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Detection of virulence and antimicrobial susceptibility testing of *Escherichia coli* O157:H7 isolated from pets and stray dogs

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Abstract
The main objective of this study was to detect the virulence of *E. coli* O157:H7 by using Congo red dye and to determine the pattern of antimicrobial susceptibility for twenty six isolates of *E. coli* O157:H7 obtained from Department of internal and preventive medicine/ College of Veterinary Medicine/ University of Baghdad isolated from diarrheic and non- diarrheic dogs found in Baghdad Province, the study extended from March 2015 to August 2015. Seven antimicrobial discs were used in this study Chloramphenicol (30µg), Ampicillin (10µg), Trimethoprim plus sulfa (1.25 µg), Ciprofloxacin (5µg), Erythromycin (15µg), Gentamycin (120 µg), Cephalexin (30 µg), Trimethoprim (5 µg). The result showed that 24 isolates gave a positive result for Congo red, whereas only two isolates give a negative result. Results of antimicrobial susceptibility showed that the isolates susceptible to Ciprofloxacin at a percentage (80.76%) followed by gentamycin and trimethoprim plus sulfa at a percentage (53.84 % ; 46.15 %) respectively. In conclusion, isolates of *E. coli* O157:H7 isolated from fecal samples from diarrheic and non-diarrheic dogs were having the multidrug resistant phenomena and most of isolates were positive to Congo red dye which indicated this organism as an invasive pathogen.

Keywords: *E. coli* O157:H7, dogs, antimicrobial susceptibility. Congo red dye, pets and stray dogs.

1. Introduction

Escherichia coli O157: H7 is regarded as an important global zoonotic food borne pathogen, which produces life threatening diseases in humans, such as hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) [1].

E. coli O157:H7 has been detected in the feces of apparently healthy cattle, sheep, goats, pigs, dogs, bats and avian species [2-4]. Also, this pathogen has been documented in dogs by [5, 6]. Dogs and puppies regarded as important reservoirs for *E. coli* O157:H7 which is one of the main causes of diarrhea and other diseases in human [7]. Also, Yousif *et al*. [8] reported that *E. coli* O157:H7 was important pathogen in dogs.

Transmission pathways of STEC were the direct contact between humans and pet animals as well as fecal and urine contamination [9, 10]. Diarrheic and non-diarrheic dogs of all ages may serve as potential sources of multi-drug resistant STEC O157:H7 transmissible to humans [11].

Escherichia coli O157:H7 were isolated from dogs fecal sample which has class 1 integrons and they conclude that the presence of class 1 integrons serving as reservoirs of antibiotic resistance genes [12]. Many microorganisms carrying integrons can change and adapt quickly to different environmental niches and remain stable in several microbial lineages highlights not only its adaptive value, but also the potential for selection and dissemination of multiresistant bacteria and it is known that antibiotic resistant bacteria will increase a lot [13].

Paula and Marin [14] concluded that the carrying of MDREC organisms by dogs represents a potential hazard for people having contact with such animals, running the risk of spreading resistance genes; they also found a high percentage of STEC strains isolated from diarrheic dogs, presenting a multidrug-resistance phenotype.

Most studies showed that *E. coli* O157:H7 isolates from different sources were resistant to most antibiotic. The study of Disassa *et al*. [15] indicated that the *E. coli* O157:H7 isolates from milk samples were resistant to most of the antimicrobials; these were resistant to tetracycline, streptomycin and kanamycin. Beyi *et al*. [16] found that *E. coli* O157 which detected in carcass swabs and cutting board swab samples from butcher shops. All isolates were susceptible to

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five drugs 100%, but five isolates were resistant to amoxicillin, two isolates to streptomycin and three isolates to chloramphenicol. One isolate was resistant to two drugs and another to three drugs. Abdissa *et al.* [17] were isolated *E. coli* O157:H7 from abattoir samples, fecal sample and the carcass swabs and found that the isolates 100% susceptible to cefotaxime, ceftriaxone, gentamycin, kanamycin and nalidixic acid.

Congo red (CR), a simple acid dye, has been used to differentiate invasive pathogens such as *E. coli* [18]. Some authors have purposed the utilization of this characteristic as virulence marker to *E. coli* strains [19, 20].

This present study was conducted to determine the in vitro virulence factors and correlation with the resistance pattern of *E. coli* O157:H7 isolated from pets and stray dogs.

2. Materials and methods
2.1 Isolates of *E. coli* O157:H7
A twenty six (n = 26) *E. coli* O157:H7 isolates obtained from Department of internal and preventive medicine/ College of Veterinary Medicine/ University of Baghdad isolated from diarrheic and non- diarrheic dogs found in Baghdad Province; the study extended from March 2015 to August 2015; these isolates confirmed in aprevious study by Hasan *et al.* [7], which possess five virulence gene (*rfb*O157, *flic*H7, *Stx*1, *Stx*2 and *eae*A), these genes confirmed by conventional and real time PCR by Yousif *et al.* [8].

2.2. In Vitro pathogenicity testing by using Congo Red dye
The Congo red agar was made by using Trypticase Soy Agar with an additional 0,15% bile salts and 0,03% dye, 0.2% sorbitol according to Zahid *et al.* [21]. Congo Red binding activity test was performed and the colonies appeared must examine at 18, 24, 48 and 72 hours post incubation.

2.3. Antimicrobial sensitivity test (Disk diffusion test)
Antimicrobial discs were purchased from Mast diagnostics company (U.K.). Susceptibility to 8 antimicrobials was determined (Chloramphenicol (30μg), Ampicillin (10μg), Trimethoprim plus sulfa (1.25 μg), Ciprofloxacin (5 μg), Erythromycin(15μg), Gentamycin (120 μg), Cephalexin (30 μg), Trimethoprim(5 μg)). The disk diffusion susceptibility method is simple and practical and has been well-standardized [22]. At least 4-5 well isolated colonies of the same morphological type were selected from chrome agar; they were transferred to a tube containing 4-5ml of nutrient broth. The inoculated broth was incubated at 35-37 °C for 18 hrs. until the appearance of visible turbidity.

A sterile swab was dipped into the standardized suspension of bacteria and excess fluid was expressed by pressing and rotating the swab firmly against the inside of the tube above the fluid level.

Then, the swab was streaked in three directions over the entire surface of the Muller Hinton agar. The inoculated plates were allowed to dry for 5 minutes. Then the discs of antibiotic were placed onto agar surface using a sterile forceps. After that the plates were inverted, placed at 37 °C incubator aerobically for 18-24hrs [23].

The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter by using a caliper. The diameter of the zone was related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug were interpreted using the criteria published by [24, 25].

2.4. Statistical analysis
All data were analyzed statistically by using the Microsoft program (SPSS).

3. Results and Discussion
The present study results showed that the differentiation between virulent and non-virulent *E. coli* O157:H7 isolates were the presence of red colonies which recorded as Congo Red positive and white to grey colonies indicate that did not bind the dye and considered as Congo Red negative (Fig. 1).
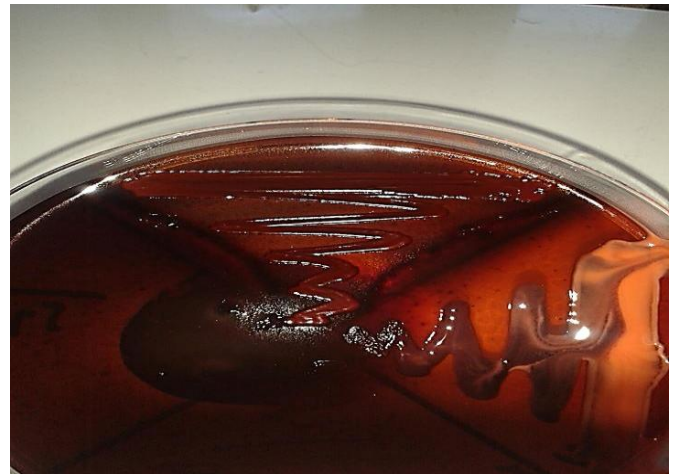

Fig 1: Congo red positive (above), Congo red negative (right).

Out of twenty six *E. coli* O157:H7 isolates, 15 isolates were given a positive result for Congo red, whereas 11 isolates give a negative result (Table 1).

Table 1: No. of isolates +ve and–ve to Congo red.

No of isolates	Congo red +	No Of Isolates	Congo red -ve
1	+	4	-
2	+	8	-
3	+	18	-
5	+	19	-
6	+	20	-
7	+	21	-
9	+	22	-
10	+	23	-
11	+	24	-
12	+	25	-
13	+	26	-
14	+		
15	+		
16	+		
17	+		
Total :-	15	Total	11

Table (2) showed the sensitivity and resistance of the 26 isolates to antimicrobial drugs, most of isolates +ve to Congo red resistant to 4 -7 antimicrobial while the isolates –ve to Congo red dye were showing resistance to 3-5 antimicrobial.

Table 2: Antimicrobial susceptibility with its relation to results of Congo red dye.

| No of isolates	Chlorm.	Ampicillin	Trim+ sulfa	Cipro.	Eryth.	Genta.	Cephalexin	Congo red
1	R	R	S	S	R	S	R	+
2	R	R	S	S	R	S	R	+
3	R	S	R	S	R	S	R	+
4	S	R	S	S	R	S	R	-
5	R	R	R	S	R	R	R	+
6	R	R	R	S	R	R	R	+
7	R	R	R	S	R	R	R	+
8	S	R	S	S	S	S	R	-
9	R	R	R	S	R	R	R	+
10	R	R	R	R	R	R	R	+
11	R	R	R	R	R	R	R	+
12	R	R	R	S	R	R	R	+
13	R	R	R	R	R	R	R	+
14	R	R	R	R	R	R	R	+
15	R	R	R	R	R	R	R	+
16	R	R	R	S	R	R	R	+
17	R	R	R	S	R	R	R	+
18	R	R	S	S	R	S	R	-
19	S	R	S	S	R	S	R	-
20	R	R	S	S	R	S	R	-
21	R	R	S	S	R	S	R	-
22	R	R	S	S	R	S	R	-
23	R	R	S	S	R	S	R	-
24	R	R	S	S	R	S	R	-
25	R	R	S	S	R	S	R	-
26	R	R	R	S	R	S	R	-

The present study results revealed that most isolates were susceptible to ciprofloxacin at a percentage(80.76%) followed by gentamycin and trimethoprim plus sulfa at a percentage (53.84%; 46.15%) respectively, whereas most isolates showed resistance to other antimicrobials at a different percentage which were mostly Congo red positive (Table 3, Fig. 2 and 3).

Table 3: Antimicrobial susceptibility testing of *E. coli* O157:H7 isolates according to its virulence

Antimicrobial agent	Sensitive isolates (%)	Congo red + (%)	Resistance (%)	Congo red + (%)
Chloramphenicol	3(11.54)D	Zero (0.0)E	23(88.46)C	15(65.2)A
Ampicillin	1(3.84)E	1 (100)D	25(96.14%)B	14(56)B
Trimethoprim+ sulfa	12(46.15)C	2 (16.66)C	14(53.85%)D	13(92.85)C
Ciprofloxacin	21(80.76)A	10(47.6)A	5(19.24%)F	5(100)E
Erythromycin	1(3.84)E	Zero (0.0)E	25(96.14%)B	15(60)A
Gentamycin	14(53.84)B	3(21.43)B	12(46.14%)E	12(100)D
Cephalexin	Zero(0.0)F	Zero (0.0)E	26(100%)A	15(57.7)A

Capital letters denote significant (p<0.05) differences between sensitive and resistant isolates
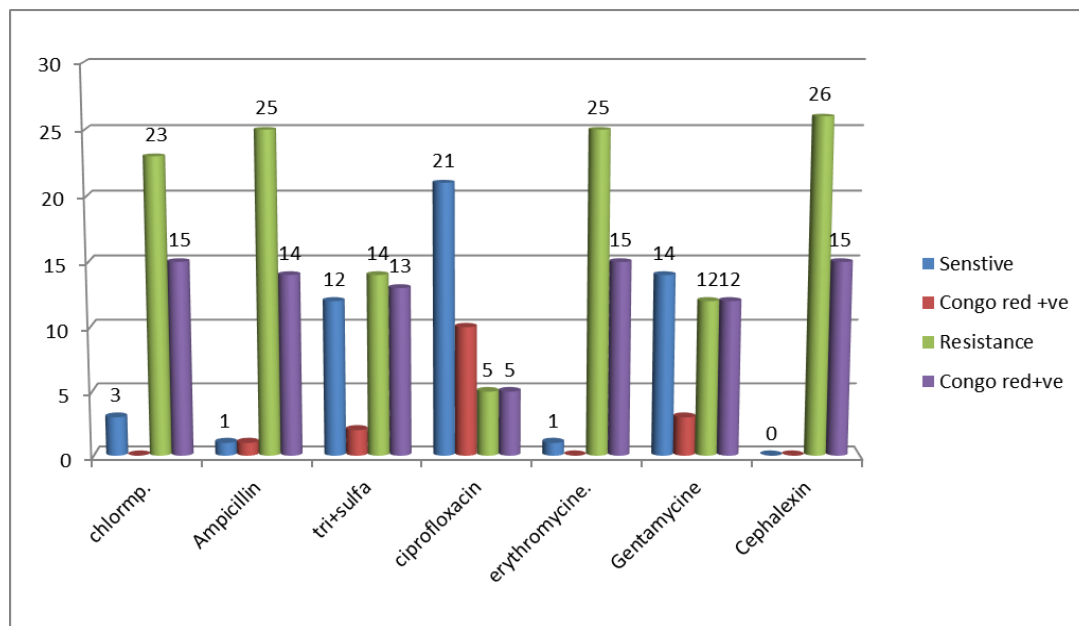
Fig 2: Resistance to antimicrobials at a different percentage which were mostly Congo red positive
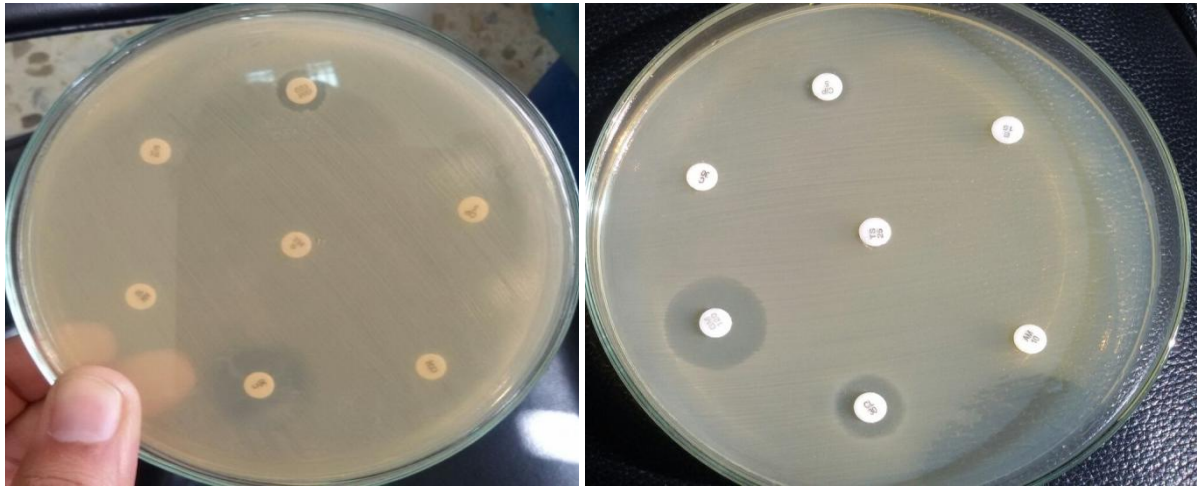
Fig 3: Antimicrobial sensitivity testing for *E. coli* O157:H7 isolates

The antimicrobial resistance of bacteria has become one of the most important therapeutic problems in human and veterinary medicine[11]. The present study analysed the occurrence of antimicrobial resistance in *E. coli* O157:H7 isolated from dogs due to its properties as a multidrug resistant bacteria. From the results of this study, most *E. coli* O157:H7 isolates which were showing a high degree of resistance and most of these isolates were Congo red positive, this result was compatible with the result of Sharma *et al*. [18] which concluded that Congo red agar was had 100% specifity for pathogenic invasive *E. coli*. Also, Corbett *et al*. [20] found that the Congo red medium was used to identify the virulent form of *Escherichia coli*. A direct correlation was found between the ability of clinical isolates of pathogenic *E. coli* to bind Congo red dye and their ability to cause infection in pigeon [26].

Zahid *et al*. [21] reported that Congo red agar can differentiate pathogenic strains from commensals. Binding of Congo Red is associated with the presence of virulence genes and genes for multiple resistance to antibiotics [27, 28].

The results of antimicrobial sensitivity test were in agreement with Osek, [29] who found that the most isolates of *E. coli* O157:H7 were sensitive to Gentamicin. And with the results of Galland *et al*. [30], that recorded most isolates of *E. coli* O157:H7 were susceptible to antibiotics used in feedlots, such as trimethoprim-sulfamethoxazole.. Antimicrobial sensitivity experiments by Shao *et al*. [31] showed that 16 of the *E. coli* O157:H7strains non-O157 STEC of different serotypes were multidrug resistant, Nalidixic acid showed the highest rate of resistance among the strains, followed by trimethoprim and sulphame-thoxazole, and sulfisoxazole, resistance to cefepime or imipenem was not detected, the 16 strains of STEC isolated from animal stools in this area were resistant to a number of antibiotics, with many strains displaying multidrug resistance. Also, the current results were similar to study of Osaili *et al*. [32] who found that *Escherichia coli* O157:H7 isolates revealed extensive resistance to Erythromycin and high susceptibility to Gentamicin and Ciprofloxacin. A study of Reuben and Owuna [33] was done to determine the antibiotic resistance patterns of *E. coli* O157:H7 from Nigerian fermented milk consumed in Nigeria, the antibiotic susceptibility profile showed that all the isolates were resistant to multiple antibiotics such as Chloramphenicol, Erythromycin, Sulphamethoxazole/Trimethoprim, while most isolates were sensitive to ciprofloxacin and gentamicin. Mora *et al*. [34] showed that a higher percentage of resistant strains of STEC O157:H7 were recovered from bovine and beef meat

human and ovine sources, the most common antimicrobial resistance was Ampicillin (10%) and Chloramphenicol (7%)

Our results compatible with a study reported in Egypt to evaluate the antibiotic susceptibilies of *E. coli* O157:H7 isolated from different water sources and establish a correlation between the presence of virulence genes of *E. coli* O157:H7 with the resistance to six antibiotic groups (amoxicillin, cefixime, ciprofloxacin, tetracycline, clarithromycin and streptomycin), the result showed that all bacterial isolates were sensitive to ciprofloxacin and showed variable degrees of resistance to other antibiotics [35].

Also, Edge and Hill [36] found low levels of resistance to ciprofloxacin. High levels of resistance to ampicillin were expected due to fact that ampicillin is older antibiotics that have been extensively used over the years [37].

The current study revealed a high rate of antimicrobial resistance among *E. coli* O157:H7 isolated from dogs, this was in agreement with a result of Ojo *et al*. [11]. The different results of resistance could be attributed to the use of non-specific antibiotic and random administration of antibiotic.

4. Conclusions

Finally, it can be concluded that, due to misuse of antibiotics, *E. coli* O157:H7 strain develop strategies for resistance to most of antimicrobial agent, only all isolates were significantly susceptible to ciprofloxacin only, so that this antibiotic can be considered a drug of choice for this bacteria. And most isolates were positive to Congo red dye which indicate that these strains isolated from dogs were virulent so Congo red agar can be used as guide for the presence of virulence factors of *E. coli* O157:H7. There is compatable results between postivetivity to Congo red dye and the resistance of bacteria to most antibiotic.

5. Acknowledgment

6. References

1. Pal Mahendra, Raj Mahendra. Escherichia coli 0517: H7: An emerging bacterial zoonotic food borne pathogen of global significance. International Journal of Interdisciplinary and Multidisciplinary Studies (IJIMS). 2016; 4(1):1-4.
2. Ojo OE, Ajwape A, Otesile E, Owoadi A. Detection of shiga toxin-producing Escherichia coli in poultry birds in

Abeokuta, Nigeria. Tropical Veterinarian. 2010a; 28(4): 1-10.

3. Ojo OE, Ajwape A, Otesile E, Owoadi A, Oyekunel M, Adetosoye A. Potentially zoonotic shiga toxin-producing Escherichia coli serogroups in the faeces and meat of food-producing animals in Ibadan, Nigeria. International journal of food microbiology. 2010b; 142(1):214-221.

4. Italia J, Rovira H, Masangkay j, Yoshikawa Y, Perez M, Reyes A, Bacticados W. Conventional isolation and polymerase chain reaction for detection of Escherichia coli O157: H7 from intestines of Philippine bats. Veterinarski Arhiv. 2012; 82(3):283-294.

5. Hancock DD, Besser TE, Rice DH, Ebel ED, Herriott DE, Carpenter LV. Multiple sources of Escherichia coli O157 in feedlots and dairy farms in the northwestern USA. Preventive veterinary medicine. 1998; 35(1):11-19.

6. Jay-Russell M, Hake AF, Bengson Y, Thiptara A, Nguyen T. Prevalence and characterization of Escherichia coli and Salmonella strains isolated from stray dog and coyote feces in a major leafy greens production region at the United States-Mexico border. PloS one. 2014; 9(11):e113433.

7. Hasan MS, Yousif AA, Alwan MJ. Detection of virulent genes in E. coli O157: H7 isolated from puppies and adult dogs by polymerase chain reaction. Research Journal for Veterinary Practitioners. 2016; 4(1):1-6.

8. Yousif A, Hasan M, Alwan M. Clinical and molecular study of E. coli O157:H7 isolated from Diarrheic and non-diarrheic dogs. Mirror of Research in Veterinary Sciences and Animals. 2016; 5(2):1-10.

9. Johnson J, Stell A, Delavari P. Canine feces as a reservoir of extraintestinal pathogenic Escherichia coli. Infection and Immunity. 2001; 69:1306-1314.

10. Bentancor A, Rumi M, Gentilini M, Sardoy C, Irino K, Agostini A, Cataldi A. Shiga toxin-producing and attaching and effacing Escherichia coli in cats and dogs in a high hemolytic uremic syndrome incidence region in Argentina. Federation of European Microbiological Societies Microbiological Lett. 2007; 267:251-256.

11. Ojo O, Bello A, Amosun E, Ajadi R. Multidrug resistant verocytotoxin-producing Escherichia coli O157:H7 in the faeces of diarrhoeic and non-diarrhoeic dogs in Abeokuta, Nigeria. Veterinarski Arhiv. 2014; 84(1):63-73.

12. Yang H, Byelashov O, Geornaras I, Goodridge L, Nightingale K, Belk K et al. Characterization and transferability of class 1 integrons in commensal bacteria isolated from farm and nonfarm environments. Foodborne Pathogens and Disease. 2010; 7(12):1441-51.

13. Colello R, Etcheverría AI, Conza JA, Gutkind GO, Padola NL. Antibiotic resistance and integrons in Shiga toxin-producing Escherichia coli (STEC). Brazilian Journal of Microbiology. 2015; 46(1):1-5.

14. Paula C, Marin J. Multidrug-resistant Shiga toxin-producing Escherichia coli in dogs with diarrhea. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2009; 61(2):511-514.

15. Disassa N, Sibhat B, Mengistu S, Muktar Y, Belina D. Prevalence and Antimicrobial Susceptibility Pattern of E. coli O157:H7 Isolated from Traditionally Marketed Raw Cow Milk in and around Asosa Town, Western Ethiopia. Veterinary Medicine International. 2017; 7581531:1-7.

16. Beyi AF, Fite AT, Tora E, Tafese A, Genu T, Kaba T, et al. Prevalence and antimicrobial susceptibility of Escherichia coli O157 in beef at butcher shops and

restaurants in central Ethiopia. BioMed Central Microbiology. 2017; 17:49.

17. Abdissa R, Haile W, Fite AT, Beyi AF, Agga GE, Edao BM et al. Prevalence of Escherichia coli O157:H7 in beef cattle at slaughter and beef carcasses at retail shops in Ethiopia. BioMed Central Infectious Disease. 2017; 17(1):277.

18. Sharma K, Soni SS, Meharchandani S. Congo red dye agar test as an indicator test for detection of invasive bovine Escherichia coli. Veterinarski arhiv. 2006; 76(4):363-366.

19. Berkhoff HA, Vinal AC. Congo red medium to distinguish between invasive and non-invasive Escherichia coli for poultry. Avian Disease. 1986; 30:117-121.

20. Corbett WT, Berkhoff HA, Vinal AC. Epidemiological study of the relationship between Congo red binding Escherichia coli and avian colisepticemia. Canadian Journal of Veterinary Research. 1987; 51:312-315.

21. Zahid AA, AL-Mossawei MT, Mahmood AB. In vitro and In vivo Pathogenicity tests of Local Isolates APEC from Naturally Infected Broiler in Baghdad. International Journal of Advanced Research in Biological Sciences. 2016; 3(3):89-100.

22. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American journal of clinical pathology. 1966; 45(4):493.

23. Markey B, Leonard F, Archambault M, Cullinane A, Maguire D. Clinical veterinary microbiology. Second edition, Mosby, Elsevier. 2013, 246-249.

24. Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards (CLSI). Performance Standards for Antimicrobial Susceptibility Testing, Wayne, PA, USA, Tenth Edition. 2011; 30(1):07-A8.

25. British Society for Antimicrobial Chemotherapy (BSAC). BSAC to actively support the EUCAST Disc Diffusion Method for Antimicrobial Susceptibility Testing in preference to the current BSAC Disc Diffusion Method. 2015, 14.

26. Hassan H, Bakeet A. Characterization Of Escherichia Coli Strains Isolated From Infected Pigeons In Assiut Province. Assiut Veterinary Medical Journal. 2014; 60(142):29-37.

27. Catana N, Popa V, Fodor I. Binding of Congo Red, Phenotypical Marker for Discrimination of APEC Strains. Lucrări Stiinłifice Medicină Veterinară. 2009; XLII:200-202.

28. Fodor I, Catana N, Herman V. Epidemiological studies on some E. coli strains inbroiler chickens. Bulletin of the University of Agricultural Sciences and Veterinary Medicine. 2010; 67:74-76.

29. Osek J. Phenotypic and genotypic characterization of Escherichia coli O157 strains isolated from humans, cattle and pigs. Veterinarni Medicina-UZPI (Czech Republic). 2004; 49(9):317-326.

30. Galland JC, Hyatt DR, Crupper SS, Acheson DW. Prevalence, antibiotic susceptibility, and diversity of Escherichia coli O157:H7 isolates from a longitudinal study of beef cattle feedlots. Applied and Environmental Microbiology. 2001; 67(4):1619-27.

31. Shao CC, Hu B, Bi ZW, Kou ZQ, Fang M, Chen BL et al. Serotype identification and antibiotic susceptibility of Shiga toxin-producing Escherichia coli in the Weishan area in Shandong Province, China. Zhonghua Yu Fang Yi

Xue Za Zhi. 2017; 51(1):70-75.

32. Osaili TM, Alaboudi AR, Rahahlah M. Prevalence and antimicrobial susceptibility of *Escherichia coli* O157:H7 on beef cattle slaughtered in Amman abattoir. Meat Science. 2013; 93(3):463-8.

33. Reuben R, Owuna G. Antimicrobial resistance patterns of Escherichia Coli O157: H7 from Nigerian fermented milk samples in Nasarawa State, Nigeria. International Journal of Pharmaceutical Science Invention. 2013; 2(3):38-44.

34. Mora A, Blanco JE, Blanco M, Alonso MP, Dhabi G, Echeita A *et al*. Antimicrobial resistance of Shiga toxin (verotoxin) procuging *Escherichia coli* O157:H7 and non-O157 strains isolated from humans, cattle, sheep and food in spain. Research in Microbiology. 2005; 156(7):793-806.

35. El-Shatoury E, Azab El-Leithy M, Abou-Zeid M, El-Taweel G, El-Senousy W. Antibiotic Susceptibility of Shiga Toxin Producing E. coli O157: H7 Isolated from Different Water Sources. Environments. 2015; 17:24.

36. Edge T, Hill S. Occurrence of antibiotic resistance in Escherichia coli from surface waters and faecal pollution sources near Hamilton Ontario. Canadian Journal of Microbiology. 2005; 51:501-5.

37. Rooklidge S. Environmental antimicrobial contamination from terraccumulation and diffuse pollution pathways. Science of the Total Environment. 2004; 325:1-13.