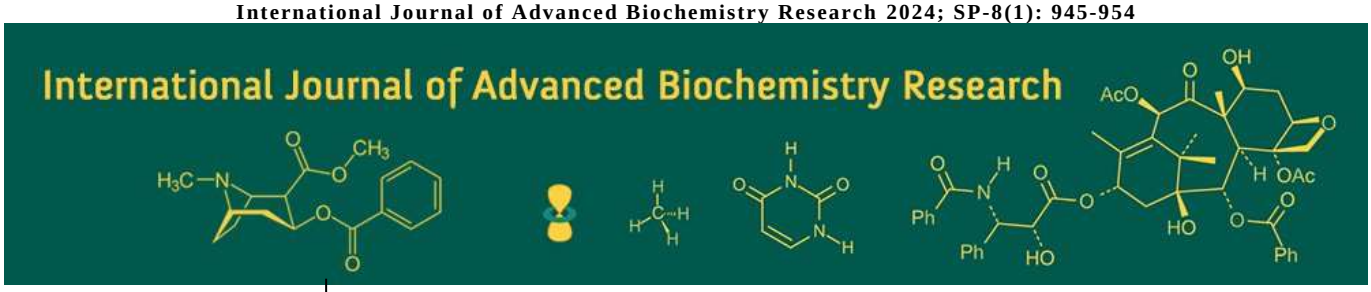

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Prevalence of *Salmonella* species isolated from poultry farms and chicken meat

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Abstract
To assess the genetic diversity of strains, genotypic characterization of foodborne bacteria such as *Salmonella* is very significant. *Salmonella* is a complex bacterial species with a wide number of serotypes and strains in different host populations, including animals, birds and humans. In total, 304 samples from poultry farms (water, feed, poultry faeces and carcass) and retail chicken meat sold in market were collected randomly from Udaipur district. Isolation of *Salmonella* strain from the samples collected from poultry farms and chicken meat sold in retail market was done according to IS 5887 (Part 3):1999. Isolation of DNA from pure culture of presumptive *Salmonella* isolates was done by using the Nucleo-pore® gDNA fungal/bacterial mini kit (Genetix) as per the instruction mentioned in the kit manual. All the *Salmonella* isolates were screened for the presence of genus specific genes by using the PCR. Out of the 304 samples from poultry farms and retail chicken meat including water (n= 29), feed (n= 29), poultry feces (n= 106), carcass (n= 42), and retail chicken meat (n= 98), a total of four samples were found to be positive for *Salmonella* spp. giving a prevalence rate of 3.44%, 0%, 1.89%, 0% and 1.02% respectively. These 4 isolates were further confirmed through PCR by targeting the genus specific primers (*invA* and *16S rRNA* gene) giving the overall prevalence to be 1.32% (4/304).

Keywords: *Salmonella*, poultry, PCR, *invA*

Introduction
Maintaining life and fostering good health need having access to adequate amounts of wholesome food. Most diseases that result from eating contaminated food are diarrheal illnesses, which afflict 550 million people and cause 2,30,000 fatalities annually. (WHO, 2019) [41].

A few common foodborne infections, including *Salmonella, Campylobacter,* and *Enterohaemorrhagic Escherichia coli*, afflict millions of people each year and can occasionally have serious or deadly consequences. The sickness manifests as fever, headache, nausea, vomiting, diarrhea, and abdominal pain. The majority of cases are brought on by eating poultry or goods made from it, or by feces contaminating foods that are of animal origin. One of the most significant zoonotic bacterial foodborne infections, nontyphoidal *Salmonella* causes 93.8 million gastroenteritis cases and an estimated 1,55,000 fatalities worldwide annually in people suffering from the illness. (Majowicz *et al*., 2010) [29]. Many factors subsidize to the spread of *Salmonella* in poultry among which the major factors are feed and water contaminated with *Salmonella* (Frederick and Huda, 2011) [18]. Furthermore, the waterer, feeders, litter and air inside poultry houses are also critical to horizontal transmission of *Salmonella* (Hoover *et al*., 1997) [21]. *Salmonellosis* in poultry is endemic worldwide, causing morbidity and mortality leading to economic loss (Abiodun *et al*., 2014; Ahmed *et al*., 2017; Akter *et al*., 2007 and Kwon *et al*., 2010) [1, 4, 5, 28].

Material and Methods
Collection of samples for isolation of *Salmonella* species
In total, 304 samples from poultry farms (water, feed, poultry faeces and carcass) and retail chicken meat sold in market were collected randomly from Udaipur district. The samples were collected in sterile container and transported to the laboratory in the Department of Veterinary Public Health, College of Veterinary and Animal Science, Navania,

Vallabhnagar, Udaipur within 2 hours in chilled condition by using ice packs. The different types of samples and the sample size are described in Table No. 1.

Table 1: Different types of samples collected for the isolation of *Salmonella* species

S. No	Type of Samples	No. of Samples collected
1.	Water	n=29
2.	Feed	n=29
3.	Poultry carcass	n=42
4.	Poultry faeces	n=106
5.	Market retail chicken meat	n=98
Total		N=304

Isolation and identification of *Salmonella* species from poultry farms and chicken meat sold in retail market

Isolation of *Salmonella* strain from the samples collected from poultry farms and chicken meat sold in retail market was done according to IS 5887 (Part 3):1999 [22]. The samples (25 ml/gm) were homogenized with 225 ml of buffered peptone water (BPW) in a sterile culture flask to obtain 1 part sample + 9 parts buffered peptone water and was incubated at 37 °C for 24 hours for pre-enrichment. Following incubation, 1 ml of inoculum was transferred into 10 ml of Rappaport-Vassiliadis (RV-10) medium for enrichment and further incubated at 42 °C for 12-14 hr. Then a loopful of the inoculum from RV-10 broth was streaked on brilliant green agar (BGA) and xylose lysine deoxycholate agar (XLD). Further, identification of *Salmonella* spp. was done on the basis of cultural, morphological, biochemical and molecular methods.

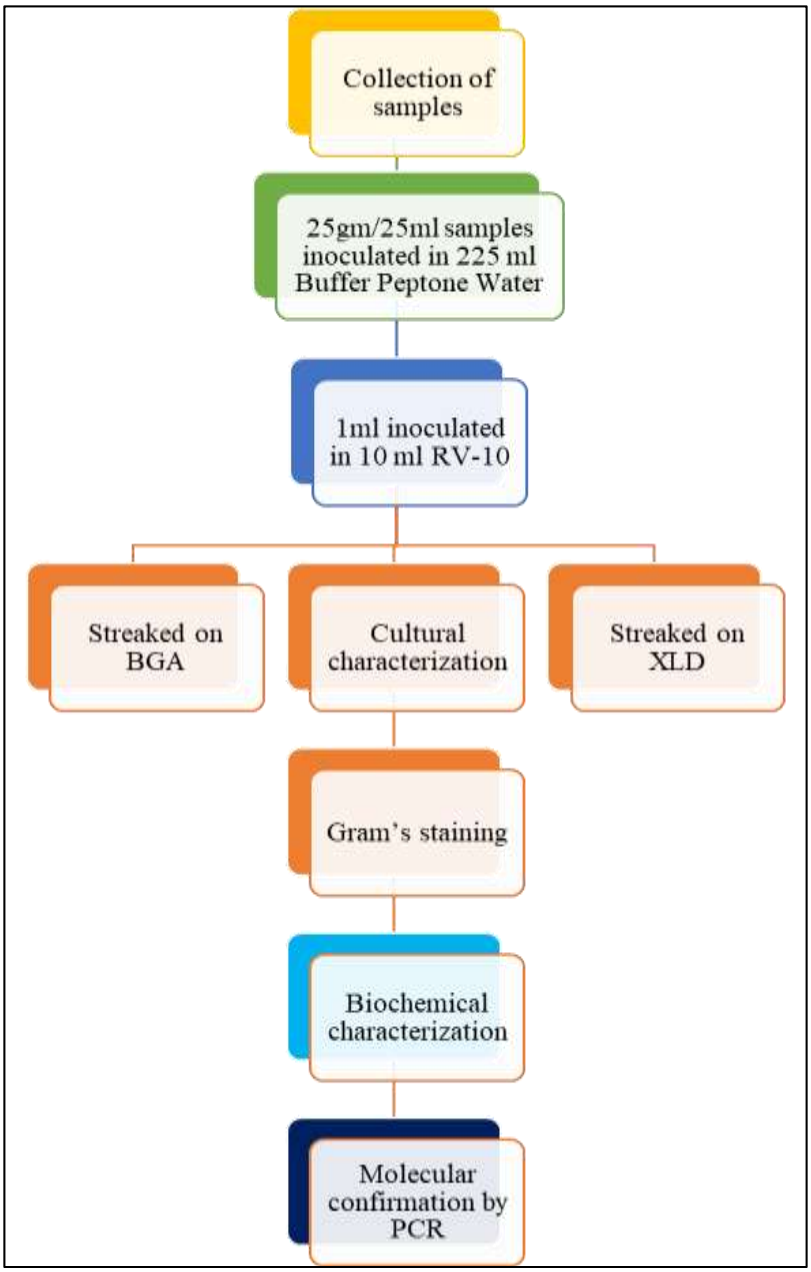

Fig 1: Flow diagram for the isolation and identification of *Salmonella* species

1. Colony characteristics and morphology of *Salmonella* spp.: Typical *Salmonella* colonies appearing as pink to red and slightly convex on brilliant green agar medium and red coloured colonies with or without black center on xylose lysine deoxycholate agar plates were selected. These putative isolates were further confirmed on the basis of their morphology by Gram's staining.

2. Biochemical characterization of *Salmonella* spp.: Various biochemical tests were performed to confirm the suspected *Salmonella* isolates *viz*., indole production test, methyl red test, voges-proskauer test, citrate utilization test, triple sugar iron agar test and urease test.

3. Molecular characterization of *Salmonella* isolates: First of all, detection of species specific and virulence gene (*invA*) was done by standardizing the PCR protocol as per the method described by Singh *et al*., 2015 with certain modifications. The primers used for the detection of *inv*A gene in test isolates and the cycling conditions for amplification are described in Table No. 3.

4. Isolation of genomic DNA: Isolation of DNA from pure culture of presumptive *Salmonella* isolates was done by using the Nucleo-pore® gDNA fungal/bacterial mini kit (Genetix) as per the instruction mentioned in the kit manual.

5. Detection of genus specific genes by polymerase chain reaction (PCR): The primers used for the detection of *inv*A and *16S rRNA* gene in *Salmonella* isolates are described in Table No. 2.

Table 2: The primers used for detection of *invA*, *16S rRNA* gene

S. No.	Oligo Name	Sequence (5'-3')	Size of amplified product (bp)	Reference
1.	*invA*	F- GTGAAATTATCGCCACGTTCGGGCAA	284	Kaushik *et al.*, 2014 [23]
		R- TCATCGCACCGTCAAAGGAACC		
2.	*16S rRNA*	F- TGT TGT GGT TAATAACCGCA	574	Mridha *et al*., 2020 [30]
		R- CAC AAA TCC ATC TCT GGA		

F= Forward, R= Reverse

6. Standardization of PCR for the detection of *inv*A gene (species specific gene)

The PCR procedure to screen the *invA* gene was standardized as described by Singh *et al.*, 2015 with certain modifications. Followed by preliminary trials, the reaction mixture was optimized to contain 12.5 µl of 2X PCR master mix, 0.5 µl of 10 nmol each forward and reverse primer, 10.5 µl nuclease free water and 1 µl of DNA template. The cycling conditions were comprised of an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minutes and final extension at 72 °C for 5 minutes (Table No. 3).

On completion of the reaction the amplified products were analysed by agarose gel electrophoresis through 1% agarose gel, visualized under UV light and the results were noted.

Table 3: Steps and conditions of thermal cycling for different virulence gene primer used in study

Primers		Cycling conditions			
(Forward and Reverse)	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
*inv*A(F) *inv*A(R)	94 °C 5 minutes	94 °C 1 minute	55 °C 1 minute	72 °C 1 minute	72 °C 5 minutes
Repeated for 30 cycles					
16S rRNA(F) 16S rRNA(R)	94 °C 5 minutes	94 °C 30second	42 °C 30 second	72 °C 30 second	72 °C 5 minutes
Repeated for 30 cycles					

7. Electrophoresis of PCR products

One percent agarose gel was prepared by adding 0.30 gm agarose in 30 ml of diluted 1X TBE buffer and boiled by using hot plate. The melted agarose was allowed to cool to about 50 °C and then 2.5 µl ethidium bromide was added. Thereafter, the gel was poured into gel tray and comb was removed after solidification of gel. During electrophoresis, the gel was placed in a horizontal electrophoresis apparatus containing 1X TBE buffer. The TBE buffer was kept at least 2-3 mm above the upper surface of gel, with orientation of wells towards the cathode end.

Amplified PCR product (5 µl) was loaded into the wells of the agarose gel to determine the size of the amplified PCR products and 100bp DNA ladder was loaded in one well. Negative control was run in the second well. Electrophoresis was carried out at 70V for 60 minutes. The amplified PCR products were visualized under transilluminator.

Result and Discussion

1. Cultural characterization of the isolates

Typical colonies of test isolate which changed the colour of the brilliant green agar (BGA) medium to pink were selected for identification (Fig. 2). Further, the putative isolates were cultivated on xylose lysine deoxycholate (XLD) agar. Colonies with transparent zone of reddish colour with black centre or pink-red zone were selected for further characterization (Fig. 3). Also, the isolates were stabbed in motility agar medium and incubated at 37 °C for 24 hours to check for the motility pattern. After 24 hours, the motility agar medium was observed with growth extending away from line of inoculation (Fig. 4). This indicated that all the isolates were motile.

2. Morphological characterization of the isolates

After the cultural characterization of the isolates, all the suspected isolates were subjected for Gram's staining, that

showed Gram negative rod shaped bacteria. These isolates were further characterized by biochemical reactions (Fig. 5).

3. Biochemical characterization of the isolates

After preliminary isolation, all the four putative isolates were subjected to different biochemical tests. All the suspected isolates (n= 4) when tested for indole showed no development of red coloured ring on the top and in MR test, red colour of culture was observed. Similarly, on testing for voges- proskauer test, no development of red colour was evident and in citrate test blue colour of the slant was observed. This indicated that all the suspected isolates were positive for MR and citrate test, while negative for indole and voges-proskauer test (Fig. 6) (Table No. 4).

Similarly, the isolates when streaked on urea agar slant and incubated at 37 °C for 24 hours were observed, no change in colour was seen, which indicated negative reaction for urease test (Fig. 7). All the isolates were stabbed and streaked on triple sugar iron agar slant and incubated at 37 °C for 24 hours. After 24 hours, the slants showed H_2S production and the red colour of slant and black butt indicated positive test for TSI reaction (Fig. 8).

Based on the cultural and biochemical tests, four isolates were considered as *Salmonella* spp. out of the 304 samples analyzed.

Table 4: Biochemical reactions of the isolates

S. No.	Biochemical Tests	Reactions
1.	Indole test	Negative
2.	Methyl red test	Positive
3.	Voges-Proskauer test	Negative
4.	Citrate test	Positive
5.	Urease test	Negative
6.	TSI test	Red colour of slant with black butt due to H_2S

Fig 2: Growth of the test culture on brilliant green agar

Fig 3: Growth of the test culture on xylose lysine deoxycholate agar

Fig 4: Motility test of the test isolate

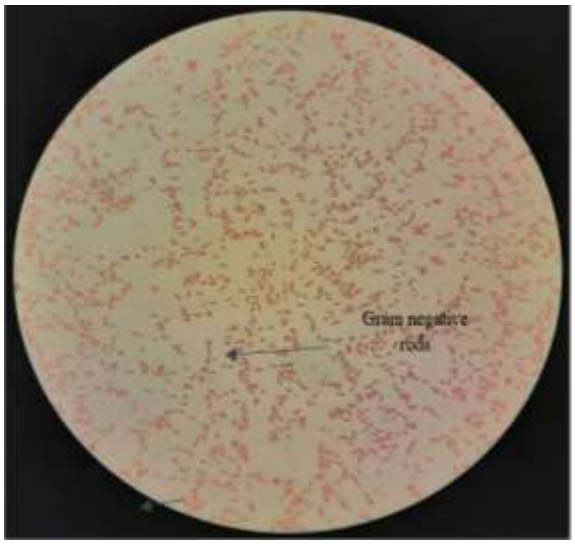

Fig 5: Gram's staining of the test isolates

Fig 6: IMViC test of the test isolates

Fig 7: Urease test of the test isolate

Fig 8: TSI test of the test isolate

4. Molecular confirmation of the isolates

The rapid diagnosis of disease is important to develop the control strategies for disease management. Polymerase chain reaction (PCR) has proved to be an efficient method for the detection of virulence and antibiotic resistant genes of the bacterial species. The *invA* gene is a conserved sequence which is prevalent in all the *Salmonella* species. Therefore, the presence of this gene help in detection and confirmation of *Salmonella* by PCR assay. Moreover, *invA* is also a virulence gene which is associated with the epithelial invasion. Similarly, genus specific *16S rRNA* gene based PCR is also used for the confirmation of the *Salmonella* isolates. In the present study, all the *Salmonella* isolates were found to possess *invA* and *16S rRNA* gene, showing a prevalence rate of 100% for both (Fig. 9 and Fig. 10). These findings are in accordance with the earlier research works reported by Awad *et al*., 2020 [8], Tarabees *et al*., 2017 [38], Das *et al*., 2012 [14], Borges *et al*., 2013 [11], Ammar *et al*., 2016 [43], Zhang *et al*., 2018 [44], Shanmugasamy *et al*., 2011 [34], Suresh *et al*., 2019 [37] and Kim *et al*., 2020 [26], who revealed 100% prevalence for *invA* gene in the *Salmonella* isolates recovered from poultry. Similarly, Chakroun *et al*., 2018 [13] and Zou *et al*., 2011 [42] also revealed 100% prevalence of *16S rRNA* gene.

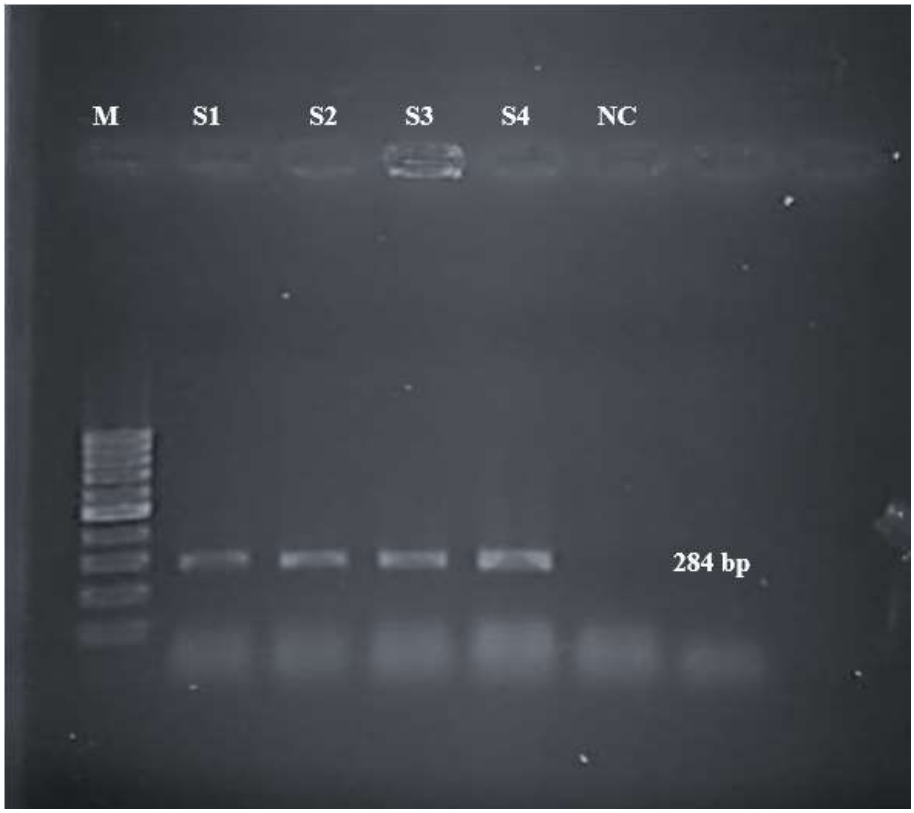

Fig 9: Agarose gel showing PCR amplified product (284 bp) for *invA* gene (M= Marker – 100 bp ladder, NC = Negative control)

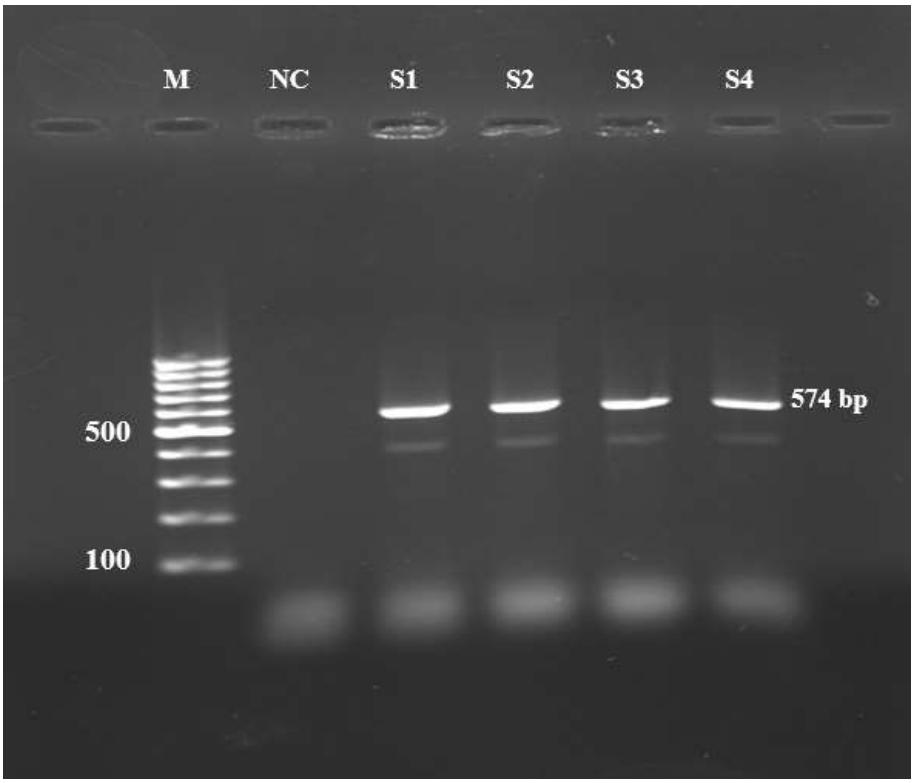

Fig 10: Agarose gel showing PCR amplified product (574 bp) for *16S rRNA* gene. (M= Marker – 100 bp ladder, NC = Negative control)

Prevalence of *Salmonella* in samples collected from poultry farms and retail chicken meat

On the basis of cultural, biochemical and molecular examination of all the 304 samples from poultry farms (water, feed, faeces and carcass) and chicken meat samples collected from Udaipur, 1.32% samples were found to be positive for *Salmonella* (Table 5). Out of the 304 samples from poultry farms and retail chicken meat including water (n= 29), feed (n= 29), poultry faeces (n= 106), carcass (n= 42), and retail chicken meat (n= 98), a total of 1, 0, 2, 0 and 1 samples were found to be positive for *Salmonella* spp. giving sample based prevalence of 3.44%, 0%, 1.89%, 0% and 1.02% respectively.

Table 5: The prevalence of *Salmonella* isolated from poultry farms and chicken meat

S. No.	Type of Samples		Total No. of Samples	No. of Samples positive for *Salmonella*	Prevalence
1.	Poultry farms	Water	n= 29	1(S1)	3.44%
		Feed	n= 29	Nil	0%
		Poultry faeces	n= 106	2(S2 and S3)	1.89%
		Carcass	n= 42	Nil	0%
2.	Retail chicken meat	Market meat	n= 98	1(S4)	1.02%
	Total		N= 304	4	1.32%

Various food borne risk including the microbiological and chemical are contributed by the poultry (Kiilholma, 2007) [25]. *Salmonella* species responsible for food poisoning in humans is attributed to more than 2500 serotypes (Guibourdenche *et al*., 2010) [19]. Identification and surveillance of *Salmonella* species can help in designing appropriate prevention and control programs. For this, both the conventional culture methods as well as the biochemical tests are applied for the isolation and identification of *Salmonella* species. *Salmonella* Enteritidis is considered as one of the important *Salmonella* serovar which causes human illness with symptoms including fever, vomiting, diarrhoea and abdominal cremps (CDC, 2010) [12].

In the present study, 3.44%, 1.89% and 1.02% of the poultry farm water samples, poultry faeces and retail chicken meat were found to be positive for *Salmonella* species contamination, respectively. For the poultry farm water samples analyzed in the present study out of the 29 water samples analyzed, *Salmonella* was recovered from only one sample giving the prevalence rate of 3.44%. This finding is an accordance with the earlier studies conducted by Ahmed *et al*., 2019 [3], Singh *et al*., 2013 [36] and Djeffal *et al*., 2018 [16], who reported the prevalence as 4.4%, 3.3% and 2.18%, respectively. Higher prevalence rates were reported by Waghamare *et al*., 2017 [40], Mridha *et al*., 2020 [30], Samanta *et al*., 2014 [33] and Balakrishnan *et al*., 2018 [9] as 16.66%, 17.19%, 8%, 20% and 60% respectively.

On analyzing the poultry faecal samples (n= 106) collected from poultry farms, the *Salmonella* species were recovered from two samples giving a prevalence rate of 1.89%. Similar findings were reported by Singh *et al*., 2013 [36], Al-Zenky *et al*., 2007 [6] and Djeffal *et al*., 2018 [16], who revealed the prevalence rates as 2%, 2.5%, 1.5% and 3.12%, respectively. On the other hand, contrasting findings showing higher prevalence rates of *Salmonella* species in poultry faeces as 16.66%, 7.9%, 8.33%, 13.24% and 14.4% were reported by Waghamare *et al*., 2017 [40], Tran *et al*., 2004, Shekhar and Singh 2014 [36], Das *et al*., 2017 [15] and Agada *et al*., 2014 [2] respectively.

On analyzing a total of 98 samples of retail chicken meat, *Salmonella* strains were isolated from only one sample giving a prevalence rate of 1.02%. Similar finding was reported by Kumar and Lakhera 2013, who found 1% samples (n=100) to be contaminated with *Salmonella* species. While, higher prevalence rates were reported by Rabins *et al*., 2018 [32], Waghamare *et al*., 2017 [40], Kaushik *et al*., 2014 [23], Naik *et al*., 2015 [31] and Awad *et al*., 2020 [8] as 10%, 7.14%, 18.42%, 7% and 15.5%, respectively.

Furthermore, in the present study it was also found that none of the poultry feed and carcass samples at the poultry farm were contaminated with the *Salmonella* species. While, earlier studies conducted by Mridha *et al*., 2020 [30], Samanta *et al*., 2014 [33], Agada *et al*., 2014 [2] have reported the presence of *Salmonella* contamination in the poultry feed samples. Similarly, Khan *et al*., 2019 [24], Mridha *et al*., 2020 [30] have revealed a significant value of contamination of *Salmonella* in poultry carcasses.

Considering the prevalence of *Salmonella* species isolated from different sources of poultry farms an overall prevalence of 1.32% (4/304) was revealed in the present study, which is considerably lower than the overall prevalence rates reported by various researchers in earlier studies. Khan *et al*., 2019 [24], Mridha *et al*., 2020 [30], Bordolei *et al*., 2018 [10] and Singh *et al*., 2013 [36] have reported higher overall prevalence rates of *Salmonella* strains in poultry farms as 12%, 31.25%, 8.49% and 3.3%, respectively. Among the bacterial diseases, *salmonellosis* is an important zoonoses causing gastrointestinal infections in humans and animals and is considered a major threat to poultry industry. Although the contamination of chicken meat and poultry house environment with *Salmonella* strains was found to be low in the present study. Still, proper cleaning and biosecurity measures can play an important role in controlling as well as reducing the infection and transmission of *Salmonella* in the poultry farms.

Conclusion

Salmonella is an important pathogen responsible for food poisoning in human beings. One of the important sources of this organism are the poultry and its products. Among the bacterial diseases, *salmonellosis* is an important zoonoses causing gastrointestinal infections in humans and animals and is considered a major threat to poultry industry. The infections are transmitted both by vertical and horizontal transmission pathways. Poultry is the main reservoir for *salmonellosis* which involves the contamination at farm as well as retail market levels (Antunes *et al*., 2016) [7]. The infections are mainly caused by the faecal contamination of foods of animal origin specially the poultry and its products (Hald *et al*., 2016) [20]. The infection of *Salmonella* species in poultry birds is also caused through the contaminated feed and water used in the poultry farms (Frederick and Huda 2011) [18]. Although the contamination of chicken meat and poultry house environment with *Salmonella* strains was found to be low in the present study. Still, proper cleaning and biosecurity measures can play an important role in controlling as well as reducing the infection and transmission of *Salmonella* in the poultry farms.

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