centrifuged at 15000 rpm after keeping it in -20 C. The supernatants were discarded and pellet was washed with 500µl of 70% ethanol and 100µl TE buffer was added.

Primers and PCR amplification

For the detection of *Inv* A gene the specific sequence to *Salmonella* genus, were used this has been proved as a suitable PCR target with potential diagnostic applications (Oliveira *et al*., 2003). Extracted DNA was subjected to PCR using one set of oligonucleotide primer as shown in Table 1. The PCR was carried out by amplifying 284-pb fragment (Rahn *et al*., 1992). Reactions and thermal cyclerconditions were set as described by (Shanmugasamy *et al*., 2012), reaction with these was carried out in a 30µl amplification mixture consisting of 15µl of PCR Master mix (Gene All), 1µl of each primer (Macrogen), 10µl of Molecular grade water and 3µl of each extraction was used.

Amplification was performed in a gradient Thermocycler. An initial incubation at 94°C for 60 seconds followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 64°C for 30 seconds, elongation at 72°C for 30 seconds, and final extension period for 10 minutes at 72°C. A 50bp DNA ladder (Gene One) was used and deionized distilled water was used as a template for negative control.

Table 1. Sequences of Oligonucleotide-primers used for amplification of Inv A gene fragments and size of amplicon produced.

Primers	Sequence 5 to 3	Target Gene	Amplicon size
Sal-F	GTGAAATTATCGCCAC GTTCGGGCAA	*InvA*	284 bp
Sal-R	TCATCGCACCGTCAAA GGAACC	*InvA*	

Electrophoresis of PCR products

Amplified PCR products were then electrophoreses in 1.2% Agarose w/v gel stained with Ethidium bromide and was documented in gel documentation apparatus (Rahn *et al.*, 1992; Salehi *et al.*, 2015).

Results

Isolation and identification of Salmonella

Total of 150 chicken meat samples were collected out of which 37(24.6%) were found *Salmonella* positive and 113(75.4%) were negative as shown in Fig 1. In raw chicken and processed chicken the contamination rate was found 26% and 22% respectively, as shown in Fig. 2.

Isolates of chicken were examined as Gram negative, rod shaped. Biochemical results of Rapid ID one system are shown in Table 2. processed chicken the contamination rate was found 26% and 22% respectively, as shown in Fig. 2.

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Table 2. Results of Biochemical test for identification of *Salmonella by* using RapidI Done system.

Test code	Test result
URE	-
ADH	-
ODC	+
LDC	+
TET	+
LIP	-
KSF	-
SBL	+
GUR	-
ONPG	-
β GLU	-
β XLY	-
NAG	-
MAL	-
ADON	-
INDOL	-
PRO	-
GGT	+
PYR	-

Antibiotic Susceptibility test

The results for antibiotic susceptibility are given in Table 3.

PCR based detection of Salmonella

The PCR amplification of *Inv* A gene 284 bp fragments of samples was positive for the isolates, shown in Fig. 3.

Table 3. Antibiotic susceptibility test for *Salmonella* from chicken

Antimicrobial agent	Code	Conc. µg	*Salmonella* spp	
			Resistant	Sensitive (diameter of inhibition zone)
Ampicillin	AMP	10	Resistant	
Gentamycin	CN	10		Sensitive (15 mm)
Kanamycin	K	30		Sensitive (18 mm)
Amikacin	AK	30		Sensitive (18mm)
Tobramycin	TOB	10		Sensitive (15mm)
Chloramphenicol	C	30		Sensitive (25 mm)
Tetracycline	TE	30	Resistant	
Doxicycline	DO	30	Resistant	
Oxytetracycline	OT	30	Resistant	
Naidixic acid	NA	30	Resistant	
Ofloxacin	OFX	5		Sensitive (19 mm)
Levofloxacin	LEV	5		Sensitive (23 mm)
Sulphamethox/trimthoprim	SXT	25	Resistant	

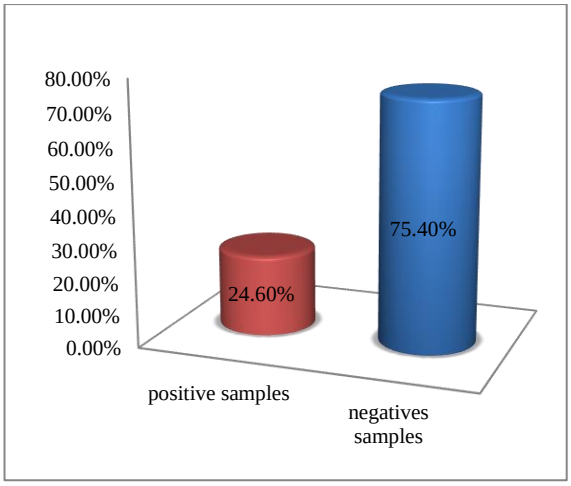

Fig. 1. Prevalence of *Salmonella* in chicken meat

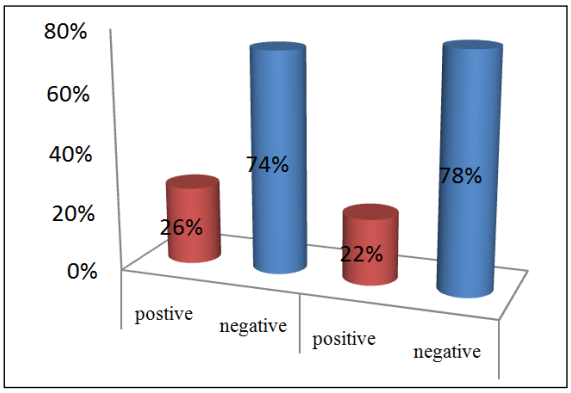

Fig. 2. Prevalence of *Salmonella* in fresh and processed chicken meat

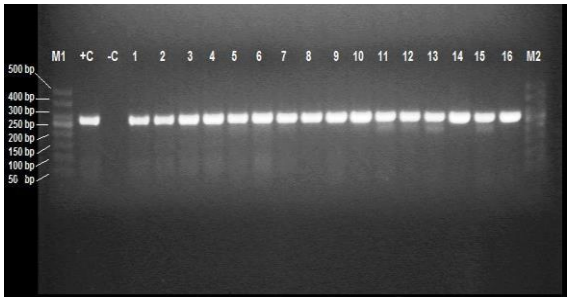

Fig. 3. PCR based identification of *Salmonella*

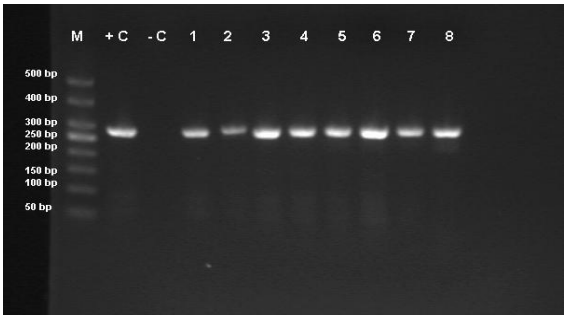

Fig. 4. PCR based identification of *Salmonella*

Discussion

The prevalence of *Salmonella* spp in raw chicken meat has been investigated in many countries including Pakistan in a range of 20 to 30%.The present study showed 26% contamination of the *Salmonella* in fresh and 22% in processed chicken meat. Difference *et al.* (2001) isolated Salmonella from fresh chicken and frozen chicken products 89% and 68% respectively in Netherlands, Dallal (2009) investigated 62.7% in Tehran, Zhu *et al* (2014) detected *Salmonella* from fresh and frozen stored poultry 28.3% and 33.5% respectively, in China, A 2016 study by Hassan *et al.* reported 76% contamination of *Salmonella* in broiler meat. Previous studies in different cities of Pakistan reported by Akhtar *et al* (2009) who reported 30% prevalence of *Salmonella* in poultry in Faisal abad. Mir *et al.*, (2010) reported 69.70% in Kashmir. Somroo *et al.* (2010) observed 38% contamination rate in poultry meat of retail markets of Sindh. A 2012 study by Shah and Kojeroob served 48.75% prevalence of *Salmonella* in Kharachi, which is higher than the prevalence in fresh and processed meat of current study.

Plummer (1995) observed 26.3% prevalence of *Salmonella*in fresh whole chicken and 26.7% in breast samples in UK which is in accordance to the current study. Adeyanjuand Ishola (2014) reported 33% of *Salmonella* in chicken obtained from retail markets that is higher than the current study and 22.6% obtained from processed meat that is in agreement with the findings of current study that showed 22% contamination in processed meat.

The current study showed the higher contamination rate than the studies by Salehi (2005) who reported 15.6%,Akbar and Anal (2013) investigated 5.26% prevalnec of *Salmonella* in thial and, Gharieb *et al.*, (2015) observed 10%, Dumen (2015) observed 15% prevalence in raw chicken, In a study conducted in Pakistan Sajid *et al.*, (2015) observed 8.04%. of *Salmonella* in chicken organs, which are lower than the current study.

In this study resistance was observed against ampicillin, tetracycline, nalidixic acid, doxycycline which is in agreement with the studies of (Li *et al*., 2014; Lu *et al*., 2014) and resistant against Sulfamethox/trimthoprime similar to the findings of (Li *etal*., 2014; Lu *etal*., 2014; Henery*etal*.,2015; El-Sharkawy *et al*., 2017).

The studies reported by (Somroo *et al*., 2010; Shah and kojero, 2012; Asif *et al*., 2016) in different cities of Pakistan exhibited resistant against ampicillin, tetracycline which is in correspondence to findings of present study. Shah and Kojero (2012) also found resistance against nalidixic acid. The sensitivity of isolates to chloremophenicol, amikacin in the present study was accord to the report of (Putturu *et al*., 2013).

In present study Gentamycin was found sensitive similar to the findings of (El-Sharkawy *et al*., 2017). The Results of the current study mostly correlate to the findings of Shah and Kojero (2012) who reported sensitivity against chlorempheniсol, of laxacin, amikacin, tobramycin and gentamycin. Levofloxacin was found sensitive in this study which is opposed to the finding of Asif *et al*. (2016); Shah and Kojero (2017), who reported resistance against levofloxacin

Variations in the results of current study and other studies might be due to sampling procedure, low hygiene measurements observed during slaughtering, processing mechanism and disinfection of processing lines, improper chilling and storage temperature. Other reasons could also be involved such as geographical, monthly and seasonal factors that prevail cross contamination (Zhu *et al*., 2014; Sajid *et al*., 2015).

Antimicrobial resistance against antimicrobial agents is an emerging problem in the world. The results ascribed by different studies could be due to use of low efficacy and frequently use of antibiotics in poultry and humans without proper prescription, which develops multidrug resistance to the *salmonella* (Hassan *et al*., 2016), which is a great public health problem, potentially affecting the medication efficacy in human (Lu *et al*., 2014).

There is a significant need of epidemiological surveillance of antimicrobial susceptibility to identify the alteration in resistance at different levels on regular basis (Putturu *et al*., 2013).

Conclusion

The study revealed that many shops may not practices good hygiene which is making *Salmonella* a potential threat to consumer's health. Maintenance of good hygiene practices in meat processing industries and slaughtering houses can reduce the chances of contamination. To control the food-borne illnesses and to keep the microbial load of raw and processed meat in check, the food safety requirements should be followed strictly in accordance with HACCP (Hazard analysis critical control point).

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