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Short Communication

Molecular Basis of Tetracycline Resistance in *Escherichia coli* Isolates Recovered from Poultry Drinking Water

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Abstract

The present study was carried out to examine the molecular basis of tetracycline resistance in the *E. coli* isolates recovered from poultry drinking water. Samples (n=50) were collected from poultry drinking water and processed for microbial and biochemical characterization. Antibiotic resistance of *E. coli* isolates was examined by disk diffusion method. Twenty five tetracycline resistant *E. coli* were isolated using 30 µg/mL antibiotic, in the growth medium. Plasmid DNA was extracted from the resistant isolates. The tetracycline resistance genes were amplified and sequenced. The prevalence of *tet*A, *tet*B, *tet*C, *tet*D, *tet*E and *tet*G genes was examined. The results demonstrated that 32% of the total isolates contained *tet*A gene where as 68% contained both *tet*A and *tet*B genes together. None of the isolates contained the *tet*C, *tet*D, *tet*E and *tet*G gene alone or in combination. We reported the existence of more than one *tet* genes in the same strain at a frequency higher than that reported in previous studies.

Keywords: Antibiotic resistance; *Escherichia coli*; Poultry drinking water; Tetracycline resistance; *tet* genes

Introduction

Escherichia coli is a common rod-shaped gram negative bacterium that commonly resides in the intestine of warm-blooded animals. Many of the strains of *E. coli* are nonpathogenic, whereas some serotypes that are pathogenic cause serious food poisoning in human. In the gut, harmless strains of *E. coli* are the part of its normal flora and thereby they can benefit their hosts by the production of vitamin K_2 and prevention of the establishment of the pathogenic bacteria inside the intestine (Hudault *et al*., 2001).

Several antibiotics are used in avian medicine for treatment of infectious diseases and fed at sub therapeutic levels as growth promoters. Due to selection pressure, antibiotic usage results in the development of antimicrobial resistance in bacteria. When animals harboring multiple antibiotic resistant bacteria are utilized as food, there is a possibility of development of antibiotic resistant bacteria in humans (Wilkerson *et al*., 2004). The growth of antibiotic resistant bacteria can be inhibited by high concentrations of antimicrobials, but the patient, sometime, would not tolerate such high concentration (Hawkey, 1998).

A low dosage, continuous administration of antibiotics is associated with the higher risk of the development of antimicrobial resistance as compared to their high dosage but a short term therapeutic use. There are also concerns about promoting the horizontal gene transfer between pathogenic and commensal bacteria due to the sub therapeutic administration of antibiotics (Mirzaagha *et al*., 2011).

Misuse of antibiotics is a major contributory factor towards the prevalence of resistance in bacteria. Tetracycline is one of the members of the family of broad spectrum antibiotics. Its low cost, efficacy together with a low frequency of side effects make this antibiotic as one of the most popularly used antibiotics in livestock and poultry farming as well as in aquaculture. Widespread and unjudicious use of tetracycline can potentially lead to emergence of antibiotic resistance in bacteria. The main mechanisms causing resistance to tetracycline are ribosomal protection, active efflux system and enzyme inactivation. Of these mechanisms in Gram negative bacteria, an efflux pump system encoded by the genes *tet*A, *tet*B, *tet*C, *tet*D, tetE and *tet*G is perhaps the most important (Skockova *et al*., 2012). The aim of the present study was to examine the presence of tetracycline resistance genes in *E. coli* isolates recovered from poultry drinking water and the prevalence of *tet*A, *tet*B, *tet*C, *tet*D, *tet*E and *tet*G genes in the local population of *E. coli.* To best of our knowledge, this is the first Pakistani report on the elucidation of molecular basis of tetracycline resistance in *E. coli* isolates recovered from poultry drinking water.

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Materials and Methods

Sample Collection

A total of 50 samples from poultry drinking water were collected in sterile glass vials from 50 randomly selected poultry farms in district Lahore, Pakistan. The samples were placed on crushed ice immediately after collection and transported to the laboratory of Department of Microbiology, University of Veterinary and Animal Sciences, Lahore within two hours.

Isolation and Identification of *E. coli* Isolates

Immediately after arrival in the lab, 0.1 mL of 1:10 dilution of each water sample in sterile water was spread on MacConkey's agar plates. The inoculated plates were incubated at 37°C for 24 h. Cultures were purified by repeated streaking. A single pure colony from each plate was inoculated onto MacConkey's agar plates containing tetracycline (30 µg/mL). The tetracycline resistant growth of suspected *E. coli* colonies was subjected to Gram staining. The presence of *E. coli* was confirmed by growing the isolates on Eosin methylene blue agar medium. Gram negative colonies that grew on this medium were subjected to biochemical tests (Indole and oxidase production tests, methyl red test, Voges Proskauer test and citrate utilization test) to confirm the colonies as *E. coli*. Furthermore, *E. coli* isolates were completely identified by following the identification flow charts of the Burgey's Manual for Determinative Bacteriology (Zarchi and Vatani, 2009).

Antibiotic Sensitivity of Isolates

The isolates were subjected to *in vitro* antibiotic sensitivity by disk diffusion method (Bauer *et al*., 1966; Clinical Laboratory Standards Institute, 2008).

Plasmid Profiling and DNA Quantification

The newly isolated tetracycline resistant *E. coli* strains were inoculated in LB medium (1% Tryptone, 0.5% Yeast extract and 0.5% NaCl) containing 30 µg/mL of tetracycline. The medium was incubated overnight at 37°C and this growth was utilized for the isolation of plasmid DNA by an alkaline lysis procedure (Birnboim and Doly, 1979; Horowicz and Burke, 1981). Plasmid DNA was electrophoresed in 1% electrophoresis grade agarose (Sigma, St. Louis, USA). Spectrophotometer (Thermoscientific, Wilmington state, USA) was used for the quantitative analysis of plasmid DNA.

Amplification of Tetracycline Resistance Genes

Tetracycline resistance genes (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E and *tet*G) were amplified by gradient PCR at a temperature range of 50-60°C using already reported primers (Zhang and Zhang, 2011). The amplified PCR product was purified from the gel by DNA purification kit (Fermentas, Life Sciences, USA) and this purified product was utilized for DNA sequencing by dideoxy chain termination method (Sanger and Coulson, 1975).

Bioinformatics Analysis of the DNA Sequences

The obtained genes sequences were utilized for comparison with the already reported tetracycline resistance genes in *E. coli* as well as with those of bacteria other than *E. coli.* Sequence homology and phylogenetic analysis was made using Bioinformatics softwares (ClustalW and MEGA 5.05 respectively).

Results

Culturing of poultry drinking water samples (n=50) on MacConkey's agar in the presence of tetracycline resulted in the appearance of growth of 25 Gram negative isolates. Their growth on Eosin methylene blue agar with appearance of green metallic sheen, positive for indole and oxidase production, methyl red test and negative Voges-Proskauer and citrate utilization tests confirmed these isolates as those of *E. coli.* All isolates were resistant to tetracycline (Fig. 1).

The PCR for various tetracycline resistance genes resulted in the amplification of *tet*A (210 bp) and *tet*B (659 bp) genes. It was found that 8 of 25 (32%) isolates contained *tet*A gene whereas 17 of 25 (68%) isolates showed the presence of both *tet*A and *tet*B genes. None of the isolates contained the *tet*C, *tet*D, *tet*E or *tet*G gene alone or in combination (Fig. 2).

The phylogenetic tree obtained as a result of comparison of *tet*A gene from the present study with the already reported *tet*A in bacteria other than *E. coli* demonstrated that the newly isolated poultry water *E. coli* isolates were close to various strains of *Acinetobacter baumannii, Salmonella enterica, Shigella sonnei, Serratia marcescens, Klebsiella pneumoniae* in clade B with a bootstrap value of 100 (Fig. 3A).

The *tet*A sequence was compared with that of already reported *E. coli* strains, (Fig. 3B) explains the relation of newly isolated poultry drinking water *E. coli* with the reported strains. The poultry drinking water *tet*A gene in *E. coli* isolates again clustered in clade B. Phylogenetic analysis of *tet*B gene with that of already reported non-*E. coli* bacteria showed a maximum homology with various species of *Actinobacillus, Salmonella, Pasteurella* and *Shigella* having a bootstrap value of 65 in clade A (Fig. 4).

Discussion

Antibiotic resistance in *E. coli* is becoming a global problem. Development of new strategies to combat this problem requires an understanding of the molecular basis of resistance, its transmission and acquisition (Angulo *et al*., 2004).

Fig. 1: Determination of tetracycline resistance by disk diffusion method. The growth was examined in LB medium. The disks contained tetracycline at a final concentration of 30 µg/disc

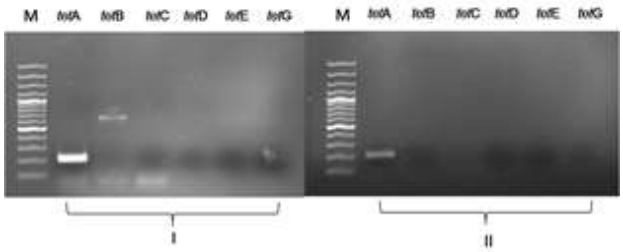

Fig. 2: Ethedium bromide stained agarose gel showing the PCR amplification of *tet*A and *tet*B genes. Lane M is the 100 bp molecular weight marker (Fermentas, Life Sciences, USA); I is the sample I showing the amplification of *tet*A and *tet*B genes and II is the second sample showing the amplification of *tet*A gene

In 2005-2006, the frequency of *tet*A and *tet*B genes was found to be 8.1 and 86.5%, respectively. This frequency changed to 81.3 and 18.8% for *tet*A and *tet*B within a period of five years (Skockova *et al*., 2012). Similarly, Korean scientists, Koo and Woo (2011) reported that *tet*A is the most frequent gene (52.4%) in tetracycline resistant *E. coli* followed by *tet*B gene (41.3%). In the present study 32% of the isolates contained *tet*A gene, while we could not detect the presence of *tet*B gene alone but existed only in combination with *tet*A gene having a frequency of 68%. We here report that more than one *tet* genes could be found within the same strain at a higher frequency as compared to *tet* genes alone. The co-occurance of *tet*A and *tet*B gene may be supported by the results of Koo and Woo (2011) but they could find only 2 isolates having both the genes. We couldn't find any *E. coli* isolate with *tet*C, *tet*D, *tet*E or *tet*G gene alone or in combination. These findings are in agreement with results reported by Skockova *et al.* (2012) whereas Koo and Woo (2011) could find the *tet*C gene but at a very low frequency of 1.7%. Our results are at a variance with those of previous reports that reported a higher frequency of *tet*B (Ryu *et al*., 2012) or *tet*C genes (Srinivasan *et al*., 2007).

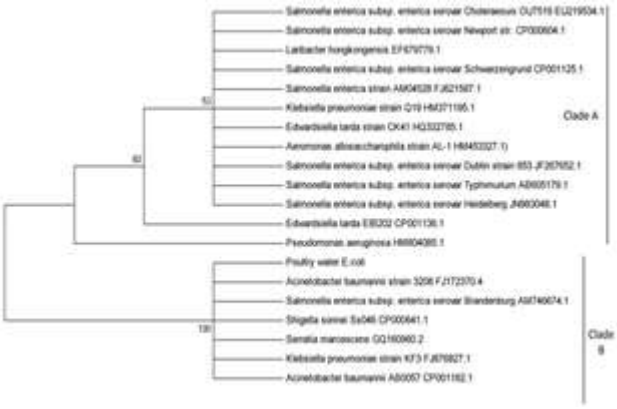

Fig. 3A: Traditional rectangular bootstrap consensus tree of *tet*A gene from poultry water *E. coli* (from present study) with the already reported bacteria other than *E. coli.* Deduced common ancestors are presented by the nodes and bootstrap value is presented by the numerical at the nodes

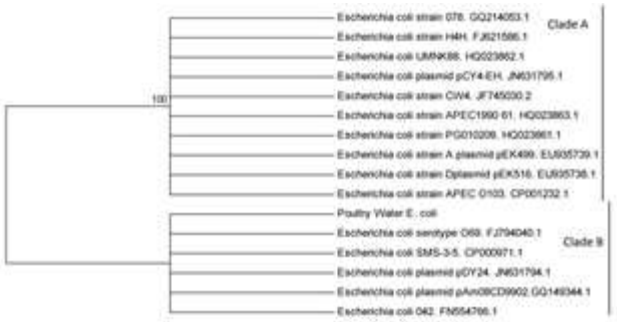

Fig. 3B: Traditional rectangular bootstrap consensus tree of *tet*A gene from poultry water *E. coli* (from present study) with the already reported *E. coli* strains. Deduced common ancestors are presented by the nodes and bootstrap value is presented by the numerical at the nodes

Fig. 4: Traditional rectangular bootstrap consensus tree of *tet*B gene from poultry water *E. coli* (from present study) with the already reported bacteria other than *E. coli.* Deduced common ancestors are presented by the nodes and bootstrap value is presented by the numerical at the nodes

In conclusion, this is the first report on the elucidation of molecular basis of tetracycline resistance in *E. coli* isolates recovered from poultry drinking water from Punjab, Pakistan. Our results are novel as we have reported

that the local population of tetracycline resistant *E. coli* isolates contained higher frequencies of *tet*A and *tet*B genes in combination rather solely.

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