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Isolation and Characterization of Salmonella Enterica Serovar Typhimurium Circulating Among Healthy Chickens of Bangladesh

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A R T I C L E I N F O	A B S T R A C T
	Salmonella is considered as a global problem ranking first among food borne diseases. All motile Salmonella of poultry origin are zoonotic and readily transmit to human via meat and eggs but reports on non - typhoidal Salmonella serovars circulating in layer chickens is very limited in South-East Asian countries including Bangladesh. *Salmonella* serovars isolated from apparently healthy chickens were characterized in the present study. Of 170 samples (cloacal swab 150 and feed 20) collected from commercial layer farms, motile Salmonella was isolated 4% (6/150) and 50% (10/20) respectively by cultural, biochemical, motility test and by detection of *hisJ* gene. About 5% (8/170) samples possessed serovar-specific gene *fimA*, suggesting that isolates were Salmonella enterica serovar Typhimurium. Antimicrobial susceptibility testing demonstrated that the isolated serovars were multidrug resistant. Therefore apparently healthy layer chickens harbour and transmit S. Typhimurium to the environment, although little is alarming since it has zoonotic significance and the isolates were resistant to commonly used first line of antibiotic in Salmonella infection.

Introduction

Food-borne bacterial pathogens are the important causes of human mortality worldwide especially in underdeveloped, least developed and developing countries. Salmonella is considered one of the most pathogenic and common zoonotic food borne bacteria (Baird-Parker, 1990). About 1.3 billion nontyphoideal salmonellosis have been reported annually in the world resulting 2 million deaths (Payment and Riley, 2002).

The major documented sources of Salmonella are the foods of animal origin. Salmonella infections might be occurring due to improper cooking methods and unhygienic handling of foods (Gorman et al, 2002). *S. enterica* serovar Typhimurium was the most commonly isolated *Salmonella* serotype from humans in the Republic of Ireland in 2001 (NDSC, 2001). As Salmonella is a pathogen resides in the gut of poultry, cross-contamination happens during processing of the Salmonella reservoir poultry for food preparation (Bryan and Doyle, 1995).

The drug resistant pathogens have been increased during recent years (Threlfall et al., 2000, Himi et al., 2015; Khatun et al., 2105, Tanzin et al., 2016). Presence of multidrug resistant bacteria in ready to eat foods is a global public health problem and sensitive bacteria readily converted to resistant one due to transfer of resistant gene.

Poultry and poultry products are the main source of human Salmonellosis since poultry are the most crucial reservoirs of Salmonella (Humphrey, 2000). In our previous report it was mentioned about 7% of healthy layer chicken in Bangladesh act as a source of multidrug resistant non motile *Salmonella* spp. during defecation (Parvej et al., 2016) but investigation on motile serovars of Salmonella in layer chickens is not reported yet. The multidrug resistant pathogenic *Salmonella* spp. circulating in some geographical areas is the great upcoming global problem because of their chances of transmission by travellers or trades of poultry and poultry products from infected countries.

Identification of the accurate source of *Salmonella* spp. at the geographical origin is only the way to control zoonotic Salmonella serovars globally for which knowledge on molecular epidemiology and local prevalence of the pathogen is very important (Collard et al., 2007). The study was conducted with a view to investigate the prevalence of S. Typhimurium in apparently healthy layer chickens of Bangladesh and also to analyse the present status of antibiotic sensitivity pattern of the Salmonella serovars circulating in the country.

Materials and Methods

Sample Collection and Processing

A total of 150 cloacal swab samples and 20 feed samples were collected from 4 commercial poultry farms located in Mymensingh, Bangladesh and inoculated into nutrient broth (Himedia, India) followed by incubation at 37°C for 2 hours for enrichment. The samples were collected following a convenience method without repetition of any bird.

Isolation and Identification of Salmonella Spp.

Isolation of Salmonella was performed according to the method of Rybolt et al. (2005) with some modification. In short, the nutrient broth (Himedia, India) containing cloacal samples were incubated for 2 hours at 37°C and then inoculated onto SS agar followed by overnight incubation at 37°C. The feed samples were weighed about 25 gm and 10% (w/v) solution was prepared using phosphate buffer saline from which 0.1 mL of each sample was inoculated into SS agar (Himedia, India) and incubated at 37°C for overnight. The plates were examined for typical colony characteristics produced by *Salmonella* spp. Salmonella suspected colonies from SS agar were sub cultured into XLD agar (Himedia, India) to obtain pure culture of the isolates. The isolated organisms were identified by Gram's staining method to determine their staining characteristics, morphology, and arrangements. Five basic sugar fermentation (glucose, maltose, lactose, dulcitol and mannitol) tests were performed to identify the organism. The pure colony was identified by indole test, Methyl red and VP test. Then the motility test was performed by hanging drop slide method (Shanson, 1989) and by culturing onto Motility Indole Urea (MIU) media (Himedia, India). Finally, the isolates were differentiated from group D Salmonella (non-motile Salmonella) by slide agglutination test using group D antigen (S and A reagent lab, Bangkok, Thailand). The isolated Salmonella were further confirmed as S. Typhimurium by PCR.

Amplification of hisJ and fimA genes by PCR

Genomic DNA of each bacterial isolate was prepared according to the method of Queipo-Ortun et al. (2008) with little modification. Briefly, 1mL of overnight broth cultures of the isolate was centrifuged at 10000 rpm for 5 minutes. Supernatant was discarded and pellet was mixed in 200 µl of sterile distilled water by pipetting. The mixture was boiled for 10 minutes followed by cooling on ice and centrifugation at 10,000 rpm for 5 minutes. The supernatants were collected to use as DNA template in PCR.

To detect and identify the isolates as Salmonella spp. performed PCR to amplify Salmonella specific gene *hisJ* using the oligunucleotides primers upper strand (5´-ACTGGCGTTATCCCTTTCTCTGGTG-3´), and lower strand was (5´-ATGTTGTCCTGCCCCTGGTAAGAGA-3´) described by Noah et al., (1993) and the amplicon size was 496 bp. To make 25 µL of reaction mixture, 1 µL template DNA, 12.5µL of 2X Master Mix (Promega,

USA), 1µL of 10 pmol/µL of each primer and 9.5µL of sterile distilled water were taken and mixed well. Thermal cycler conditions were set as 30 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 45 seconds and an initial denaturation at 94°C for 5 minutes with final extension at 72°C for 7 minutes.

To confirm the isolates as Salmonella Typhimurium another PCR was performed using the primers set of FimA-F, 5'-CCT TTC TCC ATC GTC CTG AA-3' and FimA-R, 5'-TGG TGT TAT CTG CCT GAC CA-3'(Angela et al., 2007). PCR mixture was prepared by previously describe method for *hisJ* gene detection. The *fimA* genes were amplified by 20 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds. and elongation at 72°C for 1 minute with an initial denaturation at 94°C for 5 minutes and final elongation at 72°C for 5 minutes.

The PCR products were separated by gel electrophoresis using 1.0% agarose gel stained with ethidium bromide. The stained PCR products were visualized under UV-transillumninator (UVsolo TS, BioRad, Germany) and the results were recorded.

Antibiotic Susceptibility Test

Antibiotic Susceptibility of all isolated S. Typhimurium was investigated by agar disc diffusion test on Muller Hinton agar (Himedia, India) according to CLSI (2007). The standardized commercial antibiotic discs used were : amoxycillin (10 mcg), ampicillin (10 mcg), ciprofloxacin (5 mcg), doxycycline (30 mcg), gentamycin (10 mcg), kanamycin (30 mcg), and tetracycline (30 mcg) (Oxoid, UK).

Results

In the present study, *S. enterica* serovar Typhimurium was isolated and characterized from commercial poultry farms in Bangladesh. The study demonstrated that, out of 170 samples 4% (6/150) apparently healthy layer chickens and 50% (10/20) feed samples were found positive for Salmonella serovars which was detected by both conventional and molecular method. The prevalence of *S. enterica* serovar Typhimurium was about 1.33% (2/150) and 30% (6/20) in clocal swab and feed samples respectively. On SS agar the *Salmonella* spp. produced translucent, smooth, small round colonies with black centre (Hyeon et al., 2012). Gram's staining of the suspected colonies revealed Gram-negative, pink colored short rod shaped bacteria, arranged either single or paired. All of the tested isolates were indole and VP negative whereas Methyl red positive. All the isolates fermented glucose and maltose and produced both acid and gas. The isolates were confirmed as *Salmonella* spp. by amplification of *hisJ* gene (496 bp) (figure 1 and 2). A total of eight isolates were found to be motile characterized by forming the stab line without producing turbidity in MIU medium. The *fimA* gene (amplicon sizes 85 bp) (Figure 3 and 4) were successfully amplified in all eight isolates. Antibiotic sensitivity test using 7 drugs revealed 87.5% (7/8) strains were sensitive to

Ciprofloxacin, 87.5% (7/8) isolates were resistant to ampicillin, amoxicillin, doxycycline, kanamycin, and 62.5% (5/8) were resistant to gentamycin and tetracycline.

Figure 1 Electrophoretic analysis of PCR product to detect hisJ gene (Cloacal samples). Lane M: 1 kb DNA ladder (Promega, USA), Lane PC: Positive control, Lane NC : Negative control, Lane T2,T4,T5: 3 positive Salmonella spp. of the study, Lane T1, T3 : Tested samples (Negative)

Figure 2 Electrophoresis analysis of PCR product to detect hisJ gene (Feed samples). Lane M: 100 bp DNA ladder (Promega, USA), Lane PC: Positive control, Lane NC: Negative control, Lane T1-T10: Ten Positive Salmonella spp.of the study.

Figure 3 Electrophoresis analysis of PCR product to detect *fimA* gene (Cloacal samples). Lane M: 100 bp DNA ladder (Promega, USA), Lane PC: Positive control, Lane NC: Negative control, Lane T1, T2: 2 positive S. Typhimurium strain of the study

Figure 4 Electrophoresis analysis of PCR product to detect *fimA* gene (Feed samples). Lane M: 100 bp DNA ladder (Promega, USA), Lane PC: Positive control, Lane NC: Negative control, Lane T1 –T6: Six positive S. Typhimurium strain of the study.

Discussion

Salmonellosis is the most important food borne bacterial diseases in the world. Poultry and its products are considered as the major source for human Salmonellosis. Transmission of *Salmonella* spp. in poultry generation after generation is closely associated with infected eggs (Wigley et al., 2001). About 44.4% of the chicken eggs are contaminated with *Salmonella* spp. in Bangladesh (Saha et al., 2012). Specific characterization of *Salmonella* isolates is therefore extremely important in order to control the *Salmonella* outbreak (Salehi et al., 2011; Gast, 1997). S. Typhimurium is the causal agent of typhoid fever, septicemia, and gastroenteritis in human (Tsen et al., 1994). The present study demonstrated that, about 4% of apparently healthy commercial poultry in Bangladesh carry *Salmonella spp.* which are readily spread into the environment through faeces and enter to human food chain by contaminated meats or eggs. Li et al., (2013) isolated 165 *Salmonella* isolates from 1382 samples (11.93%) in China. Rahman et al. (2014) isolated salmonella specific bacteriophage from poultry farm of Bangladesh which also indicate the presence of *Salmonella* spp. in the surrounding environment of poultry farm because bacteriophages are present in that place where respective bacteria exist. In the present study it was found that, 50% of the feed samples which were supplied to the chicken contain *salmonella.* The contaminated poultry feeds might be the main source of infection in commercial poultry farms of Bangladesh. A total of 5% S. Typhimurium were detected in both feed and cloacal samples by PCR. Separately about 1.33%-layer chicken and 30% of poultry feed carry the same serovar. Angela et al. (2007) detected S. Typhimurium from raw chicken meat by amplification of *fimA* gene. Barua et al. (2012) isolated 18% motile *Salmonella* from layer farms in Chittagong region of Bangladesh and *Salmonella* Kentucky was isolated in that geographical location of the country. In our study we have isolated and identified S. Typhimurium in the layer farms of Mymensingh region. To the best of our Knowledge, only two serovars of motile *Salmonella* spp. have been identified in Bangladesh till date. In Bangladesh contaminated animal proteins are used as feed which may be main sources of Salmonella serovars in poultry (Papadopoulou et al., 2009). Farmers purchase the ingredient separately from local markets to produce poultry feed at low-cost. There are many poultry farmers use locally produced feeds from non-registered feed company; hygienic conditions of those feeds are questionable. The *Salmonella spp.* might be entered into poultry farm through vehicles because the same vehicles are used in Bangladesh for transportation of chicken, utensils, eggs and feeds from farms to market and vice versa. These vehicles become contaminated with faeces containing salmonella and transfer the pathogen from farm to market and farm to farm within country and between countries during import and export of poultry and poultry products. Ellerbroek et al., (2010) isolated
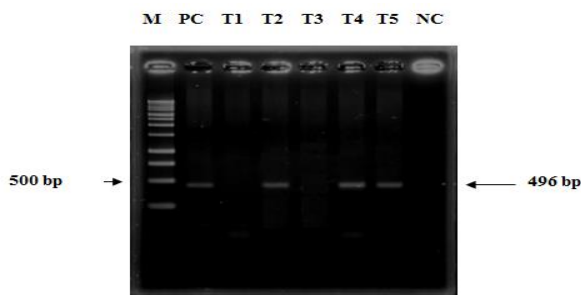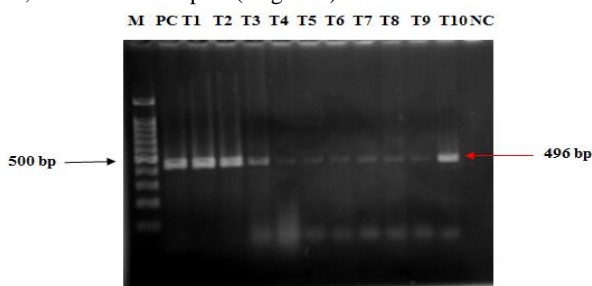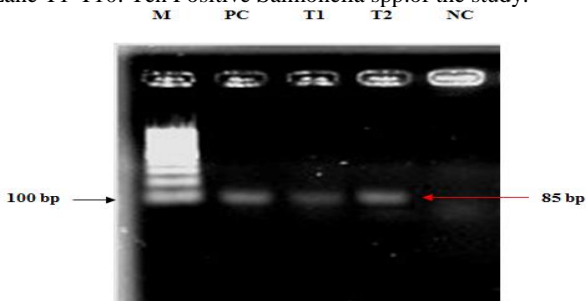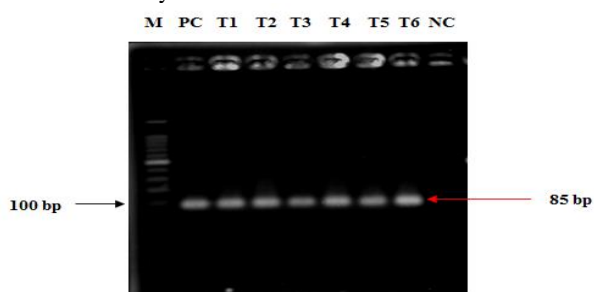
13% Salmonella from 400 imported chicken carcasses in Bhutan with 49.4% from swabs and 34.8% of the lymph nodes of pigs from Vietnam were Salmonella positive.

It is the matter of concern that, we observed high level of resistance (About 87%) toward ampicillin, which is alarming for Bangladesh because of first choice of drug for treatment of systemic salmonellosis in humans until 1980s (Karki et al., 2013). Zou et al. (2012) reported that only 2.35% of the Salmonella isolated from North Carolina, USA were resistant to ampicillin and Fernandez et al. (2003) reported 13.6% isolates from Sao Paulo State of Brazil to be resistant to the same antibiotic. They did not observe Salmonella isolates resistant to ciprofloxacin but in our study about 13% isolates were resistant to this antibiotic. This situation in Bangladesh has been arises due to continuous use of antibiotics without proper guidance from registered veterinary practitioner. The farmers use antibiotics as growth promoter and for the treatment of different diseases without any proper guideline by the respective authorities, where they don't maintain proper dose and accurate antibiotics which leads to emergence of drug resistance pathogen in the environment. The accurate dose of antibiotic to poultry or any other animal should be carefully controlled otherwise it will become a great global problem because of wide host range of *Salmonella* spp. and its serious zoonotic significance.

Conclusion

The present study showed the presence of multidrug resistant S. Typhimurium in commercial poultry farms of Bangladesh which is the matter of concern for the authorities since food contamination and food borne pathogens are the global problem. To control multidrug resistant *Salmonella* spp. farmers should careful about the appropriate use of antimicrobials in poultry and need regular monitoring by the authorities

Acknowledgements

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