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Studies on Occurrence of Invasive *Salmonella* spp. from Unorganised Poultry Farm to Retail Chicken Meat Shops in Mumbai City, India

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A B S T R A C T

The present study was planned to estimate occurrence of invasive *Salmonella* spp. in retail chicken supply chain of Mumbai and quantifying *Salmonella* at crucial stages of processing by the Most Probable Number (MPN) and confirmation by *invA* gene by PCR assay. A total of 18(n = 108) farm samples were found to be positive for *Salmonella* with prevalence of 16.66% and statistical significance was observed amongst different sources at farm (p=0.027). Highest prevalence of *Salmonella* spp. was noticed in litter samples (50.00%) followed by cloacal swabs (25.00%), water utensil swabs (25.00%), faeces (16.66%), water (16.66%), wall dust (8.33%) and worker hand (8.33%). Over all prevalence of *Salmonella* spp. amongst various samples in retail shop was found to be 19.04 %. Out of 42 different chicken retail shop samples analysed03 (7.14 %) swab samples of chopping board found positive while one sample each from water and swab samples of worker hand, platform, knife, and cloaca were found positive. Amongst 24 swab samples collected from chicken carcasses at various chicken processing stages, highest rate of contamination (50 %) was observed in post defeathering and post evisceration stages of processing with average count of 1.88 and 2.11, log MPN count/10cm^2 respectively. Out of 34 *Salmonella* isolates obtained in this study, 31 isolates showed positive amplification of 284 bp fragment specific for the *invA* gene with 91.17% detection level. Thus, study revealed that poultry litter at farm and post defeathering and post evisceration stages at retail chicken processing, are critical sources of cross contamination of invasive *Salmonella* spp.

Introduction

Salmonella serotypes are significant zoonotic pathogens and cause a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia in human and animals (Winokur *et al*., 2000 and Bennasar *et al*., 2000). Human salmonellosis is frequently associated with the consumption of poultry products (CDC, 2008; Hanning *et al*., 2009; Kang *et al*., 2009 and Pires *et al*., 2012). Contaminated poultry products are among the important sources for food-borne outbreaks in humans and *Salmonella* are isolated more often from poultry and poultry products (Habtamu *et al*., 2011; Kabir, 2010 and Linam and Gerber, 2007). As Salmonellosis is one of the most important foodborne diseases, few countries have a surveillance system that estimates the burden of salmonellosis in human populations (Flint *et al*., 2007 and WHO, 2005). Risk factors for colonization by *Salmonella* include season, hatchery of origin, feed mills, litter, water and

various hygienic measures (Rose *et al*., 1999; Skov *et al*., 1999; Cardinale *et al*., 2004). Bryan and Doyle (1995) stated that commercially reared birds are in constant contact with litter and dust, both of which can be a source of contamination. Barnes, (1972) also mentioned that *Salmonella* contamination of birds may occur before, during or after the grow-out phase of production.

Indian broiler production has been growing, with an annual growth rate of 11.44 percent, production of 3.725 million tons (Index Mundi, 2015). In India, chicken is slaughtered at both industrial as well as at retail level but 95% chicken is slaughtered at retail level, while the remaining is slaughtered at industrial level (Badhe *et al*., 2013).Probability of cross contamination of raw chicken at retail level shops increases due to poor slaughtering practices, poor personal hygiene and poor cleaning. In India common sanitary problems that occur during the slaughtering and handling of poultry are hygienic condition and cleanliness of contact surfaces. Different genes like *Inv*, *Spv*, and *Stn* have been identified as major virulence genes responsible for pathogenic salmonellosis, the chromosomally located invasion gene *invA* being thought to trigger the invasion of *Salmonella*e into cultured epithelial cells (Asten and Dijk, 2005). Therefore it is important to evaluate the food safety risks because of pathogenic *Salmonella* along the production and retail processing and identification of effective control points or control strategies on the farm and at retail level chicken meat.

The present study was conducted to estimate occurrence of invasive *Salmonella* spp. in poultry farm environment of unorganized non- integrated broiler farms and six chicken retail shops in Mumbai using cultural isolation and enumeration by the Miniature Most Probable Number (MPN) method at stages of retail chicken meat production. Positive isolates were confirmed by amplifying *invA* gene which is unique to this genus and has been proved to be a suitable PCR target with a potential diagnostic application.

Materials & Methods

Sample Collection

Sample collection from poultry farm: A total of 108 different samples were collected from randomly selected 12 unorganised non-integrated poultry farms with capacity of 1500 -2000 birds, aged between 35 to 42 days and supplying birds to retail chicken meat shops located in vicinity of Mumbai. Samples includes cloacal swab from the poultry birds, feed, drinking water, litter sample from poultry house, fresh feaces and pooled swab from hands of the personnel working in the houses, wall dust, feeder and drinker.

Sample collection from retail chicken shops: A total of 66 stage wise post processing breast swabs samples of 10cm^2 area (post bleeding, post scalding, post defeathering and post evisceration), neck skin of carcass before and after evisceration, environmental samples (washing water, scalding water and carcass contact surfaces) and cloacal swabs were collected from six chicken processing establishments identified as retails chicken shops

Swab samples from 10cm^2 area were collected aseptically as per the standard methods described by (Gill and Jones, 2005).

Cultivation and isolation of *Salmonella* spp.

Qualitative evaluation: Isolation of *Salmonella* spp. from various samples collected was carried out as per ISO 6579. In brief pre-enrichment of the collected samples

in Buffered Peptone Water as 1:10 dilution and then incubated aerobically at 37ºC for 18 hours. 0.1 ml inoculum was transferred to a tube containing 10 ml of the Rappaport Vassiliadis Soy broth and then incubated at 41.5ºC for 24 hours. From the enrichment culture, 10 µl inoculum was further inoculated onto the surface of Xylose Lysine Deoxycholate (XLD)and Brilliant Green Agar (BGA) plates then incubated at 37ºC for 24 hours. The plates containing characteristic colonies of *Salmonella* appearing as smooth colonies with black centre on XLD and red to pink on BGA were selected and the gram staining was performed. Colonies showing typical Gram negative, non spore forming short rod shaped appearance were further subjected to biochemical characterization with biochemically negative for hydrolysis of urea, positive for TSI with alkaline slant (red), acid butt (yellow) with H_2S gas production and positive citrate utilization considered as positive for *Salmonella* spp.

Quantitative evaluation by miniature MPN technique

Pre enriched swab samples and neck samples of poultry carcass collected at stages viz, before and after evisceration, were subjected for quantitative miniaturized most probable number described by Pavic *et al.*, (2009), based on ISO 6579-2002. The swab sample suspension 1 ml of a 10^{-1} dilution was pipetted into an U-bottomed 96 deep well plates (Genexy scientific, India). Serial decimal dilutions (100: 900 µl) were performed in BPW using a micropipette to the previously described final dilutions of 10^{-6} in a labelled 96 well U bottomed plates. All tubes were mixed by repeated aspiration. From each of the dilutions in the plasma tubes, 100 µl aliquots were transferred into each of three wells (i.e. A1 to A3) across a another U-bottomed 96 deep well plates with each dilution in a subsequent row (i.e. 10^{-1} in row A1–A3, 10^{-2} in row B1 to B3 to a theoretical maximum dilution of 10^{-6} in row F1–F3), producing a 3-tube MPN. The plate was then covered with adhesive paraffin wax film and incubated (37^0C for24 h). From each post incubated well, the total volume was transferred to a corresponding U-bottomed 96 deep well plates containing 500 µl MSRV and then incubated (42^0C for 24 h).

White colour change from blue to colourless in a tube was considered as a presumptive positive for the presence of *Salmonella*, with all tubes (regardless of colour development) being confirmed by subculturing onto XLD agar (37^0C for 24 h). Following incubation, typical colonies were subcultured onto nutrient agar (37^0C for 24 h) and confirmed by biochemical test and molecular characterization by PCR assay. The combination of positive and negative results yielded a MPN data set.

MPN values were calculated using MPN data by Thomas' equation in MS EXCEL data sheet developed by Division of Mathematics in FDA/CFSAN (Blodgett, 2006).

Molecular characterization of isolated strains using Polymerase chain reaction (PCR) assay

Genomic DNA of *Salmonella* spp. was extracted as per the protocol of Rawool *et al.*, (2007). Primers for *Salmonella* organism was used according Rahn *et al.*, (1992) for *invA* gene. Sequence of forward primer (*invA*) was GTGAAATTATCGCCACGTTCGGGCA A) and reverse primer was TCATCGCACCGTCAAAGGAACC). DNA samples were amplified in a total of 25 µl as the following: 2.5µl of 10x PCR Buffer, 1.5 µldNTP Mix (10mM), 2µl $MgCl_2$ (50mM) 1.25µl of forward primer, 1.25µl of reverse primer, 0.50µl *Taq* polymerase (500U) 14.0µl of PCR grade water and 2 µl of the template.

The PCR was performed under the using conditions of primary denaturation: 94˚C / 2 min., secondary denaturation: 94˚C / 30 sec., annealing: 65˚C / 1 min., extension: 72˚C / 2 min., No. of cycles: 30 and final extension: 72˚C / 5 min. Aliquots of amplified PCR products were electrophoresed in 1.5% agarose gel. The samples and a 100 bp DNA ladder were loaded in the wells in amount of 7µl of sample. A current of 90 V for 1 hour was passed on the horizontal electrophoresis unit. Specific amplicons were observed under ultraviolet transillumination compared with the marker. The gel was photographed by a gel documentation system and the data were analyzed.

Results and Discussion

Prevalence of *Salmonella* spp. from farm samples

Study revealed that 12 (16.66%) out of 108 samples were positive for *Salmonella* spp. in the environment of poultry farms located in vicinity of Mumbai city (Table: 01).There is significant difference (p=0.02) between sources in the farm and *Salmonella* occurrence. Results are comparable with study of Ahmed *et al*., (2014) who reported 11.1% of prevalence of *Salmonella* spp in the environment of broiler poultry farms of Khartoum, Sudan. Also Al-Zenki *et al*., (2007) who reported 5.4% prevalence from farm samples collected in Kuwait, this may be attributed hygienic measures applied. Kumar *et al*., (2014) in India reported 0%and15.6% prevalence of *Salmonella* under intensive production system and free-range system, respectively.

This study showed that 6(50%) *Salmonella* spp. were isolated from litter. *Salmonella* from litter can lead to heavy contamination of the bird's feathers and feet which increases the probability to recover the organism from carcasses in poultry processing plants due to fecal shedding onto the litter (Trampel *et al*., 2000). Results are in agreement with Scur *et al*., (2014) who observed 61.9 % prevalence of *Salmonella* spp. from litter samples. This study showed that there was a negative detection for *Salmonella* spp from feed and feeder swabs which confirm that use of heat treated feed material and proper storage conditions. Presence of *Salmonella* spp. in Drinker swabs (25.00%) and drinking water (16.66%) confirm that *Salmonella*e may originate either from faeces/litter or from water already contaminated by pathogenic organisms. The result pertaining to feed and water are opposite to the report of Alali *et al*., (2010) who has reported 27.5% and 0.00% prevalence of *Salmonella* from feed and water samples of conventional farms, respectively. El Hussein *et al*., (2010) who reported 7.23% prevalence from poultry drinking water which may be attributed to the variation in the numbers of collected samples.

Positive cloacal swabs (25.00%) and faeces (16.66%) indicate current infection in the flocks which is attributed to horizontal transmission from poultry environment. Dust in the poultry houses in large amount may also be a hazard, since dust has been recognized as a vehicle of transmission of *Salmonella* when large numbers of organisms are present (Harbaugh *et al*., 2006) a positive wall dust swab (8.33%) in our study confirms the same. The present result supports the report of Musa *et al*., (2014) and Corrique and Davies (2008) who reported that faeces/litter and dusts are the matrices of choice for *Salmonella* isolation and sources of cross contamination. This study also revealed that 01(8.3%) hand swabs was positive for *Salmonella* which confirms cross contamination. Similarly Ahmed *et al*., (2014) reported 01(5.6%) hand swab was positive for *Salmonella*.

The results are in agreement with Abunna *et al.*, (2016), Marin *et al*., (2011) and AL-Iedani *et al*., (2014) who recovered *Salmonella* from various environmental samples mentioned in our study.Horizontal transmission can occur by direct bird-to-bird contact, ingestion of contaminated feces or litter, contaminated water, personnel, farm and personal equipment, and a variety of other sources (Nakamura *et al*., 1997; Nakamura *et al*., 1994 and Lahellec and Colin, 1985).

Variation in prevalence were reported by Agada *et al*., (2014) 10.9% in Nigeria, Al-Abadi and Al-Mayah, (2012) 9.2% in Iraq and Jahan *et al*., (2012) 45% in Bangladesh. Abbuna *et al*., (2016) stated that differences in prevalence might be due to the difference in study design, isolation technique, different in sample type and difference in geographical location.

Prevalence of *Salmonella* spp. at chicken retail shop

Over all 42 samples comprising of washing water, scalding water, swabs of worker hand, platform, chopping board, knife, and cloacal swab were analysed for *Salmonella* spp. Out of 42 samples 08 (19.04%) samples found positive. Surprisingly in used scalding water samples were negative, that might be because of high temperature of water. Out of individual 6 samples of all category 03 (7.14 %) swab samples of chopping board found positive while one sample each from water and swab samples of worker hand, platform, knife, and cloaca were found positive. Olayinka, and Adeyanju (2014) reported 23.8, 11.90 and 0.00 per cent occurrence of *Salmonella* spp. from knives, weighing scales and wooden tables, respectively. Costerton *et al*., (1999) stated that *Salmonella* spp. noted as common contaminants of equipment used in processing of meat which are able to produce biofilm. Thiruppathi *et al*., (2004) observed *Salmonella* cross-contamination in retail chicken outlets in Chopping boards at (18.75%) and the butcher's hands (14.29%) followed by knives and the weighing balance tray. Study conducted by Ali *et al.,* (2010) to find out microbial contamination of raw meat and its environment in retail shops in Karachi, Pakistan and reported 29% distribution of *Salmonella* in meat samples but zero detection of *Salmonella* from meat cutting surfaces (knives, wooden boards, weigh scales and meat mincers) and environmental surface swabs. Higher occurrence of *Salmonella* spp. in retail chicken shop is may be due lack of adherence to good hygienic practices and poor management practices on the farms.

A total of 24 swab samples were collected from chicken carcass at various chicken processing stages at retail shop along with 6 neck samples of eviscerated carcasses. Statistically non-significant difference was observed amongst different processing stages (p=0.43). Highest rate of contamination (50 %) was observed in post defeathering and post evisceration stages of processing. While only one swab samples post bleeding and post scalding stages were positive. Out 06 post eviscerated carcass neck skin samples two samples were found positive, being at the lowest point in terms of gravity, neck skin may accumulate bacterial particles from run-off from washing (Table No: 2).The handling and processing of retail chicken needsto be improved to reduce the *Salmonella* incidence level in these stages along with washing of carcasses before and after evisceration. In similar study conducted by Morris and Wells (1970) at processing plants noticed 13.2% and 7% level of contamination after picking and after evisceration, respectively. Difference in the occurrence might be due to mechanical and non-mechanical processing operations. The level of *Salmonella* in live birds brought

for slaughter in retail markets might be very low but during processing under unhygienic stages carcasses were contamination by environmental sources. The previous studies as reported by other researchers only focused on the prevalence of *Salmonella* in chicken carcasses or chicken cuts and environment. This could explain the new way of detecting cross contamination of *Salmonella* which focused on the occurrence of *Salmonella* at different stages of retail chicken processing.

Quantification of *Salmonella* spp. by miniature MPN technique

As defeathering and evisceration are the major site of cross-contamination in poultry processing (Notermans *et al*., 1980; Clouser *et al*., 1995), each of 6 Samples at post defeathering and post evisceration stages of chicken processing were collected and subjected for quantification of *Salmonella* spp. by miniature MPN technique. Three samples each from post defeathering and post evisceration stages were positive with average log MPN count/10cm^2 of 1.88 and 2.11, respectively (Table :03). Shashidhar *et al.*, (2011) observed *Salmonell a* load in the range of 1.30 to 120 MPN/g in the retail chicken sample similarly Straver *et al.*, (2007) have reported that the number of *Salmonella* on chicken filets varied from 1 to 3.81 log MPN per filets. Very little work has been done in India on the amount of this organism present on the carcasses during the processing stages. This is first attempt in India to quantify the *Salmonella* during chicken processing stages using miniature MPN method.

Detection of invasive gene of virulent *Salmonella* (*invA*) using polymerase chain reaction (PCR)

Out of 34 *Salmonella* isolates obtained from in this study 31 isolates showed positive amplification of 284 bp fragment specific for the *invA* gene (common gene) from examined samples with 91.17% detection level (fig. 1).

Table.1 Occurrence of *Salmonella* spp. isolated from poultry farm environment and other Samples

Sr. No	Type of Sample	No of Samples Collected	No of Positive Samples	Per cent Prevalence
1	Cloacal Swab	12	3	25.00
2	Feed	12	0	0.00
3	Drinking Water	12	2	16.66
4	Litter	12	6	50.00
5	Faeces	12	2	16.66
6	Worker Hand Swab	12	1	8.33
7	Wall Dust Swab	12	1	8.33
8	FeederSwab	12	0	0.00
9	DrinkerSwab	12	3	25.00
	Total	108	18	16.66

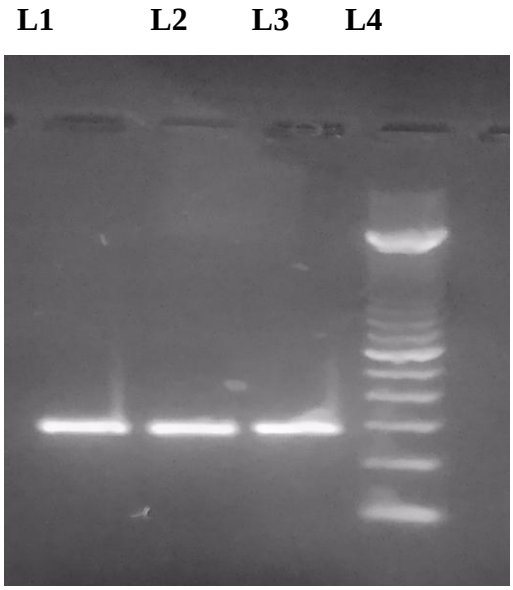
(Poultry environmental samples p = 0.02)

Table.2 Occurrence of *Salmonella* Spp. isolated from retail chicken processing shop

Sr. No.	Sample Source	Number of Samples Collected	*Salmonella* Positive Samples
A) Environmental and other samples			
1	Washing Water	6	1(2.3)
2	Scalding water	6	0
3	Worker Hand	6	1 (2.3)
4	Carcass Contact Platform	6	1 (2.3)
5	Chopping Board	6	3 (7.14)
6	Knife Swab	6	1 (2.3)
7	Cloacal Swabs	6	1 (2.3)
		42	**08 (19.04)**
B) Sampling at different Processing Stages			
8	Post Bleeding	6	1(16.66)
9	Post Scalding	6	1 (16.66)
10	Post Defeathering	6	3 (50.00)
11	Post Evisceration	6	3 (50.00)
12	Neck Sample of Post eviscerated carcass	6	2 (33.33)
		30	**10 (33.33)**

(Processing stages $p = 0.43$)

Fig.1 PCR products of 284 bp DNA fragment of *Salmonella* isolates



Lane1 and 2: 284 bp PCR products of *Salmonella* isolates recovered from poultry farm and chicken retail shop samples; Lane 3: Standard *Salmonella* Typhimurium (MTCC 3224); Lane 4 (M): 100bp DNA Ladder

Table.3 Quantification of *Salmonella* spp. in pre and post evisceration swab samples in chicken processing

Sr. No	Processing Stage	Samples	Number of Positive samples	Average log MPN Count/10cm^2
1	Post Defeathering	6	3	1.88
2	Post Evisceration	6	3	2.11
	Total	12	06	

Results are in agreement with Ohtsuka *et al*., (2005) who reported 90% detection of *Salmonella* by PCR, whereas less than Salehi *et al.*, (2005), Ozbey and Ertas (2006) and Samaxa *et al*., (2012) who reported 100% detection of *Salmonella* spp. by PCR. This may be attributed to variation due to targeting different genes and the concentration of DNA template added to the PCR reactions

In conclusion, this study revealed that the prevalence rate of *Salmonella* spp. in farms and chicken retail shops in Mumbai were 16.66% and 19.04% respectively. Highest prevalence of *Salmonella* spp. was noticed in litter samples followed by cloacal swabs and drinker swabs which would be considered as risk factors for cross contamination at farm level. Post defeathering and Post evisceration stages of processing are found critical stages of retail processing. Higher prevalence rate could be attributed to lack of adherence to good hygienic practices and poor management practices on the farms and retail chicken shop. Application of hygienic measures during farm management and processing stages may reduce the risk of Salmonellosis in human. Detection of the *invA* gene from isolated strains has revealed high risk of exposure to pathogenic strains of *Salmonella* spp. Data obtained in the study can be guide for the development of quantitative risk assessment models in chicken meat processing. The adoption of improved technology and strict hygiene measures can often reduce the risk of contamination of carcasses

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