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Antibiotic Susceptibility of Shiga Toxin Producing *E. coli* O157:H7 Isolated from Different Water Sources

Einas H. El-Shatoury[2], M. Azab El-Leithy[1,*], Mohamed A. Abou-Zeid[2], Gamila E. El-Taweel[1] and Waled Morsy El-Senousy[1]

[1]*Water Pollution Research Department, Environmental Research Division, National Research Centre, Dokki, Giza, 12622, Egypt;*

[2]*Microbiology Department, Faculty of Science, Ain Shams University. El- Khalifa El-Mamon St., Abbassia, Cairo, 11566, Egypt*

Abstract: Nowadays the extensive use of antibiotics for the treatment of humans and warm blooded animals has influenced the frequency and spread of antibiotic resistant *E. coli* O157:H7 in different aqueous sources. Thus, the main aim of this study was to evaluate the antibiotic susceptibilities 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) extensively used in Egypt. To achieve this aim 44 suspect *E. coli* O157:H7 isolates were confirmed by biochemical tests and were characterized for six virulence genes: *fliC, stx1*, *stx2*, *eae*, *rfbE* and *hlyA* using multiplex PCR. All *E. coli* O157:H7 isolates carrying three (*stx2*, *eae* and *rfbE*) or more virulence genes were resistant to amoxicillin and 77% were resistant to clarithomycin. In conclusion, it appears that the environmental *E. coli* O157:H7 strains develop strategies for antibiotic resistance more than reference strains.

Keywords: Antibiotic resistance, *E. coli* O157:H7, shiga toxins, water.

INTRODUCTION

E. coli O157:H7 causes a wide spectrum of human diseases, including bloody and non-bloody diarrhea, hemorrhagic colitis (HC), occasional kidney failure and hemolytic uremic syndrome (HUS) and *E. coli* O157 contamination of drinking, surface and recreational water has emerged as an important cause of human disease [1, 2]. There is a varying level of antibiotic resistance among *E. coli* O157:H7 isolates. Some *E. coli* O157:H7 isolates have resistance to one or more antibiotics whereas others are multidrug resistant [3]. Schroeder *et al.* [4] focused on isolates from cattle, humans, swine, and farms. Some evidence suggests that the selective pressure imposed on livestock enteric bacteria by subtherapeutic antibiotic use sets the stage for the type of genetic mutations that are required to turn harmless *E. coli* into toxin-producing, dangerous serotypes [5].

It is known that multiple antibiotic resistance genes sometimes move from one bacterium to others *via* plasmids. The genes triggering the production of *Shiga*-like toxins by certain *E. coli* serotypes may move in the same way. The role and impact of antibiotic use in triggering this process are not understood and is subject to active debate [4]. Solomakos *et al.* [6] found that all of *E. coli* O157:H7 isolates were resistant to ampicillin, an antibiotic used in human medicine for the treatment of coliform infections, and all but one isolate were also resistant to streptomycin. Also, tetracycline was found to be the most inhibitory antimicrobial in terms of the number of isolates that were inhibited (27 isolates), followed by gentamicin (26 isolates) and cefuroxime (22 isolates). Moreover, Olatoye [7] found that 100% of the *E. coli* O157:H7 isolates were resistant to one or more antibiotics. Tetracycline resistance was the highest (in 91.4% of the isolates), while 72.9% of the isolates were resistant to nitrofurantoin and chloramphenicol, 65.7% to cefuroxime, 44.3% to cotrimoxazole, 35.7% to nalidixic acid, and 11.4% to gentamicin. The main aim of this study was to determine the antibiotic resistance of *E. coli* O157:H7 isolated from different water sources, and besides that, finding if there is correlation between the presence of *E. coli* O157:H7 virulence genes and antibiotic resistance.

MATERIALS AND METHODS

E. coli O157:H7 Isolates

E. coli O157:H7 isolates were obtained during a survey of 50 Nile river water samples (Rossita Branch), 20 water samples from El-Rahawy Drain and 10 untreated hospital wastewater samples, between June of 2010 and July of 2011. The detection of *E. coli* O157:H7 was carried out using Hi-Crome EC O157:H7 selective agar base (HiMedia, India) plates supplemented with novobiocin and potassium tellurite (HiMedia, India). Typical colonies for *E. coli* O157:H7 were

*Address correspondence to this author at the Water Pollution Research Department, Environmental Research Division, National Research Centre, Dokki, Giza, 12622, Egypt; Tel: (+2) 01225110975;
E-mail: mohamedazabr@yahoo.com

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confirmed by subculturing on HiCrome MacConky Sorbitol agar base (HiMedia, India) supplemented with tellurite and cefixime (HiMedia, India) and incubated at 37°C for 24 h. Indole and oxidase tests [8] were also performed to confirm the *E. coli* O157 isolates**.**

Molecular Characterization of *E. coli* O157:H7

DNA Extraction of E. coli O157:H7 Isolates

DNA extraction was carried out according to Bai *et al.* [9]. One colony of each isolate was suspended in one mL of sterile distilled water and boiled for 10 min then preserved in ice for 5 min. After centrifugation at 12000 rpm for 10 min, 300- 500 µL of the supernatants was transferred to eppendorf tubes, and 5 µL of the supernatants was used as templates in multiplex PCR reactions.

Selection and Synthesis of Primers

The primers used for the multiplex PCR are those listed by Bai *et al.* [9] targeting six virulence genes of *E. coli* O157:H7: *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *eae* (intimin), *hlyA* (hemolysin), *rfbE* (O157 antigen) and *fliC* (flagellar antigen) in one microtube. The products sizes of the six virulence genes were at 949, 655, 477, 375, 296 and 199 bp for *fliC, stx1, stx2, eae, rfbE* and *hlyA* respectively. Primers for this study were synthesized by Bio-Basic Inc., Canada.

PCR Conditions Optimization

When multiplex PCR conditions were carried out according to Bai *et al.* [9] no PCR products were obtained, so different conditions were used to reach an optimal condition. After a series of tests, the reaction conditions were modified to target multiplex genes examined; reaction volume of 50 µL consisting of 5 µL of DNA template and 45 µL master mix (BioFlux) from 5 µL of 10X PCR buffer (containing 7.5 mM of $MgCl_2$, 50 mM of KCl, 20 mM of Tris–HCl (pH 8.4)), 0.5 µL from each primer (mixture of equal amount of the 100 mM primer stocks), 250 µM of dNTPs, and 4 units of Taq DNA polymerase (Bio-Rad, CA). The PCR program condition was modified to target multiplex genes examined; 94°C denaturation for 3 min, 35 cycles of 94°C denaturation for 30 s, 60°C annealing for 30 s, 72°C extension for 75 s, and a final step of 72°C extension for 5 min. PCR reactions were performed in a TC-S thermal cycler (BOECO, Germany). The amplified DNA was separated on 2% agarose gel and stained with 0.5 µg/ml of ethidium bromide with Ladder ΦX174 DNA/HaeIII digest (TOYOBO, Japan). The DNA bands were visualized and documented with a GelDoc UVP Fluorescent Imaging System (UVP, UK).

Antibiotic Sensitivity of *E. coli* O157:H7 Isolates

Forty four *E. coli* O157:H7 isolates and *E. coli* O157:H7 ATCC 35150 were examined for susceptibility to 6 antibiotics using the Bauer–Kirby disc diffusion method [10]. The following discs (Oxoid, UK) were used for susceptibility test: amoxicillin 10 µg (AML 10), cefixime 5 µg (CFM 5), ciprofloxacin 5 µg (CIP 5), tetracycline 30 µg (TE 30), clarithomycin 15 µg (CLR 15) and streptomycin 10 µg (S 10). The discs used belong to six antibiotic groups: penicillin, 3rd generation cephalosporin, quinolones, tetracycline, macrolides and aminoglycosides, respectively. The tested *E. coli* O157:H7 isolates were cultured on Müller Hinton agar plates and incubated at 37°C for 24 h. Isolates were classified as sensitive, intermediate or resistant to each antibiotic according to the National Committee for Clinical Laboratory Standards [11].

RESULTS AND DISCUSSION

The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognized by the WHO [12] as a serious global human and animal health problem. When manure produced in agriculture is applied to land, pollutants such as antimicrobial compounds, resistant bacteria or resistance genes concentrate and mobilize in soil and often end up in ground or surface water through runoff [13-17]. The development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon [18]. *E. coli* O157:H7 can cause HUS mainly by secretion of Shiga toxins encoded by the genes *stx1* and/or *stx2* and their variants [19, 20]. Some antibiotics may cause bacterial lysis and liberate the free Shiga toxins in the intestinal tract [21] or enhance the expression of Shiga toxin genes [22]. Antimicrobial resistance is a natural phenomenon that bacteria use to protect themselves against competitors [23]. The misuse of antibiotics in medicine and agriculture has influenced the frequency and spread of antibiotic resistant bacteria in many aquatic environments [17, 24].

In this study, 44 *E. coli* O157:H7 suspected isolates were confirmed by biochemical tests and subjected to the multiplex PCR to detect the six virulence genes in *E. coli* O157:H7. The multiplex PCR results of this work showed that twenty nine out of the 44 (66%) *E. coli* O157:H7 isolates carried five virulence genes (*flic, stx1, stx2, eae* and *rfbE*). Ten out of 44 (23%) carried four virulence genes ((*stx1, stx2, eae* and *rfbE*) in nine isolates and (*flic, stx2, eae* and *rfbE*) in one isolate). In addition to these, five out of 44 (11%) carried three virulence genes (*stx2, eae* and *rfbE*) (Table **1** and Fig. **1**). Bai *et al.* [9] used the same six primers to test 84 cattle fecal isolates and 57 human clinical isolates of *E. coli* O157. The 84 cattle strains differed only in *stx1* and *stx2* genes, and all possessed the other four genes. Among the cattle strains, 28% had *stx2*, 26% had *stx1*, and 28% had both *stx1* and *stx2*. Similarly all the 57 human strains (100%) possessed *fliC*, *eae*, *rfbE* and *hlyA* and differed in *stx1* and *stx2*. Of the 57 human strains, 38% had both *stx1* and *stx2*, 60% had *stx2* and only 2% had *stx1* alone. In the present study, the hemolysin gene (*hly*) was not detected in any tested isolates. A possible explanation for that may be that the tested isolates don't carry this gene. Another explanation might be that the amplification process requires further modification.

In Egypt, El-Safey [25] found specific Shiga-like toxin (*stx1* and *stx2*), intimin (*eaeA*) and the enterohemorrhagic *E. coli* hemolysin (*hlyA*) genes in five *E. coli* O157:H7 strains isolated from Egyptian food. El-Jakee *et al.* [26] mentioned that, from the 14 *E. coli* O157 strains isolated from different water sources in Egypt and characterized by monoplex PCR eight (57.1%) isolates carried *stx1* and four (28.6%) possessed *stx2* gene. Intimin (*eae*), *fliCh*7 and *hly* virulence genes were detected in three (21.4%), whereas the *hly* gene was found in four (28.6%) of the isolates.

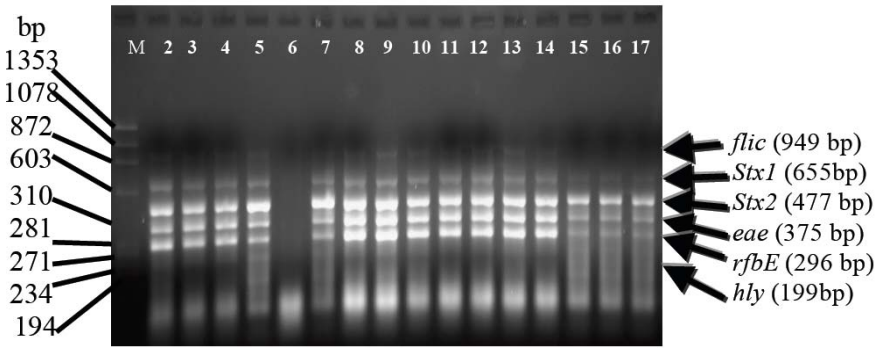

Fig. (1). Multiplex PCR of *E. coli* O157:H7 isolates from different water samples, Lane M: ΦX174 DNA-HaeIII Digest ladder, Lanes 2-5: *E. coli* O157:H7 isolates Lane 6: negative control, *Listeria monocytogenes* ATCC 25152 Lane 7-17: *E. coli* O157:H7 isolates.

Table 1. Antibiotic susceptibility of Shiga toxin producing *E. coli* O157:H7 isolates.

No. of Tested Isolates (44)	Amoxicillin (AML 10)			Cefaxime (CFM 5)			Ciprofloxacin (CIP 5)			Tetracycline (TE 30)			Clarthromycin (CLR 15)			Streptomycin (S 10)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
***flic, stx1, stx2, eae, rfbE* (n=29)**	-	-	29	24	5	-	29	-	-	27	-	2	-	4	25	4	22	3
***stx1, stx2, eae, rfbE* (n=9)**	-	-	9	9	-	-	9	-	-	9	-	-	-	5	4	-	8	1
***flic, stx2, eae, rfbE* (n=1)**	-	-	1	1	-	-	1	-	-	1	-	-	-	-	1	-	1	-
***stx2, eae, rfbE* (n=5)**	-	-	5	4	1	-	5	-	-	4	1	-	-	1	4	1	3	1
***E. coli* O157:H7 (44 isolates)**	0	0	44	38	6	0	44	0	0	41	0	3	0	10	34	6	33	5
***E. coli* O157:H7 (ATCC 35150)**			R	S			S			S					R	S		

In the present study, all *E. coli* O157:H7 isolates (44) were found to be resistant to amoxicillin (100%). Fewer isolates were resistant to clarithomycin (77%), streptomycin (11%) and tetracycline (7%). Moreover, *E. coli* O157:H7 isolates showed sensitivity to ciprofloxacin (100%), tetracycline (93%) and cefaxime (86%) (Table **1**). Maal-Bared *et al.* [27] examined the distribution of antibiotic resistant *E. coli* and *E. coli* O157 isolated from water, sediment and biofilms in an intensive agricultural watershed. They found that the frequency of resistance of *E. coli* and *E. coli* O157 to ampicillin, cefotaxime, nalidixic acid and tetracycline was significantly different among sampling sites. Also, Edge and Hill [28] found low levels of resistance to ciprofloxacin. High levels of resistance to ampicillin were expected due to fact that ampicillin are older antibiotics that have been extensively used over the years [29].

In this study, the difference between the *E. coli* O157:H7 ATCC 35150 reference strain and the environmental isolates was in the sensitivity to streptomycin. While the ATCC strain was sensitive to streptomycin, the environmental isolates showed intermediate sensitivity (75%) to streptomycin (Table **1**). In many studies, *e.g.* Edge and Hill [28] and Watkinson *et al.* [30] the antimicrobial resistance of *E. coli* isolated from water to a variety of antibiotics has been shown. Moreover, isolation of resistant *E. coli* O157 from multiple aquatic ecosystems has been reported [30]. The frequency of resistance was highest to tetracycline, followed by ampicillin and streptomycin. High levels of *E. coli* antibiotic resistance to tetracycline and ampicillin have been observed in many other studies [30-32]. Maal-Bared *et al.* [27] concluded that, there are strong relationships between the water quality and antimicrobial resistance. Furthermore, *E. coli* is a microorganism that is proficient at horizontal gene transfer that could transmit its resistance genes to other facultative or obligate pathogenic bacteria.

CDC's National Antibiotic Resistance Monitoring System (NARMS) have found widely varying levels of antibiotic resistance among *E. coli* O157 serotypes, with 10% of the *E. coli* O157 isolates being resistant to one or more antibiotics, and 7% being multidrug resistant [3]**.** A study focusing on isolates from cattle, humans, swine and farms found that 39% of the *E. coli* isolates were resistant to one or more antimicrobials [4].

Finally, it can be concluded that, due to abuse and misuse of antibiotics, environmental *E. coli* O157:H7 strains develop strategies for resistance more than reference strains, thus, restriction of antibiotics used must be enforced. Also, more investigations are required to find the correlation, if any, between the presence of pathogenic bacteria virulence genes and resistance to antibiotics. Moreover, it is very important to monitor the prevalence and persistence of *E. coli* O157:H7 organisms in aquatic environment from time to time.

According to NCCLS 2007 [11]

R: Resistant (AML 10 and CLR 15 ≤13mm; CFM 5 and CIP 5≤15mm; TE 30 and S10 ≤11mm).

I: Intermediate (AML 10 14-16mm; CFM 5 16-18mm; CIP 5 16-20mm; TE 30 and S10 12-14mm; CLR 15 14-17mm).

S: Sensitive (AML 10 ≥17mm; CFM 5 ≥19mm; CIP 5≥ 21 mm; TE 30 and S10 ≥ 15mm; CLR 15≥18 mm).

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Declared none.

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