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Study on the incidence of *Salmonella enteritidis* in poultry and meat samples by cultural and PCR methods

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Abstract

Aim: To study the incidence of *S.enteritidis* in poultry and meat samples by cultural and PCR methods.

Materials and Methods: A total of 130 samples (25 each of chicken, mutton, poultry faeces, cloacal samples and 10 each of liver, spleen and kidney) collected from different sources were subjected to cultural and PCR methods for the presence of *Salmonella* and *Salmonella enteritidis*. Primers for *invA* and *sefA* gene were used for *Salmonella* and *S.enteritidis* respectively.

Results: Out of 130 samples, 87 were positive for *Salmonella spp.* i.e. chicken-16(64%), mutton-12(48%), faeces-23(92%), cloacal swabs-23(92%), liver-5(50%), spleen and kidney samples-4(40%) each by PCR methods, whereas 77 were positive by cultural method i.e. chicken-14(56%), mutton-10(40%), faeces-22(88%), cloacal swabs-21(84%), liver-4(40%), spleen and kidney-3(30% each). Out of 87 positive for *Salmonella* by PCR method, 59(chicken-12, mutton-7, faeces-17, cloacal swabs-15, liver-3, spleen-2, kidney-3) were positive for *S.enteritidis*. High incidence of *S.enteritidis* (68%) in all the above samples are indicative of unhygienic conditions in poultry farms. Selective enrichment with Rappaport-Vassilidias (RV) broths and Tetrathionate (TT) broths were superior over Selenite-F (SF) and Selenite cysteine (SC) broths.

Conclusions: High incidence of *S.enteritidis* was seen in most of poultry samples like chicken, kidney, liver and it's faeces than mutton, which was indicative of contamination of *S.enteritidis* is more prevalent in poultry farms.

Keywords: Cultural, Incidence, PCR, *Salmonella*, *Salmonella enteritidis*.

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Introduction

Salmonella is one of the most important pathogenic genera implicated in food borne bacterial outbreaks and diseases both in developed and developing countries and constitute an important public health problem [1]. Despite global improvements in public health facilities, a marked increase in human salmonellosis has been reported in many countries including the European Union [2]. Outbreaks of *Salmonella* have been associated with wide variety of foods especially those of animal origin [3]. In many countries human salmonellosis is mainly due to consumption of eggs followed by poultry, pork, beef, and dairy products [4].

S.enteritidis is the main cause of food borne salmonellosis [5] and during the last 20 y, it has been a major causative agent of foodborne gastroenteritis in humans [6]. There is increasing evidence suggesting that the main source of human pathogens are poultry products especially eggs [7]. The symptoms in humanbeings includes diarrhoea, nausea, abdominal pain, mild fever, chills, vomition, prostration, headache, malaise etc. and the diarrhoea varies from thin vegetable soup like stools to a massive evacuation with accompanying dehydration [8].

The number of organisms, to be swallowed inorder to cause infection is rather small that is fewer than 10 [9] and it is a must for the livestock products to be tested for the presence of *Salmonella* due to it's potentially low infective dose [10]. The detection of *Salmonella* in foods is problematic due to presence of fewer number of organisms, larger number of competing microflora and due to injured organisms by different food processing methods [11]. The conventional culture method, which is routinely used for isolation of *Salmonella* is time consuming, laborious and may not be suitable for viable but non culturable (VBNC) [10].

To overcome this drawback, immunological and genetic detection methods have been developed [12]. PCR method is rapid, specific and sensitive method for the detection of food borne pathogens [13].

Table-1. Details of primers used in this study

Primer	Target Gene	Primer sequence	Amplification product (bp)	Reference
Salm-3	invA	5'-GCT GCG CGC GAA CGG CGA AG-3'	389	Malorny et. al. [14]
Salm-4	invA	5'-TCC CGG CAG AGT TCC CAT T-3'	389	Malorny et. al. [14]
SefA2	sefA[b]	5'-GCC GTA CAC GAG CTT ATA GA-3'	310	Pan and Liu, [15]
SefA4	sefA[b]	5'-ACC TAC AGG GGC ACA ATA AC-3'	310	Pan and Liu, [15]

In this study PCR method was used to detect *Salmonella spp.* and *S.enteritidis* targeting *invA* and *sefA* genes respectively.

Materials and Methods

A total of 130 different mutton and chicken related samples (25 samples each of mutton, chicken meat, chicken faeces, cloacal samples of poultry and 10 each of chicken liver, spleen and kidney) were collected from three retail markets and two slaughter houses (five replications each) in and around Hyderabad. The samples were collected in the sterile containers and transferred under cold conditions (with icepack) to the lab. Mutton, Chicken meat and chicken internal organs (10 g) samples were preenriched in 90 ml of buffered peptone water (BPW) in individual sterile polythene bags homogenized thoroughly in a stomacher for 3-5 min and incubated at 37^{0}C for 16 h. Faeces and cloacal swabs were inoculated in BPW in test tubes (50ml) and incubated at 37^{0}C for 16 h. After pre-enrichment 1 ml of each inoculum was transferred into selective broths like Tetrathionate (TT) broth, Selenite-F (SF), Selenite cysteine (SC) broths and 0.1 ml to Rappaport-Vassilidias (RV) broth, incubated at 42^{0}C for 18 h (For SC broth, 37^{0}C, 18 h). The broth cultures were spread plated onto selective media agar plates like Xylose Lysine Deoxycholate agar (XLD), Bismuth Sulphite Agar (BSA), Brilliant Green Agar (BGA), Salmonella-Shigella Agar (SSA) and Hektoen Enteric Agar (HEA) and differential agar like MacConkey Agar. Petridishes were incubated at 37^{0}C for 24 h. The presumptive colonies of *Salmonella* were taken for further confirmation by biochemical tests like IMViC (Indole, Methyl red, Voges-Proskauer, Citrate), Triple sugar iron test, sugar fermentation tests, lysine decarboxylase, ONPG(ortho-nitrophenyl galactosidase).

All the enriched samples were subjected to PCR analysis for the presence of *Salmonella spp.* using primers specific to *invA*. The samples positive for *Salmonella* by PCR method were further examined for the presence of *S.enteritidis* strains using primers specific for *sefA* gene (Table1). Primers used in this study were obtained from Bangalore Genei.

S.enteritidis strain obtained from Microbial Type Culture Collection (MTCC), Chandigarh was used as known positive strain in PCR analysis. About 1.5 ml of enriched broths were taken in eppendorf tubes and bacteria were pelleted by centrifugation at 8000 rpm for 10 min and the supernatant was discarded. Fifty µl of sterile distilled water was added to the tubes and boiled in a water bath at 100^{0}C for 10 min and immediately snap chilled to release DNA. Then centrifuged at 13,000 rpm for 5 min and the supernatants containing DNA from respective samples were used as templates for PCR analysis.

Bacterial DNA amplification was done in 20 µl reaction mixture containing 2 µl of 10x Taq DNA polymerase buffer containing 100 mM tris with p^H 9.0, 500 mM KCl, 15 mM $MgCl_2$ and 1% triton X-100), 2 µl of 10 mM dNTP mix, 0.9 U/µl of Taq DNA polymerase (Genei), 2 µl each of forward and reverse primer (4 pmol/µl) and 5 µl of crude bacterial cell lysate. This mixture was made upto 20 µl using molecular grade water. Amplification was done in thermal cycler following standardized conditions (Table-2).

Table-2. Cycling conditions

Sr.No.	Step	invA (*Salmonella spp.*)	sefA (*S. enteritidis*)
1.	Initial denaturation	95^{0}C/5 min	94^{0}C/5 min
2.	Final denaturation	95^{0}C/1 min	94^{0}C/1 min
3.	Annealing	58^{0}C/80 sec	61^{0}C/1 min
4.	Initial extension	72^{0}C/45 sec	72^{0}C/2 min
5.	Final extension	72^{0}C/7 min	72^{0}C/10 min
6.	Hold	4^{0}C	4^{0}C

The amplified DNA fragments were resolved by agarose gel electrophoresis, stained with ethidium bromide (0.5 µg/ml) and visualized with an UV transilluminater (Fig. 1).

Spiking studies: To know the sensitivity of PCR method for *S.enteritidis*, homogenized chicken was inoculated at the rate of 250 cfu, 25 cfu, 2.5 cfu and 0.25 cfu per 10 g of chicken and transferred to pre-enrichment media i.e. 90 ml of BPW, incubated at 37^{0}C for 8 h and 16 h. After incubation, inoculum transferred to selective broths like RV, TT, SF & SC broths and incubated for 12 and 18 h. The PCR and cultural testing were carried after 12 h and 18 h of incubation.

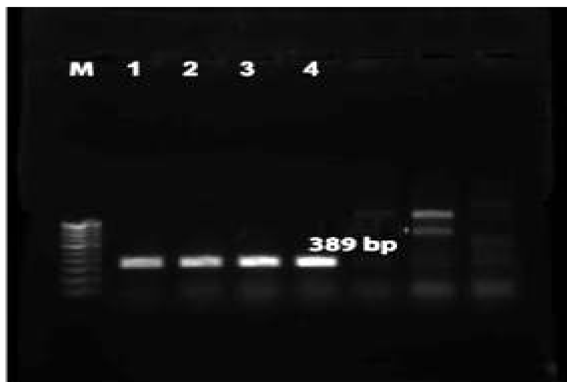

Figure-1. Results of chicken meat samples for *invA*
Lane M: 100 bp DNA Ladder, 1,2,3,4: chicken meat samples showing positive results (*Salmonella spp.*)

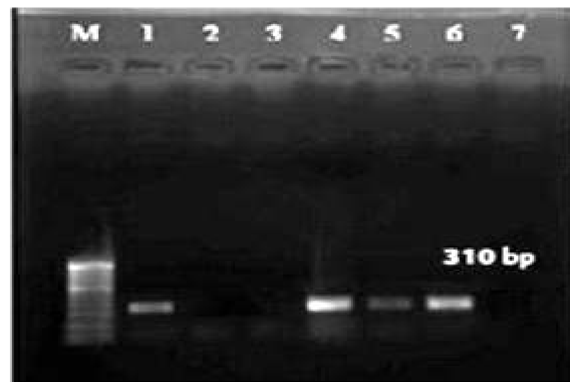

Figure-2. Results of chicken meat samples for *SefA*
Lane M: 100 bp DNA Ladder, 1,4,5,6: chicken meat samples showing positive results (*Salmonella spp.*)

Table-3. Occurrence of *Salmonella spp.* and *Salmonella enteritidis* in mutton and chicken related samples

Type of sample	Number of samples	Positive result for *Salmonella spp*				Positive samples for *S.enteritidis*		
		Cultural method		PCR assay		No.	% over total number of samples	% Over Salmonella spp. positive samples
		No	%	No	%			
Chicken meat	25	14	56	16	64	12	48	75.0
Mutton	25	10	40	12	48	7	28	58.3
Poultry faeces	25	22	88	23	92	17	68	73.91
Cloacal samples of poultry	25	21	84	23	92	15	60	65.21
Liver	10	4	40	5	50	3	30	60.0
Spleen	10	3	30	4	40	2	20	50.0
Kidney	10	3	30	4	40	3	30	75.0
Total	**130**	**77**	**59.23**	**87**	**66.92**	**59**	**45.38**	**67.82**

Results and Discussion

Out of 25 chicken meat samples, 14(56%) were positive for *Salmonella* by cultural method and 16(64%) by PCR method (Fig. 1). Out of 16 PCR positives, 12 were positive for *S.enteritidis* by PCR (Fig. 2) which accounts to 48 and 75% over total number of samples and positive samples for *Salmonella* by PCR respectively (results are shown in Table:3). Similar results were reported by Hassanien *et. al.* [16]. Low incidence of *Salmonella* in chicken meat than the present study (56%) by cultural method i.e. 40%, 26.3%, 18% and 6.79% were reported by Tavechio, [17]; Plummer *et. al.* [18]; Cortez *et. al.* [19] and Amini *et. al.* [20] respectively, whereas Blivet *et. al.* [21] reported higher incidence (97.6%). The incidence of *Salmonella* by PCR method in the present study (64%) was similar to the findings of Malkawi and Gharraibeh [22] and higher than the incidence (36.5%) reported by Uyttendaele *et. al.* [23]. The incidence of *S.enteritidis* in the present study (75%) by PCR was less than the incidence (90%) reported by Wang *et. al.* [24] and higher than the incidence (5.6% and 8%) reported by Cortez *et. al.* [19] and Hassanien *et. al.* [16] respectively.

The high incidence of *Salmonella spp.* and *S.enteritidis* in chicken might be due to not following hygienic methods in rearing, slaughtering and marketing.

Out of 25 mutton samples, 10(40%) and 12 (48%) were positive for *Salmonella* by cultural method and PCR methods. Out of 12 PCR positives, 7 were positive for *S. enteritidis* by PCR, which accounts to 28 and 58.3% over total number of samples and positive samples for *Salmonella* by PCR respectively. High incidence of *Salmonella* in meat (96.7%) by cultural method than the present study (40%) was reported by Malkawi and Gharaibeh, [22] and low incidence (0, 1.5, 6.3, 11.4 and 20%) reported by Duffy *et. al.* [25], Scanga *et. al.* [26], Stock and Stolle [27], Heredia *et. al.* [28] and Hassanien *et. al.* [16] respectively. The incidence of *S.enteritidis* in mutton samples by PCR method in this study (48%) is similar to the incidence (47.3%) reported by Malkawi and Gharraibeh [22].

The unhygienic slaughtering methods and unhygienic environment in the sale shops of mutton might be the reasons for higher incidence of salmonellosis.

Out of 25 poutry faecal samples, 22(88%) were positive for *Salmonella* by cultural method and 23(92%) by PCR method. Out of 23 PCR positives, 17

were positive for *S. enteritidis* by PCR, which accounts 68 and 73.9% over total number of samples and positive samples for *Salmonella* by PCR respectively. Low incidence (6.9% and 50%) of *Salmonella* in poultry faeces by cultural method were reported by Amini *et. al.* [20] and Mirmomeni *et. al.* [29] respectively than the present study (88%). The incidence of *Salmonella* (92%) in this study by PCR method is much higher than the incidence (1.8% and 66%) reported by Tamuly *et. al.* [30] and Carli *et. al.* [31] respectively. The incidence (73.91) of *S. enteritidis* in poultry faeces in the present study is less than the incidence (83.3% and 88%) reported by Salehi *et. al.* [32] and Mirmomeni *et. al.* [29] respectively and higher than the incidence (33.3% and 51.4%) reported by Wang *et. al.* [24] and Amini *et. al.* [20] respectively.

Out of 25 poutry cloacal samples, 21(84%) were positive for *Salmonella* by cultural method and 23(92%) by PCR method. Out of 23 PCR positives, 15 were positive for *S. enteritidis* by PCR, which accounts to 60 and 65.2% over total number of samples and positive samples for *Salmonella* by PCR respectively. The incidence (84%) of *Salmonella* in poultry cloacal samples in the present study was higher than the incidence (6.9%) reported by Amini *et. al.* [20] and Eyigor *et. al.* [33]. The incidence of *Salmonella* by PCR method (92%) was higher than the incidence (12.5%) reported by Eyigor *et. al.* [33]. The incidence of *S.enteritidis* in poultry cloacal samples by PCR method (65.2%) was almost similar to the incidence (62%) reported by Eyigor *et. al.* [33] and higher than the incidence (51.4%) reported by Amini *et. al.* [20]. Salmonella infected poultry birds will void higher levels of organisms through faeces.

Out of 10 chicken liver samples, 4(40%) were positive for *Salmonella* by cultural method and 5(50%) by PCR method. Out of 5 PCR positives, 3 were positive for *S.enteritidis* by PCR, which accounts to 60% over positive samples for *Salmonella* by PCR. The incidence of *Salmonella* was similar in spleen and kidney at 30% and 40% by cultural and PCR methods respectively. The incidence of *S.enteritidis* in spleen and kidney was 50% and 75% respectively over *Salmonella* positive samples. Mirmomeni *et. al.* [29] reported that the incidence of *Salmonella* in chicken liver and spleen were 20% and 17% respectively by cultural method and the incidence of *S.enteritidis* was 90% and 73% over *Salmonella* positive samples.

Conclusion

High incidence of *S.enteritidis* was seen in most of poultry samples like chicken, kidney, liver and it's faeces than mutton, which was indicative of contamination of *S.enteritidis* is more prevalent in poultry farms. Based on high incidence of *Salmonella* and *S.enteritidis* in mutton and poultry related samples in and around Hyderabad, strict hygienic and sanitary procedures in rearing of livestock and poultry, slaughtering and marketing of these products should be practiced.

Author's contribution

TMR participated in the preparation of experimental design and facilities of the research. P. Ramya, carried out the entire experiment. LVR helped in the analysis of the data. TMR, LVR and PR drafted and revised the manuscript. All the authors read and approved the final manuscript.

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Competing interests

Authors declare that they have no competing interests.

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