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Quantifying the *Salmonella* spp. at critical stages of poultry processing by miniature MPN techniques (mMPN)

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Abstract
In India automated, semi-automated and retails shop poultry slaughter units have different sets of practices. These various processing practices could have a significant effect on the microbial quality of the poultry meat. In poultry processing defeathering, evisceration and chlorine wash stages has considered as critical stages for control of *Salmonella* contamination. In this view present study aims quantification of *Salmonella* at critical stages of poultry processing with a different set of practices. A total of 48 swab samples were collected from critical stages of poultry processing for quantification of *Salmonella* spp.. Samples were subjected for *Salmonella* quantification by miniaturized most probable number (mMPN) based on ISO 6579-2002. Positive samples were confirmed by biochemical tests and PCR (*invA* gene) method. Twelve of the 48 (25%) samples were positive for *Salmonella* at three distinct sampling points. The range of *Salmonella* count in terms of MPN index/10cm^2 (Log MPN Count/10cm^2) of post-defeathering samples from retail shop and semi-automated processing unit were 7.33 -14.18 (0.865 – 1.17 log) and 3.05 -7.33 (0.484 – 0.865 log), respectively, whereas of post-evisceration were 19.81 – 45.36 (1.297 – 1.657 log) and 11.02- 15.04 (1.042 – 1.17 log), respectively. One sample at post chlorination stage [3.59 (0.555 log)] and post evisceration stage [19.81 (1.297 log)] were found positive at semi-automated and automated processing unit, respectively. Higher occurrence of *Salmonella* was observed at defeathering and evisceration stages of poultry processing. Incorporation of appropriate controlled chlorination stage after evisceration in retail shop and semi-automated poultry processing could be useful for the reduction of *Salmonella* load on carcasses.

Keywords: *Salmonella* quantification. mMPN techniques, defeathering, evisceration, chlorination

1. Introduction
Microbiological risk factors are so prevailing that they can be found in almost all systems of poultry production [1], if products are improperly treated while handling, cooking or post cooking and storage. *Salmonella* has been pathogen of significance, and is a major cause of gastroenteritis in humans [2, 3]. Poultry and poultry products are known reservoirs for these foodborne pathogens, and numerous reports described the prevalence of *Salmonella* associated with live poultry, production environments and processing plants [4]. *Salmonella* illness has linked with exposure to meat, a review of the Centers for Disease Control and Prevention (CDC) outbreak data from 2006 to 2011 indicated that 10 out of 25 outbreaks were related to live poultry, shell eggs, or further processed poultry products [5].
In India various types of poultry slaughter units are available which have different sets of practices. These are automated (slaughter > 6,000 birds/hour), semi-automated (slaughter 1,000-5,000 birds/hour) and retail shop (200-500 birds/day) poultry slaughterhouses [6, 7]. Initial two are equipped and capable of machine processing. The birds are hung upside down by their feet in the shackles on a conveyor and moved for slaughter process. Slaughtering can be performed manually and the birds are hold for to complete bleeding. The birds are exposed to hot water in the temperature controlled scalding tank machine (50-65 °C) and adding a continuous counter-current flow of water. Then feathers are removed with a plucking machine. In automated type of slaughterhouses, evisceration is done mechanically. In semi-automated operations, evisceration is manually. After bird washing, the carcasses are cooled down to or below 4 °C by immersion chilling by screw chiller. In retail shops poultry processing operations [6], slaughter is carried out manually using simple processing equipment. Scalding was done by dipping birds into scalding tank without temperature control while,

defeathering and evisceration was done usually by hand on table. These various processing practices have a significant effect on the microbial quality of the poultry meat.

In poultry processing defeathering and evisceration has always been considered a significant source of carcass contamination and also major points of cross-contamination [8, 9]. Hazard characterization and exposure assessment with quantification of initial number of pathogens is essential for integration of quantitative microbial risk assessment (QMRA) with HACCP [10, 11]. These quantitative data are important and used to build the growth models in various food-processing conditions [11]. Despite the literature available that implicates the post-deferathering and post-evisceration as a significant cross contamination site, the potential danger posed by these stages cannot be adequately evaluated because the samples in the previously mentioned studies tested only for the presence or absence of *Salmonella.*

Despite the large amount of research done on *Salmonella*, considering the final product prevalence but very little work has been done considering quantification of *Salmonella* spp. Therefore, this study aims quantification of *Salmonella* at critical stages of poultry processing with a different set of practices.

2. Materials and Methods

2.1 Sample collection

A total of 48 swab samples comprising six samples each of the post-defeathering, post-evisceration stages and post chlorine wash were collected from the processing unit namely retail shop, semi-automated and automated processing unit located in and around Mumbai city for *Salmonella* quantification and identification. In retail shops processing practices post chlorination process has not undertaken therefore, the samples were not collected. All the samples were collected aseptically. The swab samples were collected using sterile 10 x 10 cm steel frame to expose the area of 100 cm and placed in the 9 ml of Buffer Peptone Water (BPW) soon after collection and brought to the laboratory under refrigeration conditions.

2.2 Quantification of *Salmonella* spp. by Miniaturized Most Probable Number (mMPN)

Pre-enriched swab samples of poultry carcass collected at different stages *viz*, post de-feathering, post evisceration and post chlorination were subjected for quantitative miniaturized most probable number described by Pavic *et. al.*, [12], based on ISO 6579-2002. The test matrix suspension (1 ml of a 100 or 10^{-1} dilution) was pipetted into an empty well. Serial decimal dilutions (100: 900 µl) were performed in BPW using a micropipette to the previously described final dilutions in a labelled 96 well poly plate. All tubes were mixed by repeated aspiration. From each of the dilutions in the well, 100 µl aliquots were transferred into each of three wells (i.e. A1 to A3) across a V-bottomed 72 well poly plate 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 an adhesive paraffin wax film and incubated at 37^0C for 24 h. From each post incubated well, the total volume was transferred to a corresponding well in a plate containing 500 µl Modified Semi-solid Rappaport Vassiliadis broth (MSRV) and then incubated at 42^0C for 24 h. The detailed schematic presentation is given in Figure No 01. Change in colour of MSRV from blue to colourless indicative of positive for *Salmonella* spp.

White colour change from blue to colourless in a well was deemed as a presumptive positive for the presence of *Salmonella* spp., (Figure No 02) with all wells (regardless of colour development) being confirmed by subculturing onto BGSA agar at 37 ^{0}C for 24 h. Following incubation, typical colonies were subcultured onto nutrient agar at 37 ^{0}C for 24 h and confirmed by Biochemical tests and PCR method [13]. The combination of positive and negative results yielded a MPN data set. Those wells in which the isolation of *Salmonella* spp. was confirmed by the biochemical tests and PCR were regarded as positive. The MPN per mL and the lower and upper bounds of the 95% confidence interval (95% CI) were calculated using MPN data of Thomas' equation in MS EXCEL data sheet developed by Division of Mathematics in FDA/CFSAN [14].

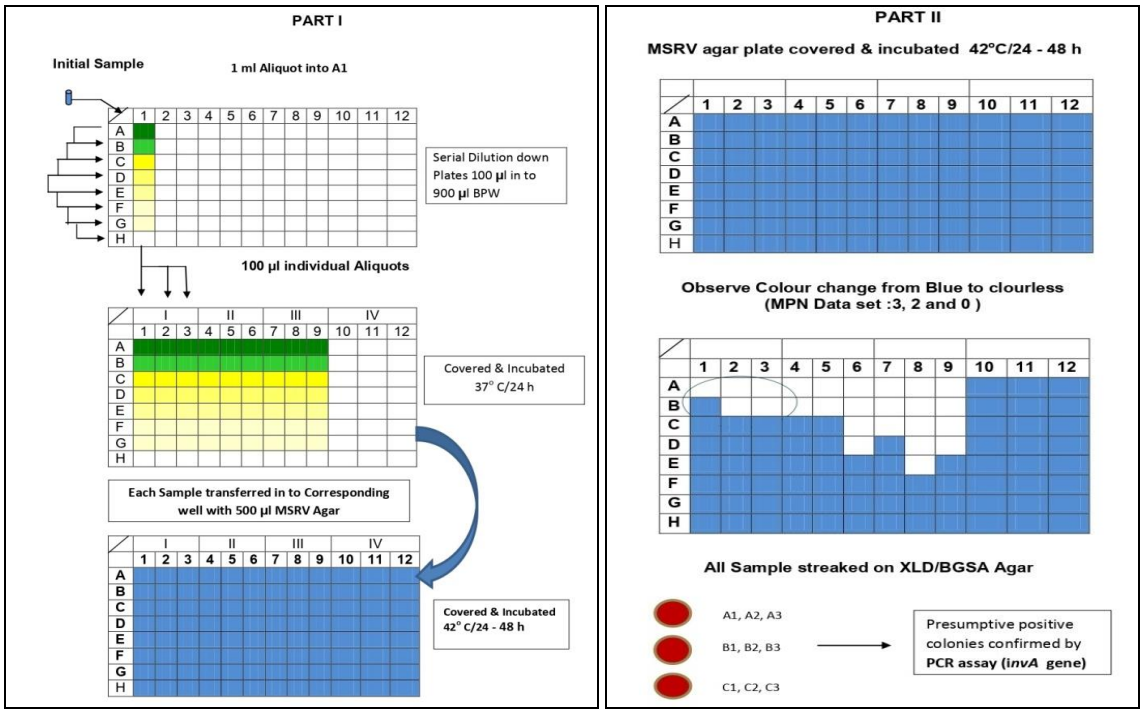

Fig 1: Schematic Presentation of Miniature Most Probable Numbers (mMPN) Methods

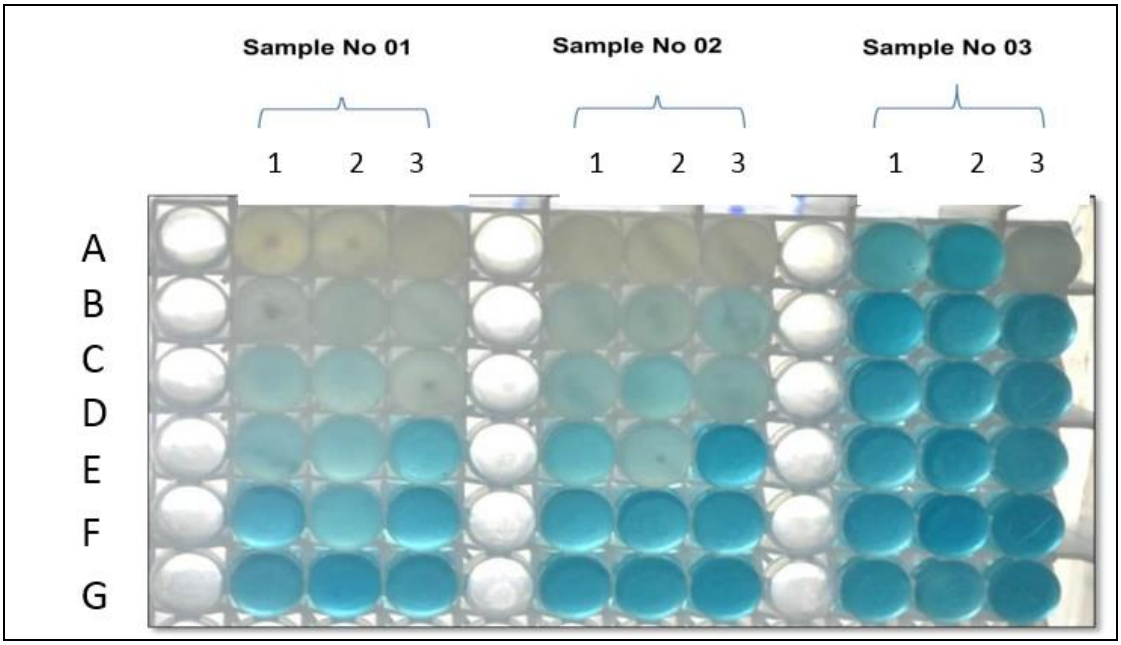

Fig 2: 2 ml well poly plate showing MSRV colour change from blue to colourless considered as a presumptive positive for the presence of *Salmonella* spp.

3. Results and Discussion

Twelve of the 48 (25%) samples evaluated by mMPN were positive for *Salmonella* at three distinct sampling points of retail shop, semi-automated and automated processing units. The results are depicted in Table 01. Higher occurrence rate was observed in post-defeathering and post evisceration. Amongst six samples, each of post-defeathering stages from a retail shop, semi-automated and automated processing unit, the *Salmonella* isolates recovered were 03, 02, and 0, respectively, whereas in post evisceration samples were 03, 02 and 01, respectively. The range of *Salmonella* count in terms of MPN index/10cm^2 (Log MPN Count/10cm^2) of post-defeathering samples from retail shops and semi-automated processing unit were 7.33 -14.18 (0.865 – 1.17 log) and 3.05 - 7.33 (0.484 – 0.865 log), respectively, whereas of post-evisceration were 19.81 – 45.36 (1.297 – 1.657 log) and 11.02- 15.04 (1.042 – 1.17 log), respectively. Automated processing unit showed only one positive sample at the post evisceration stage with MPN count 19.81 (1.297 log). Out of 12 post-chlorination samples from semi-automated and automated processing unit subjected for quantification, the one sample from semi-automated processing unit found positive with MPN count of 3.59 (0.555 log) (Table 01).

The low isolation rate of *Salmonella* in the automated processing unit can be attributed to the efficiency of control systems used in the slaughterhouse by implementation of HACCP requirements. Dickel *et al*., [15] reported that by properly managed slaughter practices, such as water replacement and temperatures lower than 4 ºC in the chiller, the initial contamination of *Salmonella* spp. can be reduced from 70% to 20%. The contamination of carcasses by *Salmonella* post chlorination might be due to the semi - automated system applying inappropriate water chlorination.

Miniaturized MPN (mMPN) methods helpful for enumeration of *Salmonella* organisms quickly, accurately and cheaply from a poultry matrices have been reported in the literature [12, 16]. Fravalo *et al*. [17] proved that mMPN technique can be efficient in the identification and quantification of *Salmonella* in poultry meat matrices. Results observed in present study are in agreement with study carried out by Svobodová *et al*. [8] who observed *Salmonella* counts as 2.11 log, 1.56 log, <1.53 log and < 1.08 log MPN per carcass after-plucking, after-evisceration, after-washing and after-chilling, respectively. Study carried out by Brichta-Harhay [18] reported 3.7 X10^1, 5.6X10^0 and 5.0X10^{-2} CFU/ml *Salmonella* load for pre-IOBW, prechill and postchill rinses, respectively. Straver *et al*. [19] reported *Salmonella* count varied from 1 to 3.81 log MPN per filets of poultry carcass. Shashidhar *et al*. [16] reported higher *Salmonella* load in chicken samples in the range of 1.30 to 120 MPN/g but no data exist on the quantification of *Salmonella* from poultry processing stage from India. In majority studies the quantification of *Salmonella* was conducted in artificially contaminated samples from different sources [12, 20] but no correlation was observed when naturally contaminated samples were assessed.

Processing stages resulted in recontamination of the carcass was reported by Morris and Wells [21]. As per the findings of study defeathering and evisceration are the two important stages of processing where cross contamination usually occurs. Chlorination of carcass is the stage where carcasses are sanitized immediately after evisceration**.** The higher number of *Salmonella* spp. in post-defeathering and post-evisceration stages of retail shop and semi-automated processing samples could be due to soiling of birds with litter or initial faecal contamination and unhygienic conditions prevailing at the processing units [9]. Immersion chilling using water with chlorine agents may decrease the prevalence of *Salmonella*-contaminated carcasses by up to 50%. Industrial studies by Stop forth *et al*. [22] demonstrated an effect of washing carcasses in hypochlorite solution on the prevalence of *Salmonella*. The handling and processing of birds also needs to be improved to reduce the *Salmonella* incidence level in these stages of processing along with reduction at farm level contamination. Incorporation of chlorination stage in retail shop processing could be useful for the reduction of *Salmonella* load on carcasses.

Table 1: MPN index and Log MPN Count/10cm^{2}of *Salmonella* spp. at selected stages of poultry processing units.

Sr. No	Stage of Processing (n=6 each stage)	Number of positive samples	Sample code	MPN index	MPN low (95%CL)	MPN high (95%CL)	Log MPN count/10cm^2	Log MPN count/10cm^2 (Low 95%CL)	Log MPN count/10cm^2 (Low 95%CL)
A) Retail Shop									
1	Post defeathering	03	1	14.80	4.462	49.138	1.17	0.649	1.691
			2	7.33	1.805	29.778	0.865	0.256	1.473
			3	14.28	4.327	47.176	1.155	0.636	1.673
			Avg.	12.13			1.063		
2	Post evisceration	03	1	45.36	9.910	207.904	1.657	0.996	2.317
			2	19.81	6.903	56.908	1.297	0.839	1.755
			3	20.58	7.130	59.473	1.313	0.853	1.774
			Avg.	28.59			1.42		
3	Post chlorination	NA***	Not Applicable						
B) Semi-automated									
1	Post defeathering	02	SCPD2	7.33	1.805	29.778	0.865	0.256	1.473
			SCPD6	3.05	0.429	21.658	0.484	-0.367	1.335
			Avg.	5.19			0.674		
2	Post evisceration	02	SCPE 4	11.02	3.490	34.826	1.042	0.543	1.542
			SCPD5	15.04	5.525	40.979	1.177	0.742	1.612
			Avg.	13.03			1.109		
3	Post chlorination	01	SCPEW4	3.59	0.500	25.762	0.555	-0.300	1.410
C) Automated									
1	Post defeathering	Nil	All Negative						
2	Post evisceration	01	ACPE3	19.81	6.903	56.907	1.297	0.840	1.755
3	Post chlorination	Nil	All Negative						

4. Conclusions

Higher concentration of *Salmonella* spp. was observed at defeathering and evisceration stages of retail shop and semi-automated processing units. Incorporation of appropriate controlled chlorination stage after evisceration in retail shop and semi-automated poultry processing could be useful for the reduction of *Salmonella* load on carcasses.

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