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ORIGINAL RESEARCH

Molecular characterization and antimicrobial resistance patterns of *Salmonella spp.* and *Escherichia coli* of laying chicken

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ABSTRACT

Multidrug resistant (MDR) Gram-negative bacteria are the most common causes of diseases in commercial poultry, and antibiotic resistance of these organisms is often plasmid mediated. In Bangladesh such types of data are very much scarce. In this study, the antibiogram profile of *Salmonella spp* and *E. coli* isolates from 20 either clinically affected or dead laying chicken obtained from 10 commercial layer farms was performed. And MDR pattern was determined by using 7 common antimicrobials followed by isolation of plasmids to correlate between plasmids and drug resistance. Of these tested samples, 70-100% of both *Salmonella Spp* and *E. coli* were resistant to β-lactam antibiotics (ampicillin, amoxicillin, and penicillin) cephalexin and cotrimoxazole while 60-90% isolates of both species were susceptible to both ciprofloxacin and gentamicin. Isolates of both *Salmonella spp* and *E. coli* contain plasmids above 10 kbp size which might contain MDR genes. This is the first report on the characterization of plasmids found in both *Salmonella spp* and *E. coli* isolates obtained from a significant number of commercial layer farms (N=10) in Chittagong District, Bangladesh. The gathered information furthers our understanding of the mechanisms of drug resistance in specific region related to other parts of the country and world. The large plasmids might be potential factors for dissemination of antibiotic resistance genes regionally.

Key Words: Chicken, Drug resistance, *E. coli*, *Salmonella spp.* Plasmid

Introduction

Antimicrobial resistance in bacterial pathogens has drawn much attention as one of the greatest threats to public health globally (Levy and Marshall, 2004). It is equally important to both human and veterinary medicine. Although, in veterinary medicine practices, commercial poultry production in most of the developing countries like Bangladesh has been playing an important role as a growing industry, hygienic standards are not strictly followed and enforced. Thus many food borne microorganisms enter into the human food chain notably *Salmonella spp*. and *E.coli.* These are the common microbial flora of gastrointestinal tract of poultry, human being and other animals and sometimes these become pathogenic to both (Jawetz *et al.,* 1984; Levine, 1987; Steve *et al.*,2005). Consequently, antimicrobials are used as therapeutic agents in clinically affected livestock, as prophylactic and sometimes as growth promoters. And these are the most important factors promoting the emergence, selection and dissemination of antibiotic resistant microorganisms in both veterinary and human medicine (Witte, 1988; Neu, 1992). Moreover, especially in poultry industry, indiscriminate use of antimicrobials for different purposes leads to emergence of multidrug resistance bacteria and caused high fatality rate especially in immune-compromised individuals (Holmberg *et al.,* 1984). Therefore, antimicrobial resistance patterns in both human and animals are imperative to characterize for control and prevention of spreading multidrug-resistant bacterial strains (Duijkeren *et al.,* 2003). A quiet number of pathogenic microorganisms are found in poultry of which both *Salmonella spp.* and *E. coli* is common food borne pathogens. Unlike other parts of the world, the genetic features of the drug resistance in Gram-negative *Enterobactriaceae* from specific locations such as Bangladesh especially in greater Chittagong district have not been studied due to constrained resources.

Dissemination of antimicrobial resistance genes among bacterial strains depends on several factors, and plasmid-mediated horizontal transfer of multidrug resistance genes has been considered as one of the most important mechanisms for obtaining drug resistance by the microbes (Zhao, *et al.,* 2010; Martinez and Baquero, 2002; Davies and Davies, 2010). Earlier studies shows that the genes found in many Gram-negative bacilli encode A, B, and D β-lactamases that mediate resistance to various β-lactam antibiotics have been found on plasmids (Navon-Venezia *et al.,* 2006; Poirel *et al.,* 2010; Carattoli *et al.,* 2012). Moreover, plasmids conferring resistance to quinolones and/or aminoglycosides have been reported (Carattoli, 2009; Miro *et al.,* 2010). In this study, we investigated the susceptibility patterns of commonly used 7 different antimicrobials to *Salmonella* spp. and *E. coli* isolates followed by plasmids isolation from both antimicrobial resistant *Salmonella* spp. and *E. coli* to identify correlations between plasmids and drug resistance attained these isolated organisms.

Materials and methods

Sample collection

Either dead or clinically affected layer chicken were collected from 10 commercial layer farms from a previously selected 30 commercial layer farms under Chittagong District. After sacrifice and postmortem examination, liver samples were collected from the suspected chicken and subjected to various biological tests.

Isolation of bacteria and bacteriological analysis

The samples were analyzed within 2-6 hours of collection. The different bacteriological culture media such as Nutrient Agar (NA), Nutrient Broth (NB), Salmonella-Shigella Agar, Brilliant Green Agar, Eosin Methylene Blue Agar, Mc Conkey Agar and Deoxycholate Hydrogen Sulfide Lactose Agar were prepared separately.

For sub culturing, the colonies of the NA media were inoculated in the selective media by looping for the identification of *Salmonella spp.* and *E. coli* from the different samples and were incubated at 37°C for overnight. On the other hand, the samples of the NB media were inoculated to all the selective media by looping from the different samples. Samples were inoculated into the selective media and incubated at 37°C for overnight.

Determination of multidrug resistance pattern

Bacterial susceptibility to different antimicrobial agents was measured *in vitro* by employing the modified Kirby-Bauer (Bauer *et al.,* 1966)

Table 1. Antimicrobial susceptibility patterns of *Salmonella spp.* and *E. coli of* chicken

Antimicrobial agents(µg)	Susceptibility patterns of bacteria to antimicrobials (N=20)									
	Strongly sensitive %		Moderate sensitive %		Weakly sensitive %		Resistant %		Total %	
	Salmonella spp	*E. coli*	*Salmonella spp*	*E. coli*	*Salmonella spp*	*E. coli*	*Salmonella spp*	*E. coli*	*Salmonella spp* (N=10)	*E. coli* (N=10)
Ampicillin	-	-	-	-	-	-	100	100	100	100
Amoxicillin	-	-	-	-	10	10	90	90	100	100
Penicillin	-	-	-	-	10	-	90	100	100	100
Cotrimoxazole	10	-	10	-	-	-	80	100	100	100
Ciprofloxacin	80	50	-	-	-	10	20	40	100	100
Gentamicin	90	50	10	-	-	10	10	40	100	100
Cephalexin	10	-	20	-	-	20	70	80	100	100

method by measuring zone sizes (in mm). Commercially available antibiotics discs (Becton Dickinson, USA) were used for the test. The antibiotics discs used in this study included ciprofloxacin (5µg), penicillin (10µg), ampicillin (10µg), and gentamycin (10µg). *E. coli* ATCC 25922 was used as control. By the standard method of inoculation, the top of a single and well-isolated colony was touched with a sterile loop and the growth was inoculated into 2 ml of Mueller–Hinton broth. The broth culture was then allowed to incubate at 37°C for 4 hours to obtain the young culture. The turbidity of actively growing broth cultures was then adjusted to a 0.5 McFarland standard and then a sterile cotton swab was dipped into the adjusted suspension within 15 minutes and excess broth was purged by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab was then spread evenly over the entire surface of the plate of Luria-Bertani agar to obtain uniform inoculums. The plates were then allowed to dry for 3 to 5 minutes. Antibiotics impregnated discs were then applied to the surface of the inoculated plates with sterile forceps. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface. Even distribution of discs and minimum distance of 24 mm from center to center were ensured. Five discs (four antibiotics discs and one blank disc as control) were placed in each petri dish. Within 15 minutes of the application of the discs, the plates were inverted and incubated at 37°C. After 16 to 18 hours of incubation, the plates were examined, and the diameters of the zones of complete inhibition to the nearest whole millimeter were measured. The zone diameter for individual antimicrobial agents was then translated into susceptible, intermediate and resistant categories according to the interpretation table of the Becton Dickinson Microbiology Company, USA.

Plasmid isolation

Plasmid DNA was extracted according to the alkaline lysis method with modification (Talukder *et al.,* 2002). The molecular weight of the plasmid DNA was determined by comparison to the electrophoretic mobility of plasmids of known molecular weight using *E. coli* PDK-9 (Haider *et al.,* 1989).

Results

A total number of 20 bacterial isolates from either clinically affected or dead laying chicken of both *Salmonella spp.* (n=10) and *E. coli* (n=10) were tested for their antimicrobials susceptibility, using the agar disk diffusion methods (Table 1). The antimicrobial sensitivity of both *Salmonella spp* and *E. coli* were categorized as strong sensitive, moderate sensitive, weakly sensitive and resistant. Of the tested samples, *E. coli* (100%) was resistant to ampicillin, penicillin and cotrimoxazole; 40% *E. coli* was resistant to both ciprofloxacin and gentamicin; and 80% *E. coli* was resistance to cephalexin. On the other hand, *Salmonella spp.* showed resistance to ampicillin (100%), amoxicillin (90%), penicillin (90%), cotrimoxazole (80%), cephalexin (70%) ciprofloxacin (20%) and gentamicin (10%), respectively.

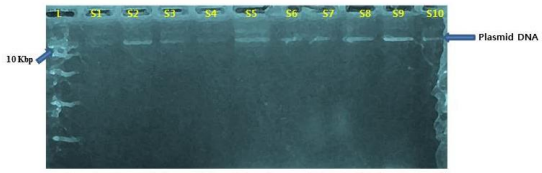

Fig. 1. Plasmid isoltaes of *E. coli* from laying chicken in 1.5% agarose gel electrophoresis. L=DNA Ladder, S1-S10= Sample 1–Sample 10: Plasmids isolated from laying chicken.

Our studies revealed that the *Salmonella* spp. and *E. coli* isolates were highly resistant to various antimicrobials, as mention in the table 1. In order to determine whether some antimicrobial resistance is plasmid-encoded or not, we extracted plasmids from different isolates (10 samples from each species). Extracted plasmids were run on 1.5% agarose gel electrophoresis. These results showed that 90% *E. coli* and 80% *Salmonella spp.* of the isolates of both *Salmonella spp.* and *E. coli* contain plasmids, which molecular weight greater than 10 kbp in size as shown in the figure 1 and 2. These types of big plasmids could contain lot of genes including antimicrobial-resistant ones (Huang *et al.,* 2012).

Discussion

To our knowledge, this is the first study shows correlation between MDR patterns and molecular size of plasmids obtained from both *Salmonella spp.* and *E. coli* isolates from commercial chicken in Chittagong region. In this study we examined antibiotic resistance patterns in *Salmonella spp.* and *E. coli* isolates from either clinically affected or dead laying hen obtained from commercial layer farms. Of the tested samples, both *Salmonella spp.* and *E. coli* isolates were highly resistant to β-lactam antibiotics (ampicillin, amoxicillin and penicillin), however, Tricia *et al*., (2006) observed both ampicillin (85.7%) and amoxicillin (96.4%) were susceptible to *E. coli* obtained from broiler chicken compared with the other antimicrobial agents used in this study, these dissimilarities might be due to long term use ampicillin and amoxicillin in layer feed as preventive measure, and low levels of resistance were also observed for ciprofloxacin and gentamicin as well. Among these tested samples, in the current study, 10-20% *Salmonella spp*. and 40% *E. coli* isolates were resistant to ciprofloxacin and gentamicin, respectively. The prevalence of gentamicin resistance in the present study does not coincided with the earlier study, who reported 100% avian isolates were susceptible to gentamicin (Tricia *et al.* 2006). In the previous study showed 20.6% and 2.9% of *E. coli* isolates of poultry were resistant to ampicillin and amoxicillin, respectively (Tricia *et al*., 2006). But in our study, *E. coli* showed 100% resistance to ampicillin and 90% resistance to amoxicillin. Likewise, *Salmonella spp.* showed similar type of resistance to both ampicillin and amoxicillin. Although, ciprofloxacin and gentamicin were highly susceptible to *Salmonella spp*. and *E. coli* isolates in broiler chicken (Tricia *et al.,* 2006), only 60% of *E. coli* isolates showed sensitivity to ciprofloxacin. Resistance of the tested antimicrobials in layer chicken could be due to repeated exposure in their lifetime.

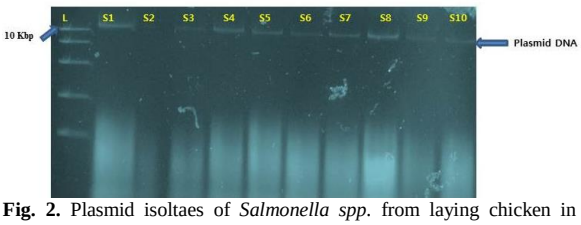

Fig. 2. Plasmid isoltaes of *Salmonella spp.* from laying chicken in 1.5% agarose gel electrophoresis. L=DNA Ladder, S1-S10= Sample1–Sample 10: Plasmids isolated from laying chicken.

There is strong evidence that the use of antimicrobial agents can lead to the emergence and dissemination of resistant *Salmonella spp.* and *E. coli* (David *et al.,*2001; Tricia *et al*., 2006) which can then be passed on to people via food or through direct contact. The present study revealed the patterns of multidrug resistance by both *Salmonella spp.* and *E. coli*

isolates of laying chicken to ampicillin (100%/100%), amoxicillin (90%/90%), penicillin (90%/100%) cotrimoxazole (80%/100%) and cephalexin (70%/80%) which are in agreement with the findings of Chowdhury *et al.*, (2009). To deal with multi-drug resistant organisms, it is usually recommended that potentially synergistic antimicrobials combinations would be useful. Both *Salmonella spp.* and *E. coli* were strongly susceptible to both ciprofloxacin and gentamicin but the same isolates were 100% resistant to ampicillin. Interestingly, both organisms showed a similar resistance patterns and possess more than 10 kbp plasmid sizes whereas Steve *et al.,* (2005) found that *Salmonella spp* and *E. coli* isolates from meat based fast food contains more than 15 kbp plasmids. It can be conferred that plasmid encoded resistance genes to antimicrobials is a significant public health concern in our country since there are possibilities to transfer of resistant genes between bacteria and natural habitats.

Conclusion

In conclusion, 70-100% of both *Salmonella spp.* and *E. coli* were resistant to β-lactam antibiotics (ampicillin, amoxicillin, and penicillin), cephalexin and cotrimoxazole while 60-90% isolates of both species were susceptible to ciprofloxacin and gentamicin. In the current study, both *Salmonella spp* and *E. coli* contains plasmids which molecular weight above 10 kbp. Further study is warranted to identification of genes responsible for antimicrobial resistance.

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Conflict of interest

The authors have declared no conflict of interest

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