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Article

Molecular characterization of *Salmonella* isolated from internal organs of dead turkey and its antimicrobial activity pattern

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Abstract: *Salmonella enterica* is a zoonotic pathogen which can readily pass from animal to man through the consumption of contaminated food. This study was designed to determine molecular characterization of *Salmonella* and antibiotic resistance profiles of *Salmonella* recovered from internal organs of dead turkey. A total of 40 internal organ samples from dead turkey were collected from different turkey farms in Dinajpur district. Among the samples 12 (30%) were positive for *Salmonella*. *Salmonella* virulence factors were determined using the polymerase chain reaction assays targeting the virulence gene &16S rRNA gene region was amplified with the universal primers, forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3') and reverse primer- 1492R (5' TACCTTGTTACGACTT 3'). PCR amplification band was found at 1470 bp. Among the different serotypes, *Salmonella enterica* was identified by using phylogenetic tree analysis. Antibiotic resistance analysis indicates that *Salmonella* spp. were 100% sensitive to Azithromycin, Kanamycin, Norfloxacin and Chloramphenicol. The isolates were 100% resistant to Cefradine, Cloxacillin, Bacitracin, Levofloxacin, Amoxicillin, Nalidixic acid and Tetracycline. In conclusion, this study provides that the isolated *Salmonella* spp. were found to AMR in response to variety of multi drugs. *Salmonella enterica* can cause a wide range of illnesses, ranging from gastroenteritis to acute, life-threatening enteric fever for turkey. This study suggests that turkeys may act as a reservoir for these strains which can be transferred to humans.

Keywords: antimicrobial resistance; *Salmonella enterica*; virulence gene**;** turkey; internal organs

1. Introduction

Salmonellosis is one of the most prevalent infectious foodborne diseases in the world (McCarthy *et al*., 2009).The primary reservoir of *Salmonella* for humans is the intestinal tract of poultry .The turkey is a large bird in the genus *Meleagris*, which is not native to Bangladesh. But nowadays it is familiar to Bangladesh. The domestic turkey is a descendant of the Wild Turkey (Polash *et al.*, 2017). For business purpose, turkeys are highly meat productive. They grow faster and become suitable for slaughter purpose earlier like broiler chickens and quails. There are several billions of bacteria present in poultry feces including pathogenic and non-pathogenic species, the normal flora and the opportunistic ones (Adegunloye, 2006). The bacteria have been observed to be attached to the enterocytes and have been associated with diarrhea in turkeys (Goodwin *et al.*, 1989). Avian pathogenic *Salmonella* strains are the etiologic agents of salmonellosis in birds and are an important problem for the turkey industry (Soon *et al*., 2008). *Salmonella* strains cause a number of diseases in domestic turkey, ultimately leading to disease and death, or to a decrease in egg and meat production or

condemning of carcasses (Sackey *et al.,* 2001). In turkey, consequences of *salmonella* infections include egg peritonitis, omphalitis, swollen head syndrome, cellulitis, and death of the birds (Hofstad *et al.,* 1992). Salmonellosis is one of the most prevalent infectious foodborne diseases in the world (McCarthy *et al*., 2009). Fewer studies have sought to understand the turkey microbiome. Some work has focused on comparison of the caecal microbiomes of wild and domestic birds (Scupham *et al.,* 2008) or examination of the turkey microbiome in relation to pathogen colonization, such as *Salmonella* spp. (Scupham, 2009). *Salmonella enterica* serovar *enteritidis* (*S. enteritidis*) and *S. typhimurium* cause the majority of human clinical cases; however, serovars of other non-typhoidal salmonellae are often more prevalent in particular countries and result in more severe infections and outcomes (Hendriksen, 2010). Outbreak data have indicated that salmonellae are strongly associated with poultry and that turkey is one of the top three foods that contribute to *Salmonella* foodborne illness. Many *Salmonella* infections occur in people who handle contaminated turkey. There is a difference in the bacterial genera present in the internal organ of different turkeys, as well as bacterial populations in the turkey intestinal tract. Bacterial disease that causes concern in the turkey industry. It results in production losses via decreased feed efficiency, slower growth rate, and increased morbidity and mortality rates, and may predispose the turkey to other diseases. Research has focused on the aerobic bacteriological (Schmidt *et al.*, 1988) etiologies of turkey enteritis. Many bacteria (Goodwin *et al.*, 1989), have been seen in the intestinal wall of the turkey in the crop, ileum, and cecum (Fuller and Turvey, 1971). Examining the antibiotic susceptibility patterns of pathogens is important toward tailoring treatment to the ever-changing resistance patterns and distribution of pathogenic bacteria (NCCLS, 2001). Investigation of the prevalence of *Salmonella* serotype that could pose threats to the turkey industry and human public health through importation of infected turkey is important (Hendriksen, 2010). To assess the virulence potential of *Salmonella* isolates from turkey. This study was designed to determine Molecular characterization of *Salmonella* and antibiotic resistance profiles in *salmonella* recovered from internal organs of turkey.

2. Materials and Methods

2.1. Collection of samples

A total number of 40 internal organs (Liver, spleen, lung, intestine) samples were collected from dead turkeys in different turkey farms of Dinajpur district. The samples were carried out at bacteriological laboratory in the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

2.2. Isolation of associated bacteria

Bacteriological examination was carried out using standard method for aerobic bacteria (Brown, 2005). For the isolation of bacteria, all samples were plated on Nutrient agar and subsequently incubated at 37^{o}C for 24 hours. Primary culture was performed in Nutrient agar and Nutrient broth media. For sub-culturing, suspected bacteria were inoculated separately onto different bacteriological agar media (MacConkey agar, SS agar, XLD agar, Tryptic soy agar, Simmons citrate agar) under aseptic condition and incubated at 37°C for 24 hours. Pure cultures were achieved as per the procedures described by OIE (2000), Merchant and Packer (1967) and Cowan (1985).

2.3. Identification of bacteria

Cultural, morphological and biochemical characteristics were studied in order to identify the bacterial flora. Gram's staining was performed to study the morphology and staining characteristics of bacteria according to the technique described by Merchant and Packer (1967). Biochemical tests, such as sugar fermentation, coagulase, catalase, MR, VP, and Indole tests were performed as per the standard methods (Cheesbrough, 1985).

2.4. *Salmonella* serotyping

Salmonella isolates were serotyped using Salmonella polyvalent "O" (A-I) antisera manufactured by Statens Serum Institute, Copenhagen, Denmark was used for the sero grouping of *Salmonella* isolates.

2.5. Antibiogram study

Antimicrobial drug sensitivity test was performed on freshly prepared, dried up Mueller Hinton agar (Oxoid) against 8 commonly used antibiotics by disc diffusion method or Kirby-Bauer method (Bauer *et al.*,1966) according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2015).

2.6. Molecular techniques

2.6.1. Materials used for bacterial genomic DNA isolation

TE buffer,10% (w\v) Sodium dodecyl sulfate (SDS), 20 mg\ml protinase k (stored in small single-use aliquots at -200C), 3M Sodium Acetate, pH 5.2,25:24:1 Phenol/Chloroform/Isoamyl alcohol Isopropanol, 70% Ethanol, 95% Ethanol & 1.5 ml microcentrifuge tubes.

2.6.2. Methods of genomic DNA Extraction and Purification

Genomic DNA from *Salmonella* isolates cultured in a sodium thioglycolate broth was extracted using the chloroform-isoamyl alcohol method. Briefly, Cells were harvested by centrifugation at 2400 g for 10 min in an IEC CL31R multispeed centrifuge (Termo Scientifc). The supernatant was discarded and the pellet resuspended in 400 L Tris-EDTA (TE) buffer, containing 0.01 M Tris-HCl pH 7.4, 0.001 M EDTA with gentle agitation. Seventy microliters of 10% SDS (sodium dodecyl sulphate) (Fisher Scientifc) was added to the suspension, followed by 5 L of (10 mg/mL stock solution) proteinase K (Ambion). The samples were then incubated for 1 hr at 65º C in hybridization oven (Biometra OV2, Anachem, UK). After incubation, 100 L of 5 M NaCl followed by 100 L CTAB/NaCl (prewarmed at 70º C) was added to the solutions. The solution was gently inverted for 10 sec, incubated at 65 ˚C for 20 min, and then cooled at room temperature for 5 minutes. Seven hundred and fifty microliters of chloroform-isoamyl alcohol (24 : 1, Sigma-Aldrich) was added and centrifuged at 1300 ×g for 15 minutes. The supernatants were transferred to new tubes, treated with 5 L RNase A (5 mg/mL in RNase A buffer containing 0.5 M NaCl, 0.01 M EDTA), and incubated at 37 ˚C for 30 minutes. The quality of DNA was examined by running 4 L of the DNA sample on 0.85% agarose gel. The DNA was subsequently stored at -20 ˚C 37 ˚C awaiting further analysis.

2.6.3. Materials used for polymerase chain reaction

dNTP, MgCl2, Forward Primer (27F), Reverse Primer (1492R), Nano Pure Water, DNA Taq DNA Polymerase, Final Volume, Thermal Cycler (Thermo cycler, ASTEC, Japan), 0.85% agarose gel, Gel casting tray with gel comb, TAE buffer, Microwave oven, Conical flask, Electrophoresis apparatus (Biometra standard power pack P 2T), Bromphenicol blue of loading buffer, Ethidium bromide (0.5 µg/ml), Distilled water, UV trans-illuminator.

2.6.4. Procedure of polymerase chain reaction

The PCR reaction was performed in 25 µl reaction scale. The reaction consisted of 12.5 µl of 2x master mix (GENE Amp Fast PCR Master mix (2x)). About 2µl sample (samples were diluted at 50 ng/µl), 0.2 µl Taq DNA polymerase, 0.5µl forward primer, 0.5µl reverse primer were used. 9.3µl molecular grade water was added to make final volume of 25µl.For Mx-Sironi primer samples were subjected to initial denaturation for all 95ºC for 10 minutes; followed by 35 cycles of denaturation at 94ºC for 1 minute; annealing at 53ºC for 1 minute; extension at 72ºC for 1 minute; and a final extension at 72ºC for 10 minutes on Gene Atlas (Model: G02, Japan).

3. Results

3.1. Cultural characteristics

Cultural characteristics of each type of bacteria isolated from internal organ of turkey were studied for the examination of size, shape, colony characteristics, and pigment production in various solid media. The pure cultures of the organism from each mixed culture were obtained by repeated streak plate method by using different simple, enriched and selective solid media for study. The individual culture characteristics of bacterial isolates are presented in Table 1.

Table 1. Cultural characteristics of the organisms isolated from internal organ of turkey.

Isolated organism	Nutrient agar	MacConkey agar	SS agar	EMB Agar	SCA
***Salmonella* spp.**	Circular, smooth, opaque and translucent	Smooth and circular white/transparent colony	Black centered, smooth, small round colony	Pink color, circular and smooth colony	Green color converted into bluish color

Legends: NA = Nutrient Agar, MC = MacConkey, EMB = Eosin Methylene Blue, SS = Salmonella- Shigella, SCA= Simmons citrate agar.

3.2. Staining characteristics

The staining characteristics of the isolated organisms were determined according to Gram's staining technique and the results are presented in Table 2.

Table 2. Morphology, staining and motility characteristics of bacterial isolates.

Bacterial isolates	Shape	Arrangement	Gram's staining reactions	Motility characteristic
Salmonella spp.	Small rod	Single	Gram negative	Non motile

3.3. Biochemical tests

Bacteria isolated from internal organs were subjected to various types of biochemical tests such as Triple sugar iron agar, methyl red test, Voges–Proskauer test, MIU test and buffer peptone water test (Indole test) in order to determine their biochemical characters and degree of variation in their reactivity pattern. The result was presented in Table 3.

Table 3. Biochemical tests of the isolated *Salmonella* spp. from internal organ of turkey.

Isolated bacteria	MR	Indole	VP	TSI	MIU
Salmonella spp.	+	+	-	Butt-Yellow Slant-Red	+

Legends: MR= Methyl Red, VP= *Voges–Proskauer*, TSI= Triple Super Iron, " +"= Positive, "-"= Negative, Y= Yellow, R= Red, Indole= Buffer Peptone water, MIU= Motility Indole and Urease test.

3.4. Bacterial flora isolated from turkeys

Salmonella spp. were isolated from the 40 internal organ samples of turkey. Out of 40 samples, 12 were positive for *salmonella* of which 6 pathogens were isolated from liver, 4 pathogens were isolated from intestine, 1 pathogen were isolated from lung & 1 pathogen were isolated from spleen.

3.5. Results of antibiotic sensitivity assay of isolated bacteria

The isolated bacterial pathogens were selected randomly for the antibiotic sensitivity and resistance patterns against commonly used antibiotics. The results of sensitivity against antibiotic discs (zone of inhibition) were categorized as resistant (-), intermediate (++) and sensitive (+++).

3.5.1. Antibiotic sensitivity pattern of *Salmonella*

The antibiotic sensitivity pattern of *Salmonella* under the study (Table 4) revealed that all of the isolates (12) were 100% sensitive to Azithromycin, Kanamycin, Norfloxacin and Chloramphenicol. The isolates were 100% resistant to Cefradine, Cloxacillin, Bacitracin, Levofloxacin, Amoxicillin, Nalidixic acid and Tetracycline.

Table 4. Antibiotic sensitivity pattern of *Salmonella* (n = 5).

Antibacterial agents	No. of isolates			Percentages (%)		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Azithromycin (AZM)	5	0	0	100	00	00
Chloramphenicol	5	0	0	100	00	00
Cefradine (CH)	0	0	5	00	00	100
Cloxacillin (COX)	0	0	5	00	00	100
Bacitracin	0	0	5	00	00	100
Erythromycin (E)	3	2	3	60	40	00
Levofloxacin (LE)	0	0	5	00	00	100
Kanamycin (k)	5	0	0	100	00	00
Amoxicillin	00	00	05	00	00	100
Tetracycline	00	00	05	00	00	100

3.6. PCR amplification, sequencing of 16S rRNA genes with universal primers and phylogenetic analysis of *Salmonella* spp.

Out of 40 samples, *Salmonella* spp. were present in 12 cases. 16S rRNA gene region was amplified with the universal primers, forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3') and reverse primer- 1492R (5' TACCTTGTTACGACTT 3'). PCR Amplification band was found at 1470 bp as shown in Figure 1.

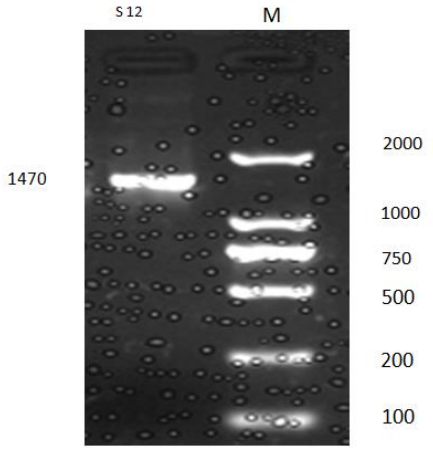

Figure 1. Result of amplification of 16S rRNA gene region of Salmonella by PCR.
M: Marker, 2kb DNA ladder Note: PCR= Polymerase Chain Reaction, kb= kilo base.

3.7. Phylogenetic tree analysis of *Salmonella*

Phylogenetic tree analysis of *Salmonella* is shown in Figure 2.

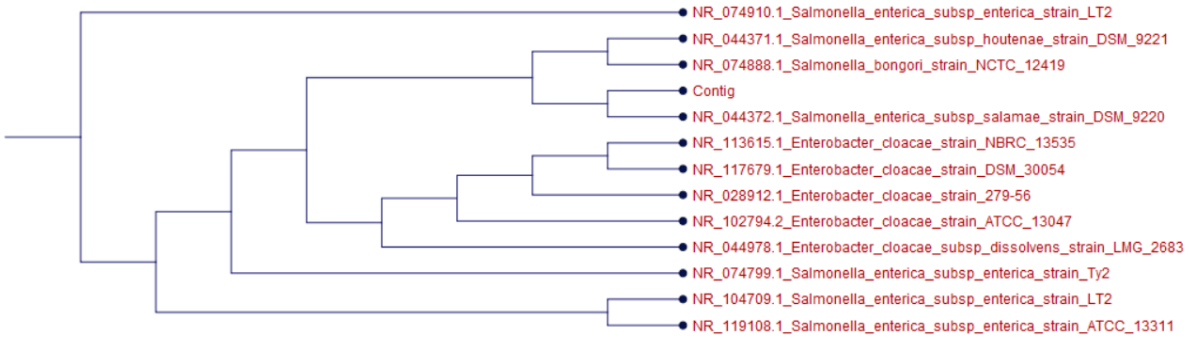

Figure 2. Phylogenetic tree analysis of *Salmonella.*

4. Discussion

As a zoonotic bacterium, *Salmonella* has reservoirs in various animals, including turkey, which are considered a reservoir for salmonella and a potential cause of disease outbreaks in the human population. This study was performed to determine molecular characterization of *Salmonella* and antibiotic resistance profiles recovered from internal organs of turkey. For this purpose, 40 internal organ samples of dead turkey were collected & 12 (30%) samples were positive for *Salmonella*. This observation is slightly higher than the result of Polash *et al.* (2017). The incidence of *Salmonella* spp. isolated from internal organ samples collected from turkey compared with the findings of Tiffany (2014), Jessica *et al.* (2013), Bielke *et al*. (2003) and Boyer *et al*. (1962) with slight variation due to the samples of this research work has been collected from internal organs of turkey. The isolates of *Salmonella* spp. showed identical results in different biochemical tests including Methyl-Red, Voges-Proskauer, and Indole test and similar type of biochemical reaction as reported by Menconi *et al.* (2011) and Bryan *et al*. (1965). In this study, the colony characteristics of *Salmonella* spp. observed in NA, SS agar, were similar to the findings of Potturi *et al*. (2005). In Gram's staining, the morphology of the isolated bacteria exhibited Gram negative small rod arranged in single or paired and motile which was supported by several authors Kumar *et al.*(1971); Tempe *et al.*(2003). *Salmonella* strains of turkey origin are also often resistant to a variety of antimicrobials approved for use in poultry; these include tetracycline, chloramphenicol and amino

glycosides Poppe *et al*. (1995). Antibiotic resistance analysis indicates that *Salmonella* spp. were 100% sensitive to azithromycin, kanamycin, norfloxacin, chloramphenicol. The isolates were 100% resistant to cefradine, cloxacillin, bacitracin, levofloxacin, amoxicillin, nalidixic acid & tetracycline. The results obtained from antibiotic sensitivity analysis is more or like similar to (Polash *et al*., 2017). Nevertheless, the relatively common resistance of turkey isolates to nalidixic acid is considered alarming, as fluoroquinolones are used for the treatment of invasive salmonellosis Iseri *et al*. (2010). Salmonella virulence factors were determined using the polymerase chain reaction assays targeting the virulence gene & 16S rRNA gene region was amplified with the universal primers, forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3') and reverse primer- 1492R (5' TACCTTGTTACGACTT 3'). PCR Amplification band was found at 1470 bp. Among the different serotypes, *Salmonella enterica* was identified by using phylogenetic tree analysis.

5. Conclusions

Salmonella species continue to be one of the major causes of bacterial illnesses. Salmonellosis occur due to linked with foodborne outbreaks, live animal contact, poor hygiene, and environmental exposure. With the emergence of antimicrobial resistance, the pathogenicity and virulence of certain *Salmonella* serotypes have increased and treatment options are decreasing and becoming more expensive. In the context of this study, it may be concluded that the internal organ samples were collected from turkeys contain *Salmonella enterica*. That might make the birds vulnerable for easy access of infection and also the bacterial pathogens may pass through the feces to the environment and cause a potential human health hazards.

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Conflict of interest

None to declare.

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