ORIGINAL ARTICLE

Biochemically and Genetically Evaluation of Antibiotic Resistance of *E. coli* Isolated from Egg Yolk Distributed in Guilan Province, Iran

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ABSTRACT

Aim: The use of antibiotics in poultry can lead to the public health risks.In this study, the antibiotic resistance of *E. coli* pathogens isolated from commercial eggs distributed in Guilan province (Iran) is investigated.
Methodology: Sample of eggs' shells are used for general identification of *E. coli.* Twelve*E. coli*pathogens are tested for resistance to *Chloramphenicol* (CAP) and *Fluorophenicol* (FP)antibiotics by the disk diffusion method on Mueller-Hinton agar plates. Isolates that shows the highest degree of resistance are examined to detect of antibiotic resistance genes, *floR*, *cmlA* and *cat1* genes, based on PCR method and gel electrophoresis.
Results: It can be observed that seven and ten *E. coli* isolates demonstrate resistance to CAP and FPantibiotics, respectively, based ondisk diffusion method. In contrast, seven and three *E. coli* isolates demonstrate resistance gene to FP and CAP antibiotics. The results show good correlation between antibiotic resistance phenotype and genotypes in these *E. coli* isolates.
Conclusion: It seems we should set rules and provide routine monitoring for testing and controlling the unnecessary and unconscious use of antibiotics in the diet of chicken breeding.
Keyword: Antibiotic resistance; E. colipathogens; Egg yolk; Biochemistry; Genetic

INTRODUCTION

The food industry in Iran is expanding dramatically with everyday new technologies;nevertheless, there are many cases of food poisoning which is more than the developed countries[1,2,3].These adverse health and nutritional consequences of food contamination are associated with highlyeconomic cost concerns.In the poultry industry, the low doses of antibiotic compounds as prevention of infections and growth promoters wereconducted with the WHO license[4,5,6].

However, many practical researches and experiences have shown that the use of antibiotics resulted in significant improvement in animal productivity and animal health due to prevent and treat bacterial infections[7],butthe number of antibiotic-resistant bacteria is increasing rapidly[8]. These antibiotic resistant bacteria that are new serotypes with different pathogenesis result in allergies in sensitive individuals [9].Reducing the efficacy of antibiotics in medicine and veterinary medicine, and also the occurrence of microbial resistance in humans may be related to the use of antimicrobial compounds in animals [10,11]. Microbiological and clinical evidences have shown that a large number of resistant bacteria weretransmitted from animal to human and caused infection in humans which do not respond to treatments[12].Undoubtedly, the most important way to transfer microbial resistance from animals to humans is to eat foods such as milk and dairy products, eggs and meat [13,14].

It has been shown that the gram-negative bacteria play a more important role in the development of infection [15].In a survey conducted in 2015, on meat products distributed from poultry meat factories in Tehran, it was observed that 27 percent of the produced chicken sausages contain high levels of bacterial contamination, including*Escherichia coli (E. coli)*[16]. In another research, Angulo et al. performed an Epidemiological studies ondairy and meat products and found (or isolated)food contamination with resistant strains the *Entrobacteriacea* family [17].Due to the consumption of contaminated dairy and meat products, *Escherichiacoli* and other antibiotic resistant forms are replaced in the human intestines and can transfer the drug resistance factor to susceptible pathogens such as *Salmonell*a and *Shigella*. In recent decades, antibiotic resistance in bacteria has been raised as a major public health problem. Therefore, determination of antibiotic resistance pattern in all isolated forms of these products is important in terms of public health. The aim of this study is to investigate the antibiotic resistance of *E. coli* pathogens isolated from commercial eggs distributed in Guilan province, Iran.

MATERIAL AND METHODS

To conduct the current study, 1536 pooled samples and 1002 individual samples of 5683200 commercial eggs were randomly bought from different shops of Gilan province (Iran)from January up to October 2017, and then they were transferred to a 4 °C refrigerator. In order to sample the eggs' shells, initially, each egg was placed on a surface, and a circle was drawn on the bottom of its shell with the use of a sterile swap which had been inoculated with betadine before. After this stage, a mild tap was applied on the center of the circle, and parts of the shell were taken and transferred to peptone water and sterile 2%glycerinemicrotube, stored in -20 °C for microbiologic tests.
The specimens were directly cultured in Tryptic Soy Broth (TSB) medium and cultured for agar and sorbitol agar media for general identification of *E. coli* and incubated for 24 hours at 37 ° C. The isolated colonies were cultured in Trypticase Soy Agar (TSA) medium and biochemical tests, such as indole, methyl red, Vogesproskauer and citrate utilization (IMViC), were performed on suspect specimens.

Among examined samples, only 12 isolates of *E. coli* demonstrated high resistance to the antibiotics were

targeted in this study. Twelve *E.coli* isolates were tested for resistance to antibiotics *Chloramphenicol* (CAP) and *Fluorophenicol*(FP) (Padtan Teb Co, Iran) by the disk diffusion method on Mueller-Hinton agar plates [18,19,20,21]. The standard procedure of the Clinical and Laboratory Standards Institute (CLSI) were followed throughout the testing procedure [22].

Twelve multi-resistant isolates, which showed the highest degree of resistance against CAP and FP, were examined for antibiotic resistance. To this purpose specific primers were designed according to Van's results[23]to detect *floR*, *cmlA* and *cat1* genes. The DNA sequence of such primers has been summarized in Table 1.

Table 1: Primers used to detect the presence of*floR*, *cmlA*and*cat1*genes.

Gene	DNA Sequence 5' →3'	Amplified Product (bp)
floR	F:TATCTCCCTGTCGTTCCAG R:AGAACTCGCCGATCAATG	399
cmlA	F:CCGCCACGGTGTTGTTGTTATC R:CACCTTGCCTGCCCATCATTAG	698
cat1	F:AGTTGCTCAATGTACCTATAACC R:TTGTAATTCATTAAGCATTCTGCC	547

The boiling method was used for DNA extraction from fresh colony cultures of *E. coli* isolates. To this purpose, 3 to 5 colonies of each sample were dissolved in a 1.5 mL volumetric flask containing 200 µL of distilled sterilized water and were placed in boiling water (95 ° C) for 10-15 minutes. Then, the samples were centrifuged at 14000 rpm for 10minutes, and the supernatant which contain DNA were transferred to the sterilized micro vials for the PCR reaction. These micro vials (50 µL) containing KCl(10 mM), dNTPs(0.2 mM), $MgCl_2$(3 mM), Tris-HCL (10 mM), 1 µl of Taq DNA polymerase, 20 picomoles from each primer and 4 microliters of extracted DNA. Thermo-cycler (SensoQuest GmbH, Germany)with summarized applied conditions intable 2 was used for PCR. Finally, 10 µl of PCR product was transferred to a 1.5% agarose gel containing ethidium bromide and after electrophoresis; a transilluminator device was used to detect *floR*, *cmlA* and *cat1* genes. It should be mentioned that the all data were analyzed by SPSS software version 19 and chi-square test at 95% confidence level (0.05).

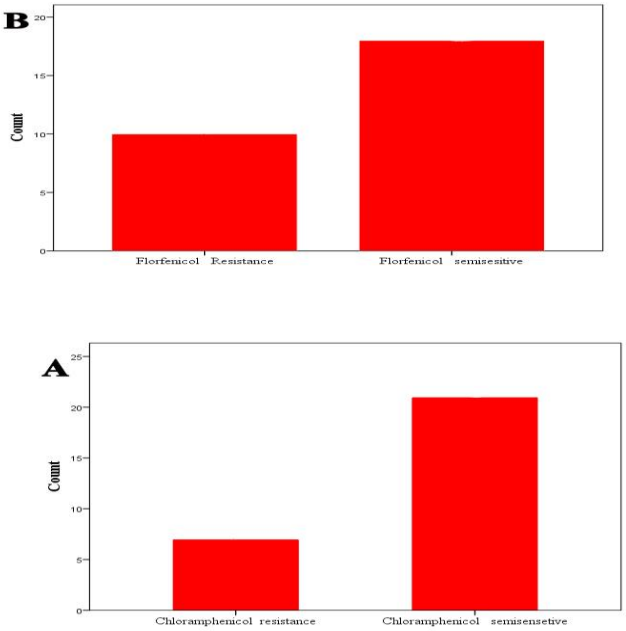
Table 2: The applied conditions of thermo-cycler program for PCR

Process	Temp.	Time	No of cycles
Initial denaturation	95 °C	3 minute	1
Denaturation	95 °C	30 Sec	35
Annealing	55 °C	45 Sec	35
Extension	72 °C	1 minute	35
Final extension	72	5 minute	1
Stop	4 °C		

RESULTS AND DISCUSSION

Figure 1 shows the results ofdisk diffusion method on Mueller-Hinton agar plates to determine resistance of *E. coli* isolates to CAP (Fig **1A**) and FP (Fig **1B**) antibiotics. It can be observed that seven and ten of twelve *E. coli* isolates demonstrate resistance to CAP and FP antibiotics, respectively.

Figure 1: Resistance of twelve E. coli isolates to CAP(A) and FP(B) antibiotic based on disk diffusion method on Mueller-Hinton agar plates.

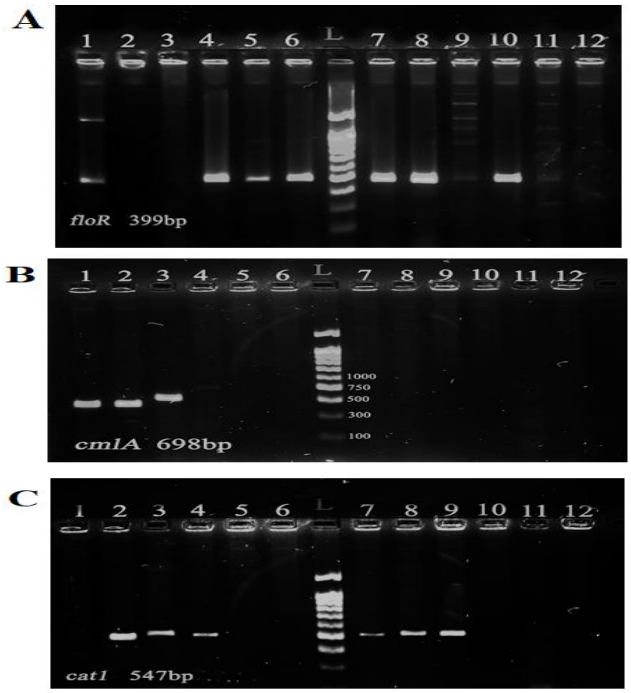


Genes responsible for a variety of antibiotic resistance characteristics have been investigated by PCR method from twelve *E. coli* isolates that showed the highest resistance. Figure 2 shows the presence of the antibiotic resistance genes band (*floR*, *cmlA* and *cat1*) in the agarose gel. Based on this method, figure 3 shows the number of antibiotic resistance genes in twelve *E. coli* isolates. *floR*, *cmlA* and *cat1*gen were observed in seven, four and six*E. coli* isolates, respectively. The results showed good correlation between antibiotic resistance phenotype and genotypes in these *E. coli* isolatesMore than 50% of animal protein needed by the communities is supplied by poultries [24]. Moreover, more than 800 common diseases exist between humans and animals[25]. Nevertheless, emerging new diseases indeed increases the risk of disease transmission. For example, Kaleidari et al. (2006) stated that bacilli are naturally present in feces and that the egg shell contamination through feces is responsible of increasing the isolation rate of these bacilli [26]. The point to be considered is that the contamination of the shell with feces can increase the number of bacteria on the shell and increase the risk of contamination of the contents. De Reu et al. [27]concluded that bacterial contamination in the content of egg was significantly higher in the cracked egg shell in comparison to the eggs without cracked shell. Jones et al.[28]showed that commercially-washed eggs have a significantly lower bacterial contamination rate than sow eggs. Another point that is important in terms of community health is the fact that the high levels of shell contamination when egg shells break down to use eggs as raw materials can contaminate the contents and the incidence of contamination.

Antibiotics are critically important for treating serious infections in humans, and the resistance to themis induced by the use of these antibiotics in food animals. The results

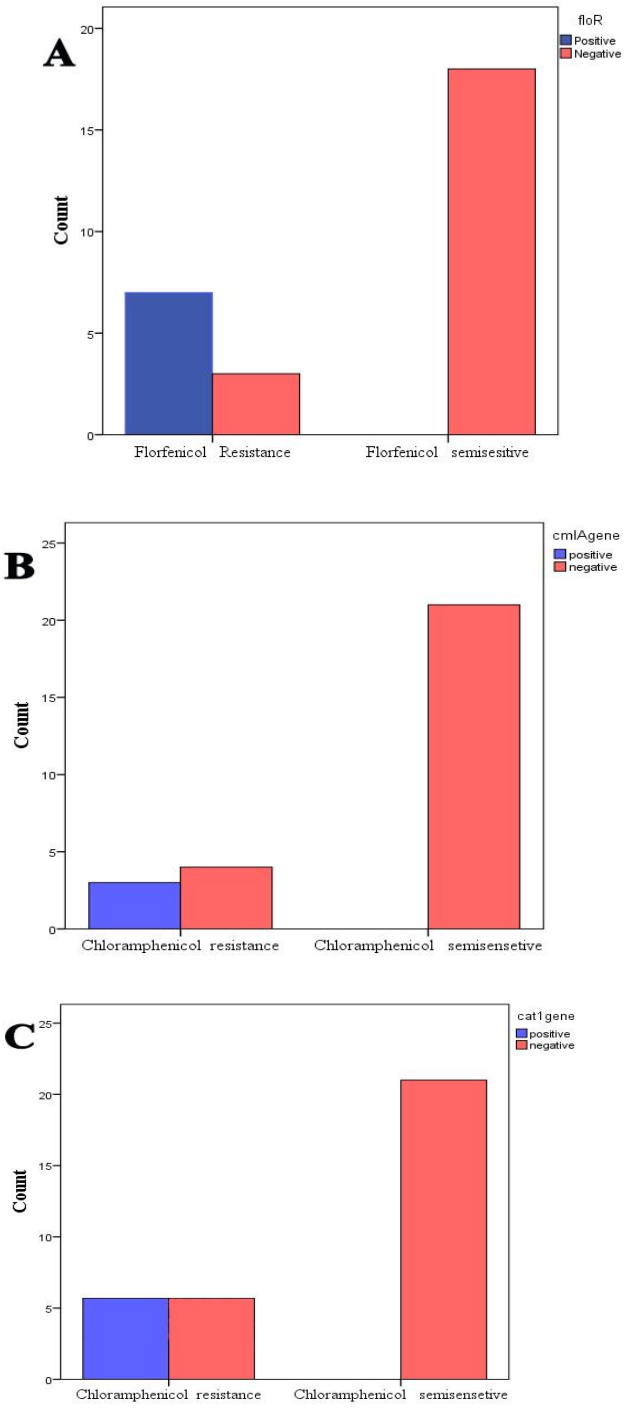
demonstrate that high individual and multiple resistances to antibiotics in *E. coli* and the 12 isolates showed resistance to all 2 antibiotics tested (figure 3). In contrast to this study, resistance to CAP and FP in *E. coli* isolates from poultry was reported as either non-existent or low in developed countries, perhaps due to restricted uses of them in animal husbandry in these countries. In a country such as Canada, such antibiotics are not registered for use in pigs, therefore *E. coli* isolated from this source showed very little resistance to these antibiotics. Santanilio et al. [29]collected 504 samples of pigeon clown swab from the city of Napoli, Italy, in which 4 samples were infected with O157: H7.Also, Wallace et al. [30] found out 99 birds near the dairy factories in the US state of Wisconsin in a pigeon contaminated with O157: H7.

Figure 2: The antibiotic resistance genes band, A) *floR*, B) *cmlA* and C) *cat1*in the agarose gel



The process of producing new antibiotics in world markets is much less than cardiovascular, nervous and psychoactive drugs, and even chemotherapy drugs. Pharmaceutical companies have little desire to invest in antibiotics because the period the use of antibiotics is limited and has high costs in production[31]. Therefore, an overabundant use of antibiotics results in microbial resistance which provides highly health and economic cost concerns. These isolated bacteria from eggs, especially antibiotic-resistant one with an extremely high antibiotic resistance, can be considered as an alarm for the uncontrolled administration of antibiotics. According to our findings, it seems that whether or not contaminated shell eggs in feces cannot consume raw or under cooked is reassuring. The presence of fecal contamination on egg shells will increase the number of bacteria on it and will increase the risk of contamination of egg contents, especially during egg breaking. Therefore, careful washing of eggs before breaking them to access the contents can be considered to reduce the risk of transmitting bacterial agents to the contents. In addition, we should set rules and provide routine monitoring for testing and controlling the unnecessary and unconscious use of antibiotics in the diet of chicken breeding.

Figure 3. Number of antibiotic resistance genes A) *floR*, B) *cmlA* and C) *cat1* in twelve *E. coli* isolates.



CONCLUSION

The results of this study showed that 11.2% of the collected egg samples were contaminated with *E. coli* and 10.7% demonstrated dangerous antibiotic resistant strains. Twelve isolates of *E. Coli* showed high resistance against both CAP and FPantibiotics.In addition, PCR resultsconfirmed the existence of the resistance genes, i.e., *FloR*, *cmlA* and *cat1* in isolated *E. coli.*These results confirmed highly and unconscious use of antibiotics in the diet of chicken breeding which will result in antibiotic resistance infections in humans.

REFERENCES

1. Shiferaw, B.;Smale, M.; Braun, H. J.;Duveiller, E.; Reynolds, M.;Muricho, G. Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. Food Security.2013, 5(3), 291-317.
2. Caplan, P. Approaches to the study of food, health and identity. Food, health and identity.1997, pp.1-31.
3. Armar-Klemesu, M. Urban agriculture and food security, nutrition and health. Growing cities, growing food. Urban agriculture on the policy agenda.2000, pp.99-118.
4. Assessment, M.E. Ecosystems and human well-being (Vol. 5). Washington, DC: Island press.2005.
5. Buzby, J.C.; Roberts, T. Economic costs and trade impacts of microbial foodborne ilness.1997.
6. Ansari-Lari, M.;Soodbakhsh, S.;Lakzadeh, L. Knowledge, attitudes and practices of workers on food hygienic practices in meat processing plants in Fars, Iran. Food control. 2010 Mar 1; 21(3):260-3.
7. Parvez, S.; Malik, K.A.; Ah Kang, S.; Kim, H.Y. Probiotics and their fermented food products are beneficial for health. Journal of applied microbiology. 2006, 100(6), pp.1171-1185.
8. Marshall, B.M.; Levy, S.B. Food animals and antimicrobials: impacts on human health. Clinical microbiology reviews. 2011, 24(4), pp.718-733.
9. LeBlanc, S.J.;Lissemore, K.D.;Kelton, D.F.; Duffield, T.F.; Leslie, K.E. Major advances in disease prevention in dairy cattle. Journal of dairy science.2006, 89(4), pp.1267-1279.
10. Wierup, M.. The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microbial Drug Resistance.2001, 7(2), pp.183-190.
11. Van den Bogaard, A.E.;Stobberingh, E.E. Epidemiology of resistance to antibiotics: links between animals and humans. International journal of antimicrobial agents. 2000, 14(4), pp.327-335.
12. Heuer, O.E.; Kruse, H.; Grave, K.; Collignon, P.; Karunasagar, I.; Angulo, F.J. Human health consequences of use of antimicrobial agents in aquaculture. Clinical Infectious Diseases.2009, 49(8), pp.1248-1253.
13. Jiménez-Colmenero, F.;Carballo, J.; Cofrades, S. Healthier meat and meat products: their role as functional foods. Meat science. 2001, Sep 1; 59(1):5-13.
14. McMichael, A.J.;Powles, J.W.; Butler, C.D.;Uauy, R. Food, livestock production, energy, climate change, and health. The lancet. 2007, Oct 6; 370(9594):1253-63.
15. Singer, R.S.; Finch, R.; Wegener, H.C.; Bywater, R.; Walters, J.; Lipsitch, M. Antibiotic resistance—the interplay between antibiotic use in animals and human beings. The Lancet infectious diseases. 2003, Jan 1; 3(1):47-51.
16. So, A.D.; Shah, T.A.; Roach S.; Chee, YL.; Nachman, K.E. An integrated systems approach is needed to ensure the sustainability of antibiotic effectiveness for both humans and animals. The Journal of Law, Medicine & Ethics. 2015, Aug; 43(3_suppl):38-45.
17. Kabir, S.M. The role of probiotics in the poultry industry.International Journal of Molecular Sciences. 2009, Aug; 10(8):3531-46.
18. Montgomery.; Lenaruzi,A. Consequence to chicks hatched from Escherichia coli inoculated embryos. Avian disease. 1999,43:553-563.
19. Levy, S.B.; Marshall, B. Antibacterial resistance worldwide: causes, challenges and responses. Nature medicine. 2004, Nov 30; 10(12s):S122.
20. Huyghebaert, G.; Ducatelle, R.; VanImmerseel. F. An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary Journal. 2011, Feb 1; 187(2):182-8.
21. Paruch, A.M.; Mæhlum, T. Specific features of Escherichia coli that distinguish it from coliform and thermotolerant coliform bacteria and define it as the most accurate indicator of faecal contamination in the environment. Ecological Indicators.2012, Dec 1; 23:140-2.
22. Van, T.T.H.; Chin, J.; Chapman, T.;Tran, L.T.; Coloe, P.J. Safety of raw meat and shellfish in Vietnam: an analysis of Escherichia coli isolations for antibiotic resistance and virulence genes. International journal of food microbiology.2008, 124(3), pp.217-223.
23. Reij, M.W.; Den Aantrekker, E.D. ILSI Europe Risk Analysis in Microbiology Task Force. Recontamination as a source of pathogens in processed foods. International journal of food microbiology. 2004, Feb 15; 91(1):1-1.
24. Ventola, CL. The antibiotic resistance crisis: part 1: causes and threats. Pharmacy and therapeutics. 2015, Apr; 40(4):277.
25. Kalidari, G.A.; Darzi, H.P.; Hashemtabar. G.H. Isolation and identification of facultative aerobic-anaerobic gram-positive bacteria in hatching eggs. Iranian Journal of Veterinary Research, 2006. 61-67.
26. De Reu, K.; Grijspeerdt, K.; Messens, W.; Heyndrickx, M.; Uyttendaele, M.; Debevere, J.; Herman, L. Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including Salmonella enteritidis. International journal of food microbiology. 2006. 112(3), pp.253-260.
27. Jones, D.R.; Musgrove, M.T. Pathogen prevalence and microbial levels associated with restricted shell eggs. Journal of food protection.2007, 70(9), pp.2004-2007.
28. Santaniello, A.; Gargiulo, A.; Borrelli, L.; Dipineto, L.; Cuomo, A.; Sensale, M.; Fontanella, M.; Calabria, M.; Musella, V.; Francesca Menna, L.; and Fioretti, A. Survey of Shiga toxin-producing Escherichia coli O157: H7 in urban pigeons (Columba livia) in the city of Napoli, Italy. Italian Journal of Animal Science.2007, 6(3), pp.313-316.
29. Wallace, J.S.; Cheasty, T.; Jones, K. Isolation of Vero cytotoxin-producing Escherichia coli O157 from wild birds. Journal of Applied Microbiology.1997, 82(3), pp.399-404.
30. Gilchrist, M.J.; Greko, C.; Wallinga, D.B.; Beran, G.W.; Riley, D.G.; Thorne, P.S. The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance.Environmental health perspectives. 2006, Nov 14; 115(2):313-6.
31. Lyte, M. Microbial endocrinology and infectious disease in the 21st century. Trends in microbiology. 2004, Jan 1; 12(1):14-20.