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Prevalence of Chicken Host and Nonhost Adapted Salmonella in Retail Outlet of Chennai, India

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ABSTRACT

In India, most of the consumers procure the chicken as a raw meat in small retail outlets (slaughter house). But, there is no stringent regulations are available to monitor hygienic status of retail chicken outlets. Hence, the present study was planned to assess the existence of Salmonella in retail market in Chennai. To carry out the study, 422 samples were collected and analyzed by conventional and molecular techniques, then grouped based on O9, O4 and A-G serological reaction. Polymerase Chain Reaction (PCR) targeted on 'repeat sequence' and *invA* gene was carried out for confirmation of Salmonella genus. To differentiate serotype multiplex PCR was carried out for *phoP*, *hin* and *h-li* genes. The study revealed that, highest recovery of *Salmonella* sp. were recorded in cloacal swabs. Totally, 11 (2.6%) samples were contaminated with Salmonella consist of 1.9% of Non-Chicken-Host-Adapted (Non-CHA) and 0.7% Chicken Host Adapted (CHA). The CHA Salmonella were further identified upto species level as S. Pullorum by *rfbs* gene polymorphism. It was observed that high percentage of Non-CHA Salmonella than CHA Salmonella in the retail market may possibly due to the carrier birds in the retail butcher outlet. Hence, strict regulation has to be implemented to control the Salmonella in retail outlets i.e., retailers should receive the birds after the Salmonella screening test. In addition controlling authority has to cancel the license of retail outlet/farm if they found positive for the carrier birds.

Key words: Salmonella, *invA* gene, multiplex PCR, allele specific PCR

INTRODUCTION

Salmonellosis is one of the major concern in poultry industry, because, it has been frequently identified as common etiological agent for food borne illness (Crump *et al*., 2011; CDC., 2013). Hitherto more than 2500 serotypes of Salmonella have been reported in worldwide and more than 203 serotypes have been reported in poultry. It has been reported that, in every-day, there is a continuous emergence of new serotypes and bio-vars within the serotype (Guibourdenche *et al*., 2010). In Salmonella, some of the species are specific to host e.g., *S. choleraesuis* is origin of pigs; likewise, S. Pullorum and S. Gallinarum are originated from poultry, these two Salmonella

bio-vars are called as Chicken Host Adapted (CHA), other than these two bio-vars are called as non-CHA species. Vertical transmission of CHA species makes the Salmonella hard to eradicate from farm even after frequent screening and culling of serological positive birds. In other side, horizontal gene transfer play a major role for the emergence of new serotypes of Salmonella in poultry.

In poultry, S. Enteritidis is accounted for most of the outbreak (Hendriksen *et al*., 2011; Andino and Hanning, 2015) Hence, FDA recommends frequent monitoring of S. Enteritdis in poultry houses and egg is needed to control Salmonellosis (EFSA., 2011). Rapid slide agglutination test has been traditionally used as an easy andinexpensive test for screening of Salmonella infection in poultry, but, it lacks sensitivity. Conventional methods such as isolation, identification and characterization by cultural methods are laborious and more time consuming. But, the molecular technique viz., Polymerase Chain Reaction (PCR) istrouble-free and highly sensitive to detect Salmonella, moreover, variation in species level can alsobe detected (Shivaprasad and Barrow, 2008).

In India most of the consumers prefer to purchase chicken as a raw meat from small butcher's retail outlet (retail outlet). But, there is no stringent regulation to monitor the hygienic status of the small retail butcher outlets. Generally, the chicken host adapted Salmonella such as S. Pullorum and S. Gallinarum are arise from the poultry farm mostly by trans-ovarian infection (Eswarappa *et al*., 2009). But, the other non-CHA Salmonella comes from the infected/carrier birds. These carrier birds continuously secrete the contaminated faecal materials from farm to retail outlets, thus acts as a main source of contamination in the retail outlet samples. Inbreeder farm most of the CHA Salmonella infected birds are regularly culled by screening. But, the non-CHA Salmonella usually infect the birds in farm, which leads to prolonged shedding of Salmonella upto the retail outlet. Hence, the research work was planned to assess the prevalence status of Salmonella in retail butcher out let in Chennai by collecting different samples in live and dead birds in the retail outlets of Chennai region and confirmed using serological and molecular techniques.

MATERIALS AND METHODS

Total of 422 samples consist of cloacal swabs (75), liver (69), ovary (58), intestinal swabs (80), muscle (75) and eggs (65) were randomly collected from retail outlets in Chennai, Tamil Nadu, India and screened for the presence of Salmonella. Cloacal swabs were taken from the live birds, where as other samples were taken from culled birds.

Conventional method of isolation: Liver, ovary and egg samples were processed as per Bacteriological analytical manual (Andrews *et al*., 2007) protocol. For cloacal and intestinal swabs 10 mL of buffered peptone water was used as a pre-enrichment medium. The presumptive Salmonella were subjected to initial screening tests (Grams staining and motility) followed by series of biochemical tests viz., urease, oxidase, triple sugar iron agar, lysine iron agar and IMViC tests.

Isolates which exhibited negative for urease and oxidase were taken further for biochemical confirmation by 'Hi-Salmonella identification kit' (Hi-Media, Mumbai). Biochemically confirmed cultures were sent to Gangagen Biotechnologies limited, Bangalorefor characterization using O9, O4 and A-G anti-sera.

Table 1: Details of primers and reaction conditions used in different PCR

Target region	Primers	Thermal cycling conditions	Product size	Reference
Repeat sequence	F GATCATCCATTCGGCATTAAACA R TTCTCAGCGACGGAAGGGTAAATC	Initial denaturation 90°C/3 min Followed by 40 cycles of Denaturation 90°C/30 sec Annealing 60°C/30 sec Extension 72°C/30 sec	199 bp	Kim *et al*. (2014)
*inv*A gene	F GTGAAATTATCGCCACGTTCGGGCAA R TCATCGCACCGTCAAAGGAACC	Initial denaturation 94°C/5 min Followed by 35 cycles of Denaturation 90°C/1 sec Annealing 55°C/1 sec Extension 72°C/21-sec	284 bp	Abd El-Ghany *et al*. (2012)
Multiplex PCR *pho*P	F ATGCAAAGCCCGACCATGACG R GTATCGACCACCACGATGGTT	Initial denaturation 94°C/2 min Followed by 30 cycles	*pho*P-299 bp hin-236 bp	Gunaselan *et al*. (2012)
Hin	F CTAGTGCAAATTGTGACCGCA R CCCCATCGCGCTACTGGTATC	Denaturation 94°C/1.5 min Annealing 62°C/30 sec	H-Li-173 bp	
H-li	F AGCCTCGGCTACTGGTCTTG R CCGCAGCAAGAGTCACCTCA	Extension 72°C/1.5 min Final extension 72°C/7 min		
Differentiation of S. Pullorum and S. Gallinarum	F GATCGAAAAAATAGTAGAATT R GCATCAAGTGATGAGATAATC	Initial denaturation 94°C/5 min followed by 30 cycles Denaturation 94°C/1 min	147 bp	Desai *et al*. (2005)
Allele specific PCR	F GTATGGTTATTAGACGTTGTT R TATTCACGAATTGATATACTC	Annealing 62°C/1 min Extension 72°C/5 min		
		Initial denaturation 94°C/5 min Followed by 30 cycles Denaturation94°C/1 min Annealing 60°C/1 min Extension 72°C/1 min Final extension 72°C/5 min	187 bp	Shah *et al*. (2005)

Molecular detection and characterization: All biochemically confirmed Salmonella isolates were further confirmed by Polymerase Chain Reaction (PCR) using primer targeted specific to Salmonella conserved region i.e., *invA* (Invasive gene) of 284 bp (Abd El-Ghany *et al*., 2012); repeat sequence of 199 bp (Kim *et al*., 2014). Primers and thermal cycling conditions were summarized in the Table 1. Template DNA was prepared by cell lysate method (Desai *et al*., 2005). Amplification obtained from these two PCR were electrophoresized in 2% agarose gel (Sigma) with 1X Tris acetic acid EDTA (TAE) buffer and the banding pattern were analyzed by alpha gel documentation system. All PCR reactions were performed using 1XPCR Mastermix (Bangalore GENEI).

Multiplex PCR: Multiplex PCR technique has been used in this study to differentiate different of serotypes (Way *et al*., 1993; Gunaseelan *et al*., 2012) using primers targeted to amplify *pho*P gene, Hin and H-li genes. *Pho*p gene were selected from the loci of *pho*P/*pho*Q responsible for regulation of expression of genes responsible for virulence, which play a major role to survive with in macrophage (Gunaseelan *et al*., 2012). The Hin and H-li genes were specific and sensitive in detection of Salmonella species; these genes are responsible for control of phase variation of *Salmonella* sp. used to confirm species such as S. Typhimurium and S. Enteritidis. Thermal cycling condition and primers used for the multiplex PCR were summarized in the Table 1.

Differentiation of Salmonella Pullorum and Salmonella Gallinarum: The allele specific PCR were carried out as per, Desai *et al*. (2005) and Shah *et al*. (2005), which were based on *rfbs* gene polymorphism, specific for S. Pullorum (nucleotide at 237 position, Guanidine in place of adenine) and S. Gallinarum (nucleotide at 598 position, Adenine in place of guanidine) (Table 1).

RESULTS

Among the 422 samples, 11 samples contained Salmonella and it has been observed that highest recovery of Salmonella in cloacal swabs (5.3%), followed by eggs (4.6%), liver (2.9%) and muscle (2.6%). None of the isolates were recovered from the ovary and intestinal swabs. Repeat sequence and *invA* gene specific for Salmonella genus were amplified i.e., 199 and 284 bp, respectively (Fig. 1). Out of eleven isolates, three (S11, S13 and S40) were found to be non-motile by hanging drop method.

Based on the Hi-Salmonella identification kit (Himedia, Mumbai) and interpretation chart, it is inferred that isolates S1, S2, S9, S21, S26, S33, S38 and S39 are all falls under most common serotypes of Salmonella (Table 2). The remaining isolates S11, S13 and S40 cannot be typed using this method. Slide agglutination and sero-grouping results showed that isolates S1, S2, S9 and S21 categorized under serogroup B and isolates S11, S13, S26 and S40 falls under serogroup D. The isolates S33, S38 and S39 do not falls under either Serogroup B or D. Slide agglutination or sero grouping results were furnished in Table 2. In total Among the 11 isolates 3 (0.7%) isolates are Chicken Host Adapted (CHA) others (1.9%) are non-chicken host adapted (non-CHA).

Table 2: Slide agglutination and serogrouping test results

Identity of the isolates	Poly A-G	Poly O_4	Poly O_9	Serogroup
S1	+	+	-	Group B
S2	+	+	-	Group B
S9	+	+	-	Group B
S11	-	-	+	Group D
S13	-	-	+	Group D
S21	+	-	-	Group B
S26	+	-	+	Group D1
S33	+	-	+	Untypable
S38	+	-	-	Untypable
S39	+	-	-	Untypable
S40	+	-	+	Group D1

+: Present, -: Absent

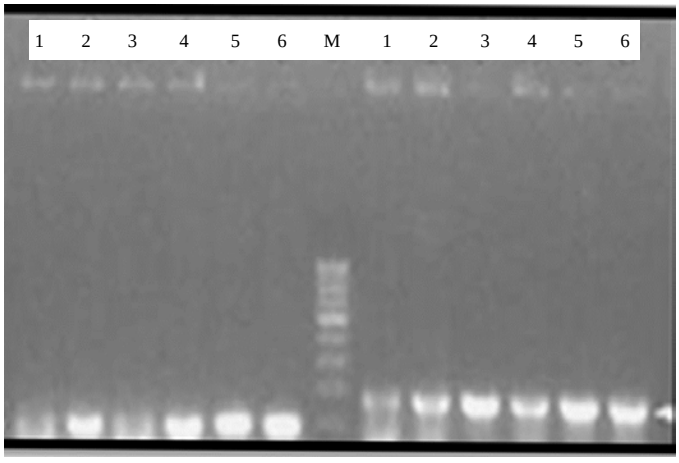

Fig. 1: Genus specific PCR for Salmonella. Amplification of 199 bp (left side) for genus specific repeat sequences, Amplification of 284 bp (right side) for *invA* gene

DISCUSSION

Out of 422 samples, 11 samples contained Salmonella; so, incidence rate of the Salmonella in Chennai region retail outlet is 2.6%. These findings are in accordance with the report of Saravanan *et al*. (2012); they reported that 0.72% of poultry samples are contaminated with *Salmonella* species in farms. Similar study was carried out by Abd El-Ghany *et al*. (2012) in Egypt. They collected 293 samples from the live, diseased and dead birds and found that 51 samples (17.4%) of were contaminated with Salmonella. Yang *et al*. (2011) reported that more than 50% of the retail markets in China are contaminated with Salmonella. Another study carried out in China by Zhu *et al*. (2014), they found that 41% of the retail market are contaminated with Salmonella. Jinu *et al*. (2013) collected 510 samples and screened by PCR, they found that 5.88% of samples were positive of the Salmonella. It has been observed that the retail market are highly contaminated than farm i.e. Singh *et al*. (2010) also reported that retail market eggs are having higher percentage of contaminated than the farm. The present study insists that the retail market is favourable place for the Salmonella to spread by improper handling techniques. WHO-INFOSAN (2010) reported that most of street vendors are lack of knowledge on food safety, are responsible for the disease outbreak.

Highest recovery of *Salmonella* species were observed in cloacal swabs (5.3%), followed by eggs (4.6%), liver (2.9%) and muscle (2.6%). None of the isolates were recovered from the ovary and intestinal swabs. Based on these observations, cloacal swabs are more suitable sample for isolation of the Salmonella. Similarly, highest recovery of Salmonella in cloacal swab was reported by Rahman *et al*. (2011), they isolated 80 Salmonella from 96 cloacal swabs. Similarly, Saad *et al*. (2007) reported the 4.87% of the cloacal samples were contaminated with Salmonella in Saudi Arabia. Limited reports are available for the presence of Salmonella in ovary and oviduct (Nief and Hoop, 1998; Bygrave and Gallagher, 1989); so, based on the present study we can able to assess those ovaries are rarely a source for Salmonella. Intestinal swab method is not suitable sample method for the isolation of Salmonella, because none of the Salmonella was recovered. This may be due to the presence of high level of inhibitors and enzyme may prevent the isolation.

All the isolates were confirmed molecularly as *Salmonella* sp. using genus specific primers targeted against repeat sequence and *invA* gene specific for Salmonella revealed specific band pattern of 199 and 284 bp, respectively (Fig. 1). For further identification upto the species, multiplex PCR technique was used; the interpretation was carried out based on the different band pattern observed on the agarose gel. The product sizes are 299, 236 and 173 bp for *pho*P, hin and H-li genes, respectively. The Multiplex PCR result shows 5 isolates such as S1, S2, S9, S21, S26 showing double band (Fig. 2), hence, these isolates either S. Enteritidis or S. Typhimurium. Remaining six isolates such as S11, S13, S33, S38, S39 and S40 exhibiting single band; among the 6 isolates which showing single band in multiplex PCR, three were non-motile (S11, S13 and S40) which was again tested for the two allele specific PCR to identify polymorphism at *rfbs* gene. PCR results of allele specific PCR revealed all non-motile isolates were S. Pullorum (Fig. 3) and others were un-typable using this method. Based on this study we could able to identify Salmonella showing phase variation and cannot be differentiated upto serotype level.

Out of eleven isolates, three (S11, S13 and S40) were found to be non-motile by hanging drop method. Results of biochemical characterization with series of test and comprehensive Hi-Salmonella identification kit were depicted in Table 3 and 4. It is inferred from the findings that isolates S11, S38 and S39 were non-H_2S producer, isolate S40 was negative for methyl red test and isolates S11, S13 were weak citrate utilizers. In the present study, three strains were isolated from

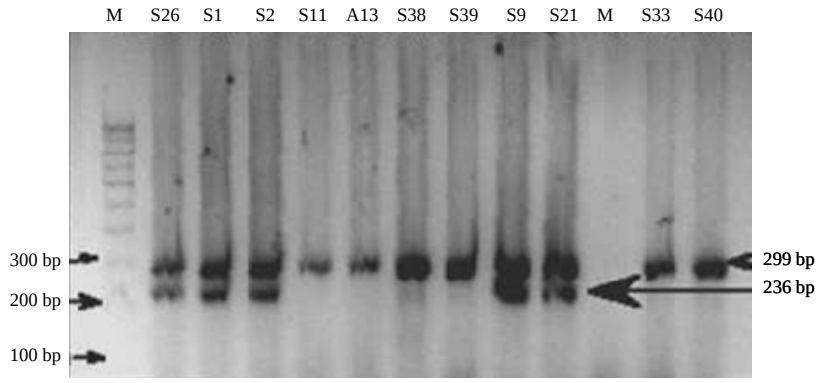

Fig. 2: Multiplex PCR to differentiate the for Salmonella serotype

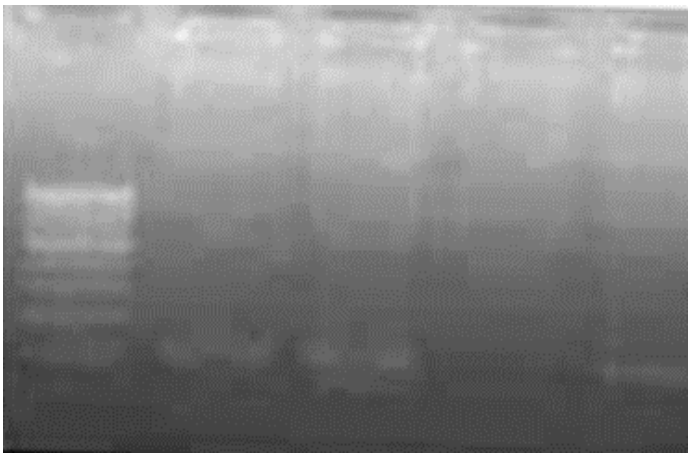

Fig. 3: Allele specific PCR for *rfbe* gene polymorphism. Amplification 147bp specific for S. Pullorum

Table 3: Biochemical characterization

	Triple sugar iron agar test (TSI)				Lysine iron agar test (LIA)								
Identity of isolates	S	B	H_2S	G	S	B	H_2S	G	U	I	M	V	C
S1	K	A	+	+	K	K	+	+	-	-	+	-	+
S2	K	A	+	+	K	K	+	+	-	-	+	-	+
S9	K	A	+	+	K	K	+	+	-	-	+	-	+
S11	K	A	-	+	K	K	-	+	-	-	+	-	W
S13	K	A	+	+	K	K	+	+	-	-	+	-	W
S21	K	A	+	+	K	K	+	+	-	-	+	-	+
S26	K	A	+	+	K	K	+	+	-	-	+	-	+
S33	K	A	+	+	K	K	+	+	-	-	+	-	+
S38	K	K	-	+	K	K	-	-	-	-	+	-	+
S39	K	K	-	+	K	K	-	-	-	-	+	-	+
S40	K	K	+	-	K	K	+	-	-	-	-	-	+

K: Alkalinity-TSI (Pink), LIA (Purple), A: Acidity-both TSI and LIA (yellow), S: Slant, B: Butt, G: Gas, +: H_2, S: Blackening of the medium, W: Week citrate utilization, I: Indole production test, M: Methyl red test, V: Vogesproskauer test, C: Citrate utilization test, U: Urease test

Table 4: Hi-Salmonella identification kit results

Identity of the isolates	MR	VP	U	H_2S	C	Ly	O	L	A	M	S	D
S1	+	-	-	+	+	+	-	-	+	+	+	+
S2	+	-	-	+	+	+	-	-	+	+	+	+
S9	+	-	-	+	+	+	-	-	+	+	+	+
S11	+	-	-	-	W	+	+	-	+	+	+	-
S13	+	-	-	+	W	+	-	-	+	+	+	-
S21	+	-	-	+	+	+	-	-	+	+	+	+
S26	+	-	-	+	+	+	-	-	+	+	+	+
S33	+	-	-	+	+	+	-	-	+	+	+	+
S38	+	-	-	+	+	+	-	-	+	+	+	+
S39	+	-	-	+	+	+	-	-	+	+	+	+
S40	-	-	-	+	+	+	-	-	+	-	-	-

MR: Methyl red test, VP: Voges-proskauertest , U: Urease test, C: Citrate utilization, Ly: Lysine utilization , O: ONPG test, L: Lactose fermentation, A: Arabinose fermentation, M: Maltose fermentation, S: Sorbitol fermentation, D: Dulcitol fermentation

egg yolk are belongs to S. Pullorum. The result also accorded with the report of Rahman *et al*. (2006), they reported that, only trans-ovarian Salmonella are present in yolk, if it is horizontal transfer most of the *Salmonella* species are present egg membrane, mostly it will not penetrate upto yolk. In the present study stateall S. Pullorum samples are isolated from the egg only, which clearly attributes that all eggs are vertically contaminated from the hen.

Among the 11 isolates 3 (0.7%) isolates are Chicken Host Adapted (CHA) others (1.9%) are non-chicken host adapted (Non-CHA); thus, clearly shows that high prevalence of Non-CHA Salmonella were found more than CHA Salmonella. This may be due to the CHA Salmonella viz., S. Pullorum and S. Gallinarum were mostly controlled by the screening test in the breeder farm itself. But, in farm, the birds are infected by the S. Enteritis or S. Typhimurium, it persistently releases the Salmonella via., faecal material even after the complete recovery from the illness. Hence, the higher incidence of Non-CHA Salmonella is due to slaughter house contamination by carrier bird. So, the present study clearly indicates the carrier birds continue spillage of infected faecal material in the slaughter house (retail outlet) favours contamination. Saad *et al*. (2007) clearly reported that slaughter house are major source of contamination of Salmonella. Suresh *et al*. (2011) also reported that the persistent environmental contamination of housing is reported to be another important factor in increasing Salmonella infection in poultry. Moreover, the presence of Multiple Drug Resistant (MDR) Salmonella in the environment may further increase the hazards (Taddele *et al*., 2012).

CONCLUSION

It is concluded that, totally, 2.6% of the chickens are contaminated in the retail chicken outlets in Chennai region and high prevalence of non-CHA Salmonella was observed in this study, which is due to carrier birds present in the chicken retail out lets play a major role for infection. Multiplex PCR is more efficiently discriminate the Salmonella than conventional and we could able to identify upto species level. So, the retail outlet owner has to purchase the chicken from the Salmonella free farm or he has to receive the birds after the Salmonella screening test. Food Safety Standards and Authority of India (FSSAI) has to give the licence to the retail owners who procure the Salmonella free birds. Stringent monitoring of S. Pullorum and S. Gallinarum has to be done at farm level to avoid vertical transmission. Hence, those farms having Salmonella, that farm licence has to be cancelled for producing the chicken, likewise, those retails outlets are having the Salmonella, its licence has to be cancelled for selling the poultry products.

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