RESEARCH ARTICLE AGROBIOLOGICAL RECORDS
ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

STUDY ON THE GENOTYPIC AND PHENOTYPIC RESISTANCE OF TETRACYCLINE ANTIBIOTIC IN *ESCHERICHIA COLI* STRAINS ISOLATED FROM FREE RANGING CHICKENS OF ANHUI PROVINCE, CHINA

Khalid Mehmood [1], Rana Muhammad Bilal[1] and Hui Zhang [2],*

[1]Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur-63100, Pakistan
[2]College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, Guangdong, China

*Correspondence: hz236@scau.edu.cn

ABSTRACT

Poultry industry is growing day by day and it is a major and cheap source of animal protein for human beings. Various bacteria cause serious illness and variety of diseases in poultry birds. The use of tetracycline antibiotics has been increased 4 times in recent years to treat such diseases, leading to the development of drug resistant bacteria in veterinary medicine. This study was conducted to evaluate the resistance profile of tetracycline in avian *Escherichia coli* strains isolated from free ranging chickens of Anhui province, China. For this purpose, disk diffusion method was used to examine the antimicrobial susceptibility of *Escherichia coli* strains (n=203) against tetracycline, minocycline, and doxycycline. However, PCR analysis was utilized for detection of the tetracycline resistant genes; *tetA, tetB, tetC, tetG* and *tetM*. The overall frequency of phenotypic resistance was 29.1%, which was highest to tetracycline (14.3%), followed by minocycline (6.9%), and doxycycline (7.9%). Whereas the genotypic resistance rate was 24.1%, which include 10.3%, 7.9%, 4.4%, 1.0% and 0.5% resistance rate of *tetA, tetG, tetC, tetA + tetC + tetG, and tetA + tetC* genes, respectively. Conversely, no isolates were positive for the *tetB and tetM* genes. The rate of phenotypic resistance (29.1%) was almost in line with genotypic resistance rate (24.1%). Our study demonstrates that chickens are not important contributors to bacterial resistance in an extensive farming system. As such, restrictions on the use of antibiotics could prevent the emergence of resistant pathogens. Furthermore, this is first study of the occurrence of antibiotic resistant *E. coli* strains in free ranging chickens of Anhui province, China.

Keywords: Avian *Escherichia coli*; disk diffusion method; tetracycline resistance; PCR

Article History (2020-0723) || Received: 07 Jul 2020 || Revised: 23 Jul 2020 || Accepted: 31 Jul 2020 || Published Online: 10 Aug 2020

1. INTRODUCTION

Poultry industry is growing day by day and it is a major and cheap source of animal protein for human beings (FAO 2015; Ayandiran et al. 2018). *E. coli* is a Gram-negative bacteria which belongs to genus *Escherichia*. It is an important part of normal micro-flora of many species specially chickens (Sciberras et al. 2019). This bacterium is a major threat to commercial poultry production along with public health and food safety issues worldwide. It is also considerably contributing towards economic losses in poultry and turkey farming by producing variety of disease in these animals (Mohammadi et al. 2018; Sciberras et al. 2019). *E. coli* can commonly produce swollen head syndrome, colibacillosis and yolk sac infection in chickens (Gharajelar and Zare 2017).

Antibiotics are those medicines which are widely used worldwide to handle and treatment of bacterial disease. In poultry industry, antibiotics are commonly used as an antimicrobial agents, growth promotors and to enhance the production in commercial farming (Gharajelar and Zare 2017). The long term and rational use of antibiotics may lead to development of resistant bacterial strains and antibiotics resistance (Theobald et al. 2019). The antibiotic resistance in commercial poultry farms can spread to humans' beings through food chain and contamination of poultry waste materials through environment (Velhner and Milanov 2015). The predictable usage of antibiotics in food animals is shocking as shown by the growing amount of manure pollution; 298 million tons were estimated for 2020. So, lacking any actual control, antibiotic consumption in chickens is probable to be increased by 143% from 2010 to 2030 (Van Boeckel et al. 2015; Xu et al. 2020).

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

Tetracycline is one of those broad-spectrum antibiotics, which is widely used in the prevention and control of poultry diseases. Thus, more than 40 kinds of tetracycline resistant genes have been identified, including inactivated enzyme gene, ribosomal protection gene and so on (Ljubojevic et al. 2016). Such imprudent tetracycline usage has increased the resistance to tetracycline antibiotics in animals by 4 times. The resistant genes of tetracycline (*tetA, tetB*) have been found in healthy chickens (Zhang et al. 2010). Therefore, the study on the resistance pattern of antibiotics is of great importance for researcher to control and prevent human and animal diseases. Furthermore, the results will help veterinarians and relevant authorities in development of sensible guidelines to reduce the emergence and spread of antibiotic resistance in bacteria originated from free-range poultry.

Antibiotic resistance is not monitored in many countries and accessible data is quite scattered. Currently, it is difficult to assess the impact of free ranging livestock-related antibiotic resistance on public health due to limited data and only a few studies are available about antibiotic sensitivity in free range poultry birds. Therefore, this study was planned to characterize phenotypic and genotypic characteristics of antimicrobial resistance and also compare the rate of tetracycline resistance in extensive farming system with intensive farming system.

2. MATERIALS AND METHODS

2.1. Samples Collection and Processing

In this study, a total of 203 *E. coli* strains were isolated from fecal samples of free ranging chicken of Anhui province (Huaibei, Suzhou, Bengbu, Bozhou and Fuyang), China between August 2015 to December 2015. It is mentioned that all the free ranging chickens were not exposed to antibiotics as growth promoter or disease prevention. However, raw water was their drinking source and grain or human's food waste as feed source. We examined *E. coli* isolate in each fecal sample. Initially, all the samples were cultured on nutrient broth for enrichment purpose and then MacConkey agar was used for streaking purpose. After incubation, we selected pink color colonies from MacConkey agar and for inoculation purpose on eosin methylene blue (EMB) agar, when greenish metallic color colonies were seen on the EMB agar, it was confirmed *E. coli.* One suspected colony from each plate was collected and stored in glycerol at -80 °C. *E. coli* strains were further confirmed by Gram staining method and biochemical tests which include citrate utilization, urease production, catalase, indole production, motility, methyl red and Voges-Proskauer.

2.2. Antibiotics Testing

The profile regarding sensitivity of antibiotics for each *E. coli* strain was confirmed through disk diffusion methods as per given standards defined by Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2014) for the tetracycline, minocycline, and doxycycline antibiotics. Briefly, Muller-Hinton Agar plates containing tetracycline (30µg), minocycline (30µg), and doxycycline (30µg) disks (placed 25 to 30mm intervals on the medium) were incubated at 35 °C in 5% CO_2 for 20-24 hr, as per the criteria described by CLSI. Plates were evaluated for inhibition zone diameters by measuring the zone of inhibitions (mm) through the scale according to previous studies (Seifi and Khoshbakht 2016).

2.3. PCR Amplification of Resistance-Associated Genes

Genomic DNA extraction was performed from each samples and strain through boiling method according to previous study (Yu et al. 2015). All samples were then processed for PCR examination for recognition of tetracycline resistant genes (*tetA, tetB, tetC, tetG* and *tetM*) by applying previous primers and methodology with some modifications (Dayao et al. 2016). Amplification process of DNA was performed in 25µL volumes comprised of 5µL DNA, 12.5µL 2X reaction buffer, 1µL of very primer, 0.2µL golden DNA polymerase, and 5.3µL ddh_2O by using thermocycler (Applied Biosystems, Waltham, MA, USA). The situations of different steps were following denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C and each cycle was 40 sec, annealing at 55°C for 40 sec, and finally extension was performed at 72°C for 50 sec, while final extension was at 72°C for 10 min.

2.4. Gel Electrophoresis and DNA Sequencing

Gel electrophoresis was performed for the separation of amplified product, which was performed and quantified in 1% agarose gel. The marker was also run in agarose gel to determine and compare the band size. The desire band was obtained and observed in gel doc apparatus (SynGene, UK). The forward and reverse strands of all PCR products were sequenced by a commercial company (Qingke, China) and the nucleotide sequences obtained in this study were compared with previously reported tetracycline resistant sequences available at NCBI database by using the DNASTAR software suite (version 3.3.8; DNASTAR).

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

3. RESULTS

3.1. Antibiotic Resistance Profiles of Avian *E. coli* Isolates

Among the 203 faecal *E. coli* isolates tested, we observed high rates of resistance to tetracycline (14.3%), followed by minocycline (6.9%), and doxycycline (7.9%). The overall rate of resistance to antibiotics was 29.1% (59/203). The antibiotic susceptibility pattern of tetracycline antibiotics is listed in Table 1. From 203 isolates, 161 (79.3%) were sensitive while 13 (6.4%) isolates were intermediate sensitive to tetracycline. Similarly, among 203 isolates 181 (89.2%) were sensitive while 8 (3.9%) were intermediate sensitive to minocycline. In case of doxycycline, 183 (90.1%) were sensitive and 4 (2.0%) were intermediate sensitive.

3.2. Detection and Distribution of Tetracycline Resistant Genes

Of the 203 avian *E. coli* isolates tested, 46 (93.9%) were confirmed for one resistance gene, from which 21 were positive for *tetA*, while 16 were positive for *tetG* and 9 were positive to *tetC*. On the other hand, all the isolated strains were negative for *tetB* and *tetM*. Notably, 3 isolates (6.1%) were positive for two or more virulent genes: *tetA* + *tetC* + *tetG* (2 isolates); *tetA* + *tetC* (1 isolates). Our result indicated that *E. coli* isolates were more resistant to single gene as compared to two or more genes. Table 2 summarizes the distribution and combination patterns of tetracycline resistance genes noticed in *E. coli* strains obtained from free range chickens.

3.3. Sequence Analysis

All the tetracycline resistant isolates shared 97% to 99% nucleotide sequence identity with the previously reported resistant strains (Accession number: KF240812 KX462013 and KJ603216, for *tetA*, *tetC* and *tetG*, respectively). Among these isolates, the similarity for *tetA*, *tetC* and *tetG* genes was 99%, 97% and >98%, respectively (Fig. 1).

Table 1: Antimicrobial resistance rate of *Escherichia coli* strains (n=203) isolated from the feces of free ranging chickens of Anhui province, China.

Antibiotics	Sensitive		Intermediate		Resistant	
	Number	%	Number	%	Number	%
Tetracycline	161	79.3	13	6.4	29	14.3
Minocycline	181	89.2	8	3.9	14	6.9
Doxycycline	183	90.1	4	2.0	16	7.9

All methods were opted according to the standards of Standards Committee of Clinical Labs of USA (CLSI).

Table 2: Distribution and combination patterns of tetracycline resistance genes detected in *E. coli* strains isolated from free range chickens (n=203)

Resistance genes	Resistance rate		Negative isolates	
	Number	%	Number	%
tetA	21	10.3	182	89.7
tetG	16	7.9	187	92.1
tetC	9	4.4	194	95.6
tetA + *tetC* + *tetG*	2	1.0	201	99
tetA + *tetC*	1	0.5	202	99.5

4. DISCUSSION

China's economic growth in animal production make it largest producer and user of antibiotics in the world, and its usage in animals share about more than half of the total consumption (Hvistendahl 2012). So, the high amount of antibiotic practice has a significant effect on the appearance of resistant bacteria spread between animals and humans by direct contact, food borne or indirect contact through the environment (Krishnasamy et al. 2015; Zhang et al. 2015). Antimicrobial resistance is a usual spectacle. The use of tetracycline antibiotics has been increased 4 times in recent years, leading to the development of drug resistant bacteria in veterinary medicine (Ljubojevic et al. 2016). In this study, resistance against tetracycline, minocycline and doxycycline antibiotics was 14.3%, 6.9% and, 7.9%, respectively. Alike to our findings, similar level of resistance to different antibiotics was observed in free ranging poultry from Australia and Bangladesh (Hasan et al. 2011; Obeng et al. 2012). However, in contrast to our findings, high level of tetracycline resistance was observed in *E. coli* strains obtained from farm chickens, in China and different other countries (Table 3).

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

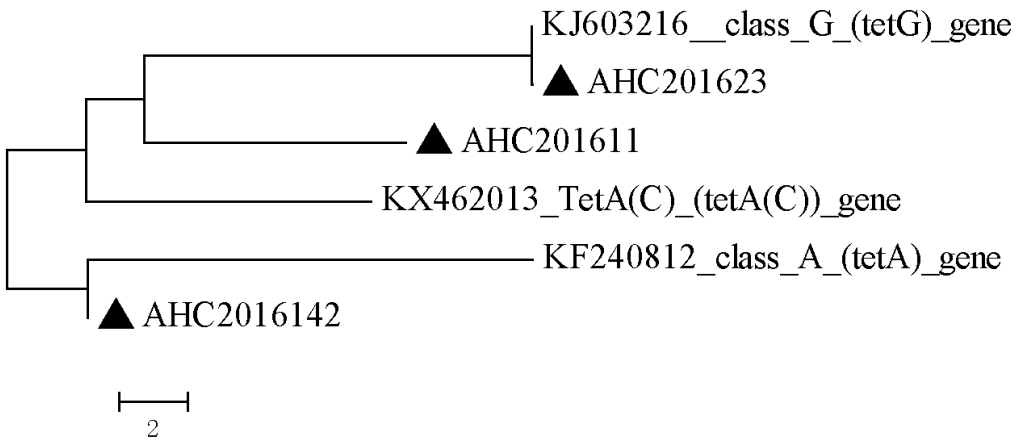

Fig. 1: Phylogenetic tree constructed by the neighbor joining method using MEGA software.

In Brazil it was reported 92.86% by Koga et al. (2019), in China 89.63% (Zhang et al. 2012), in India 88% (Balasubramaniam et al. 2014), in Nigeria 77% (Ayandiran et al. 2018), in USA 71.1% (Tadesse et al. 2012) and in Pakistan 68% (Naseer et al. 2014). Similarly, in other countries like Iran, Korea and South Africa it was reported 54.5%, 46.7% and 36.22-98.4% respectively (Seifi and Khoshbakht, 2016; Hwang et al. 2017; Mohammadi et al. 2018; Theobald et al. 2019). Our study demonstrates that resistance against antibiotics can be reduced through discouraging the overuse and rational use of antibiotics in commercial poultry farming. Moreover, antibiotic resistance up to some extent is also noticed in free range poultry due to use of some antibiotics as growth enhancer, and for the treatment and prevention of important infectious disease in poultry birds in China (Gharajelar and Zare 2017). Secondly, it has been also noticed that this resistance can also be spread through direct contact and contamination of environment, water, food and feces among chickens and between the farm present in the vicinity of the study area (Velhner and Milanov 2015).

Table 3: Distribution of tetracycline resistance (phenotypic or genotypic) detected in poultry from different countries.

Countries/regions	Surveillance (%)	References
Brazil	92.86	Koga et al. (2019)
China	89.63	Zhang et al. (2012)
India	88	Balasubramaniam et al. (2014)
Nigeria	77.0	Ayandiran et al. (2018)
USA	71.1	Tadesse et al. (2012)
Pakistan	68	Naseer et al. (2014)
Iran	54.5	Mohammadi et al. (2018)
Korea	46.7	Hwang et al. (2017)
Bangladesh	45.5	Hasan et al. (2011)
South Africa	36.22-98.4	Theobald et al. (2019)

In this study, we also observed relatively low prevalence (24.1%) of tetracycline resistance genes as compared to intensively farm chickens (Zhang et al. 2012). Notably, the rates of phenotypic and genotypic resistance were almost same; indicating that the disk diffusion method comprises a convenient and effective method for use in analysing *E. coli* strains. The resistance to *tetA*, *tetC* and *tetG* in our study is basically in line with the findings of those other researcher in *E. coli* isolates from chickens and pigs (Wang et al. 2007; Zhang et al. 2010). However, in contrast to our finding, no isolate was positive for *tetC* genes in *E. coli* isolates from chickens, in North China (Zhang et al. 2012). This difference may be because of the difference in region and farming system because in commercial farming more quantity of antibiotics is used as prophylactic measures and growth enhancer compared to free range poultry rearing. However, the low prevalence of tetracycline resistance genes recognized was in consistent with the phenotypic appurtenance of the isolates inspected in this study. The ill use of antibiotics in food animals highlight the suggestions of the development of different types of antibiotic resistant bacteria and their

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

distribution among animals, humans and the environment. The condition has been described in many earlier studies from numerous countries, which have introduced the One Health concept for better considering and addressing health issues round the world (Rousham et al. 2018; WHO, 2014; Xu et al., 2020).

Conclusion: In conclusion, the prevalence of resistant-associated genes among the isolates tested was low compared to that observed in intensively farmed animals in China. Prophylactic use of antibiotics could prevent the emergence of resistant pathogens in farming system.

Author's Contribution: HZ designed the study, conduct analysis, perform the experiment, and write initial draft; KM conduct analysis and write final draft. HZ and KM handled the revision.

ORCID

Khalid Mehmood 0000-0003-4720-8792
Hui Zhang 0000-0002-6844-4375

REFERENCES

Adelaide OA, Bii C and Okemo P, 2008. Antibiotic resistance and virulence factors in *Escherichia coli* from broiler chicken slaughtered at Tigoni processing plant in Limuru, Kenya. East African Medical Journal 85: 597-606.

Ayandiran TO, Falgenhauer L and Schmiedel J, 2018. High resistance to tetracycline and ciprofloxacin in bacteria isolated from poultry farms in Ibadan, Nigeria. The Journal of Infection in Developing Countries 12: 462-470.

Ayeni FA, Odumosu BT, Oluseyi AE and Ruppitsch W, 2016. Identification and prevalence of tetracycline resistance in enterococci isolated from poultry in Ilishan, Ogun State, Nigeria. Journal of Pharmacy & BioAllied Sciences 8: 69-73.

Balasubramaniam A, Arthanari EM, Suresh P and Sukumar K, 2014. Detection of tetracycline resistance determinant tetA gene and antimicrobial resistance pattern in *Escherichia coli* isolates recovered from healthy layer chickens. Veterinary World 7: 635-638.

CLSI, 2014. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; twentieth information supplement. CLSI document M100-S24. Pennsylvania.

Dayao D, Gibson JS, Blackall PJ and Turni C, 2016. Antimicrobial resistance genes in *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, and *Pasteurella multocida*, isolated from Australian pigs. Australian Veterinary Journal 94: 227-231.

Food and Agriculture Organization of the United Nations. Poultry Development Review, 2015. Available at: www.fao.org/docrep/019/i3531e/i3531e.pdf

Gharajelar SN and Zare P, 2017. Monitoring the prevalence of the tetracycline efflux genes among *E. coli* isolated from chicken colibacillosis. Iranian Journal of Veterinary Medicine 11: 235-241.

Hvistendahl M, 2012. Public health. China takes aim at rampant antibiotic resistance. Science 336(6083): 795.

Hwang D, Kim SM and Kim HJ, 2017. Modelling of tetracycline resistance gene transfer by commensal Escherichia coli food isolates that survived in gastric fluid conditions. International Journal of Antimicrobial Agents 49: 81-87.

Koga VL, Maluta RP, da Silveira WD, Ribeiro RA, Hungria M, Vespero EC, Nakazato G and Kobayashi RKT, 2019. Characterization of CMY-2-type beta-lactamase-producing Escherichia coli isolated from chicken carcasses and human infection in a city of South Brazil. BMC Microbiology 19: 174.

Krishnasamy V, Otte J and Silbergeld E, 2015. Antimicrobial use in Chinese swine and broiler poultry production. Antimicrobial Resistance & Infection Control 4: 17.

Ljubojević D, Velhner M, Todorović D and Pajić M, 2016. Tetracycline resistance in *Escherichia coli* isolates from poultry. Arhiv Veterinarske Medicine 9: 61–81.

Lutful-Kabir SM, 2010. Avian colibacillosis and salmonellosis: A closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. International Journal of Environmental Research & Public Health 7: 89-114.

Mohammadi V, Ghaniei A and Sepehrnia P, 2018. Antimicrobial resistance profile and prevalence of tetracycline resistance genes in Escherichia coli isolates from broiler chickens, Northwestern Iran. Bulgarian Journal of Veterinary Medicine 21: 169-175.

Naseer H, Firyal S, Yaqub T, Tayyab M, Anjum AA, Zahoor MY, Wasim M, Mukhtar N and Awan AR, 2014. Molecular basis of tetracycline resistance in *Escherichia coli* isolates recovered from poultry drinking water. International Journal of Agriculture & Biology 16: 227-230.

Obeng AS, Rickard H, Ndi O, Sexton M and Barton M, 2012. Antibiotic resistance, phylogenetic grouping and virulence potential of *Escherichia coli* isolated from the faeces of intensively farmed and free-range poultry. Veterinary Microbiology 154: 305-315.

Rousham EK, Unicomb L and Islam MA, 2018. Human, animal and environmental contributors to antibiotic resistance in low-resource settings: integrating behavioural, epidemiological and one health approaches. Proceedings of the Royal Society B Biological Sciences 285(1876): Article ID: 20180332. https://doi.org/10.1098/rspb.2018.0332

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

Sciberras M, Pipova M, Regecova I, Jevinova P and Demjanova S, 2019. Antibiotic resistance of *Escherichia coli* isolated from broiler chickens. Folia Veterinaria 63: 1-8.

Seifi S and Khoshbakht R, 2016. Prevalence of tetracycline resistance determinants in broiler isolated *Escherichia coli* in Iran. British Poultry Science 57: 729-733.

Tadesse DA, Zhao S, Tong E, Ayers S, Singh A, Bartholomew MJ and McDermott PF, 2012. Antimicrobial Drug Resistance in *Escherichia coli* from Humans and Food Animals, United States, 1950–2002. Emerging Infectious Diseases 18: 741-749.

Theobald S, Etter EMC and Gerber D, 2019. Antimicrobial Resistance Trends in Escherichia coli in South African Poultry: 2009–2015. Pathogens and Disease 16: 652-660.

Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A and Laxminarayan R, 2015. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences 112: 5649–5654.

Velhner M and Milanov D, 2015. Resistance to tetracycline in Escherichia coli and Staphylococcus aureus: brief overview on mechanisms of resistance and epidemiology. Archives of Veterinary Medicine 8: 27-36.

Wang XQ, Wang YH, Wu S, Jiao XA, Pan ZM and Liu XF, 2007. Distribution and spread of tetracycline resistance genes among *Salmonella enterica* isolates from chicken. China Poultry 9: 10-12.

WHO, 2014. Antimicrobial resistance global report on surveillance. Geneva. Available from: https://www.who.int/drugresistance/en/

Xu J, Sangthong R, McNeil E, Tang R and Chongsuvivatwong V, 2020. Antibiotic use in chicken farms in northwestern China. Antimicrobial Resistance and Infection Control 9: 10. https://doi.org/10.1186/s13756-019-0672-6

Yu-Xi Z, Xian-Gan H, Jia-Kun Z, Jian-Sen G, Yue H and Guo-Bo F, 2015. Distribution of lipopolysaccharide core type in avian pathogenic *Escherichia coli* and its correlation with virulence gene. Microbiology China 42: 1619-1625.

Zhang CP, Ning YB and Song LB, 2010. Resistance to tetracycline and distribution of tetracycline resistance determinants in commensal *Escherichia coli* isolated from clinically healthy chickens and pigs. Scientia Agricultura Sinica 12: 2578-2583.

Zhang QQ, Ying GG, Pan CG, Liu YS and Zhao JL, 2015. Comprehensive evaluation of antibiotics emission and fate in the river basins of China: source analysis, multimedia modeling, and linkage to bacterial resistance. Environmental Science and Technology 49: 6772-6782.

Zhang T, Wang CG, Lv JC, Wang RS and Zhong XH, 2012. Survey on tetracycline resistance and antibiotic-resistant genotype of avian *Escherichia coli* in North China. Poultry Science 91: 2774-2777.