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ORIGINAL RESEARCH

Virulence and antimicrobial resistance of *Escherichia coli* isolated from Tigris River and children diarrhea

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Objective: To investigate the virulence factors including hemolysin production, β-lactamase production, and biofilm formation. Antimicrobial resistance and plasmid content of 20 *Escherichia coli* isolates obtained from feces and Tigris water were screened.

Methods: Ten clinical and ten environmental *E. coli* isolates were collected from children diarrhea and swim areas on Tigris River in Baghdad city, Iraq, respectively. The bacterial isolates were identified by cultural characteristics, Gram stain, biochemical tests, and screened for the presence of *E. coli* O157:H7 serotype. Bacterial *E. coli* isolates were investigated for hemolysin production, biofilm formation, and β-lactamase production. Antibiotics susceptibility and plasmid content were determined.

Results: A total of ten clinical and ten water *E. coli* isolates were studied. Results showed that all *E. coli* isolates give negative results for latex O157:H7. Virulence factors analysis showed that 6/10 water isolates and 2/10 clinical isolates were hemolytic, 5/10 water isolates and 3/10 clinical isolates were biofilm formation, and 7/10 water isolates and 4/10 clinical isolates were β-lactamase producer. Antibiotics profile showed that all bacterial isolates were multidrug resistant. All *E. coli* isolates (100%) were resistant to carbenicillin, cefodizime, imipenem, and piperacillin. The plasmid DNA analysis showed that all *E. coli* isolates contained plasmid with molecular weight range between 4.507 kbp and 5.07 kbp, but clinical isolates contained multiple small and mega plasmids.

Conclusion: Our study revealed that *E. coli* isolates from river water exhibit a higher level of hemolysin production, β-lactamase production, and biofilm formation than feces isolates may be due to long adaptation. On the other hand, clinical *E. coli* isolates from feces showed higher level of antibiotic resistance and have multiple plasmids.

Keywords: *E. coli*, hemolysin, β-lactamase, biofilm, multidrug resistance, plasmid

Introduction

Escherichia coli is a gram-negative bacteria belonging to the Enterobacteriaceae family, short bacilli, non-spore forming, facultative anaerobic, and it is grown on a simple media.[1] *E. coli* is a major component of the human normal intestinal flora. Among the intestinal pathogens, there are six well-described categories: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli*, enteraggregative *E. coli*, enteroinvasive *E. coli*, and diffusely adherent *E. coli*.[2] There are several virulence factors that contribute to *E. coli* pathogenicity, such as pilli, enterotoxins (LT, ST), shiga-like toxins, endotoxin (lipopolysaccharide), hemolysin, aerobactin, cytonecrotizing factor, intimin, and biofilm formation.[3,4] Plasmid profile analysis is useful in determining the epidemical strain in outbreaks caused by multiple species: *Escherichia*,

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Klebsiella, *Pseudomonas*, *Serratia*, *Streptococcus*, and so on.[5] The adherence plasmid of EPEC strain is associated with the ability of EPEC to adhere to tissue culture cells in a pattern called localized adherence.[6,7] EHEC also carry a large plasmid (91.2 kbp) that is associated with the presence of fimbriae.[8] Plasmids allow the movement of genetic material, including antimicrobial resistance genes, between bacterial species and genera.[9] The widespread development of resistance to several different antibiotics is generally as a result of lateral or horizontal gene transfer.[10] Many studies have demonstrated that plasmid transfer between bacteria occurs in diverse environments. Various diverse phenotypic characteristics are encoded by plasmids; these include antibiotic and metal resistance, degradation of complex organic compounds, production of enterotoxins and colicins, and the production of restriction enzymes.[11] Most of the transfers are described using bacteria from the same group or ecological niche.[12] Plasmids size range from 1 kbp to 2,000 kbp resistance plasmids code for enzymes that can inactivate antibiotics, prevent the uptake of an antibiotic, or pump out the particular antibiotic.[13] Gram-negative bacilli causing nosocomial infections are usually multiple-antibiotic resistant, resulting in significant problems for treatment.[14] The aim of this study is comparing virulence factors and plasmid content among *E. coli* isolates from clinical and environmental sources.

Materials and methods

Specimens

In this study, 20 *E. coli* isolates were analyzed, ten *E. coli* isolates were obtained of the Tigris River water in Baghdad city and the other ten bacterial isolates were collected from children diarrhea from Children Hospital Center in Baghdad city. Samples collections were carried out from May to July 2013.

Isolation and identification of *E. coli*

Membrane filter technique

A known volume (30 mL) of water samples collected from swim areas on the Tigris River in Baghdad city was filtered through sterile 47 mm diameter membrane filters with 0.45 μm pores.[15] The membrane filters were placed on the surfaces of eosin methylene blue agar plates for isolation of enteric bacteria and on MacConkey agar plates for isolation of lactose and nonlactose-fermenting bacteria.

E. coli isolation from clinical samples

Ten *E. coli* isolates were collected from children (aged under 4 years) diarrhea. Fecal samples were cultured on MacConkey agar and eosin methylene blue agar plates.[16]

Biochemical tests

Enteric bacteria isolated on respective selective and differential media were identified on the basis of colonial characteristics, Gram stain and biochemical tests, IMViC, Urea, and Kligler Iron Agar.[16]

Latex O157:H7

All *E. coli* isolates were investigated with latex O157:H7 (Well Colex) slide agglutination test.

Detection of hemolysin production

The *E. coli* isolates tested for blood hemolysis were streaked on blood agar plates containing 5% (v/v) human blood and incubated aerobically at 37°C for 24 hours. The clear zones around the growth colonies indicate a positive reaction.[17]

Detection of β-lactamase production

All *E. coli* isolates were tested for production of β-lactamase enzyme using iodometeric test method.[18]

Detection of biofilm formation

The ability of *E. coli* isolates to colonize abiotic surface was investigated by using Christensen et al's method.[19] The *E. coli* isolates were cultivated in tubes with Tryptone soy broth and incubated aerobically at 37°C for 48 hours and thereafter the culture tubes were emptied carefully and stained with 1% crystal violet solution for 30 minutes, and then the tubes were rinsed with distilled water and left to dry at room temperature. Results were compared with negative control, and biofilm formations as a layer at the internal wall of the tubes noted by the naked eye indicate a positive result.

Antibiotic sensitivity test

Antibiotic susceptibility profiles of *E. coli* isolates were determined by the standard Kirby–Bauer disk diffusion method.[20] The antibiotics with their respective disk concentrations are as follows: amoxicillin–clavulanic (30 μg), carbenicillin (100 μg), cefodizime (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), imipenem (10 μg), norfloxacin (10 μg), piperacillin (100 μg), rifampicin (5 μg), and pefloxacin (5 μg). Bacterial cultures suspension equivalent of 0.5 tube McFarland turbidity standards were spread on Mueller-Hinton agar plates using sterile swabs and incubated aerobically at 37°C for 24 hours, and then the diameter of inhibition zones around antibiotic disks were measured. Results were expressed susceptible or resistant according to the criteria recommended by the Clinical and Laboratory Standards Institute.[21]

Plasmid DNA isolated procedure

All *E. coli* isolates were screened for plasmid content by the alkaline method of Brinboim and Doly[22] and separated on a 1% agarose, at 50 vol for 1 hour and 1.5 hours. The DNA bands were visualized and photographed under UV light after the gel had been stained with ethidium bromide.

DNA purity

DNA purity was estimated by NanoDrop method (ACT gene, USA). The absorbance at 260 nm (A_{260}) and at 280 nm (A_{280}) for DNA was measured to check its purity. The ratio of A_{260}/A_{280} was found to be between 1.65 and 1.84.

Results and discussion

In this study, ten *E. coli* isolates obtained from the Tigris River in Baghdad city and ten *E. coli* isolates collected from children diarrhea from Children Hospital Center in Baghdad city were tested for hemolysin production, β-lactamase production, biofilm formation, antibiotic sensitivity, and plasmid content.

Latex O157:H7

Results showed that all *E. coli* isolates investigated with latex O157:H7 slide agglutination test gave a negative reaction. Chigor et al[23] found that rate of *E. coli* O157 in surface waters was 2.2% and its prevalence in children with diarrhea was 5.4%. Baqir et al[24] revealed that *E. coli* O157:H7 was isolated from four (1.14%) children with diarrhea.

Hemolysin production

The hemolytic toxin α-hemolysin belongs to the RTX toxin family[25] and is an important virulence factor produced by several strains of *E. coli*. It is involved in human diseases such as urinary tract infections (UTIs), peritonitis, meningitis, and septicemia.[26] Results illustrated in Figure 1 showed that eight (40%) *E. coli* isolates (six isolated from river and two isolated from children diarrhea) were producing hemolysin, while the remaining 12 (60%) isolates showed γ-hemolysis (no hemolysis) and were considered as non-hemolysin producers. Martinez-Martinez et al[27] found that 56 (27.3%) of clinical *E. coli* isolates were hemolytic. Al-Chalabi[28] recorded that 57.1% of uropathogenic *E. coli* produced hemolysin and Abdel Rahman[29] revealed that 70.5% of *E. coli* isolated from chronic UTIs were hemolytic. The difference percentage of hemolysin production by *E. coli* isolates may be due to source of blood, type of hemolysin produced, source of bacteria, and screening method.

β-lactamase enzyme production

Extended spectrum β-lactamases have emerged as a major threat worldwide with limited treatment options.[30] The results showed that eleven (55%) *E. coli* isolates (seven isolated from river and four isolated from children diarrhea) were β-lactamase enzyme producers (Figure 2). This result disagreed with the result recorded by Panus et al,[31] who found that 21 (14.66%) aquatic *E. coli* isolates (seven isolated from drinking water and 14 from sea water) were positive for β-lactamase test. Results illustrated in Figure 3 showed that all *E. coli* (100%) isolates were resistant to β-lactam drugs, such as carbenicillin, piperacillin, cefodizime, and imipenem. In gram-negative bacteria, a variety of plasmid-determined β-lactamases can hydrolyze ampicillin, carbenicillin, cephalothin, and related drugs.[32,33] The "newer β-lactamases" that consist of plasmid-mediated AmpC β-lactamases (eg, CMY types), extended spectrum β-lactamase (eg, CTX-M types),

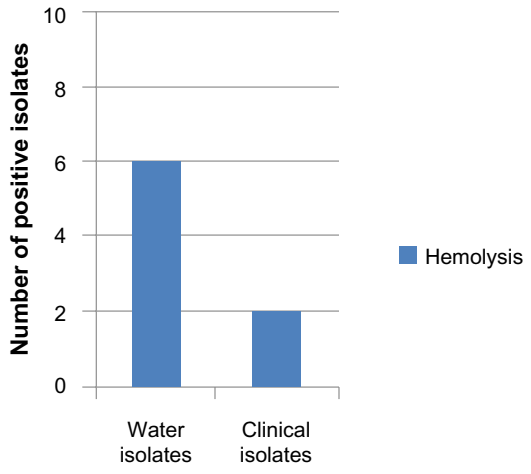

Figure 1 Number of *E. coli* isolates producing hemolysin.

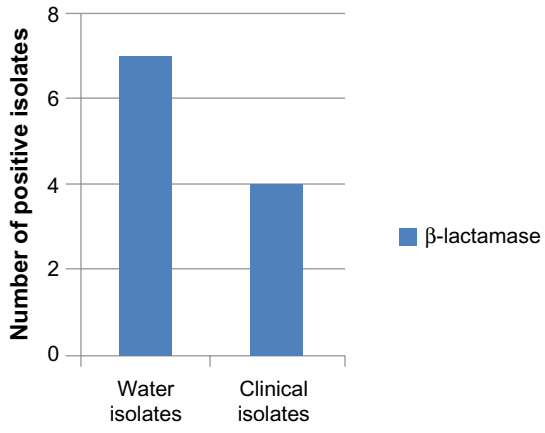

Figure 2 Number of *E. coli* isolates producing β-lactamase.

and carbapenemases (eg, NDM) are important causes of resistance to β-lactam antibiotics among extraintestinal pathogenic *E. coli*.[34]

Biofilm formation

The biofilm formation is considered to be a two-step process in which the bacteria first adhere to a surface mediated by capsular antigen or flagellar antigen followed by multiplication to form a multilayered biofilm, which is associated with production of exopolysaccharide matrix. The ability of bacteria to form biofilms helps them to survive hostile conditions within host and is considered to be responsible for chronic or persistent infections.[35]

Five of the ten water *E. coli* isolates and two of the ten clinical *E. coli* isolates showed biofilm formation (Figure 3). Al-Chalabi[28] found that 90% of *E. coli* isolated from UTI were producing biofilm. There are complexities of genetic and environmental effectors of the biofilm phenotype within the species *E. coli*.[36]

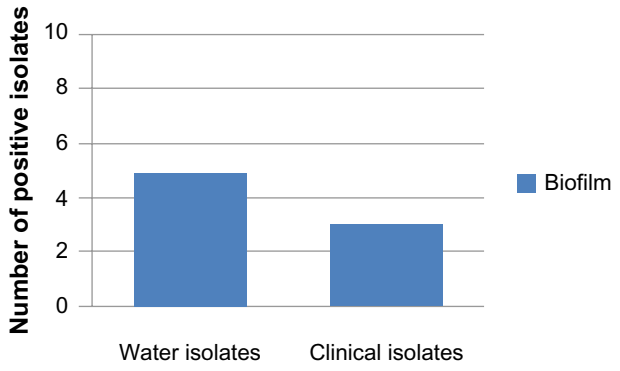

Figure 3 Number of *E. coli* isolates producing biofilm.

Antibiotic sensitivity

There was a high correlation between water isolates and clinical isolates of different sources in a pattern of antibiotic resistance, which implies that the water sources might have been contaminated with mixed contaminants originating from human and animal excreta.[37] All *E. coli* isolates showed multidrug resistance (Figure 4). Overall resistance was most frequently observed with antibiotics carbenicillin, cefodizime, imipenem, and piperacillin (100%) for both clinical and water *E. coli* isolates; chloramphenicol showed 100% for clinical isolates and 80% for river isolates; norfloxacin showed 100% for clinical isolates and 90% for water isolates; gentamicin showed 90% for clinical isolates and 80% for water isolates; amoxicillin–calvulanic showed 90% for clinical isolates and 70% for water isolates; and pefloxacin showed 90% for clinical isolates and 50% for water isolates; water isolates showed 90% resistance to rifampicin and clinical isolates revealed 80% resistance to rifampicin. Other researchers revealed that multidrug resistance (MDR) was higher among aquatic isolates than the clinical isolates.[38] The presence of antibiotic-resistant bacteria in water and surface water is a subject of public health concern; this leads to a real risk factor of acquiring such bacteria from the environments.[39]

Plasmid DNA

All *E. coli* isolates had plasmids bands that ranged from 4.507 kbp to 5.07 kbp (Figure 5). Loss of plasmids (>2.1 kbp) due to treatment with sodium dodecyl-sulfate correlated with loss of resistance to antibiotics, suggesting that the observed MDR was plasmid mediated.[38] Our results may be referring

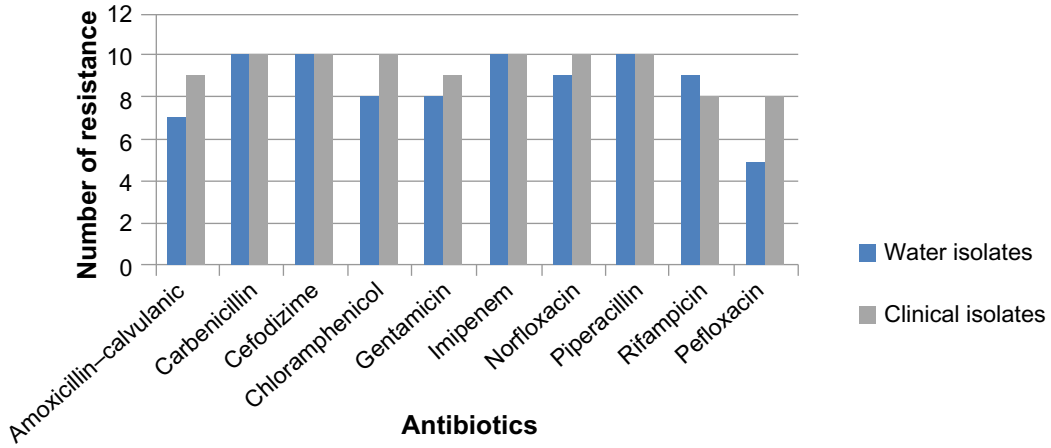

Figure 4 Number of *E. coli* isolates resistant for antimicrobial agents.

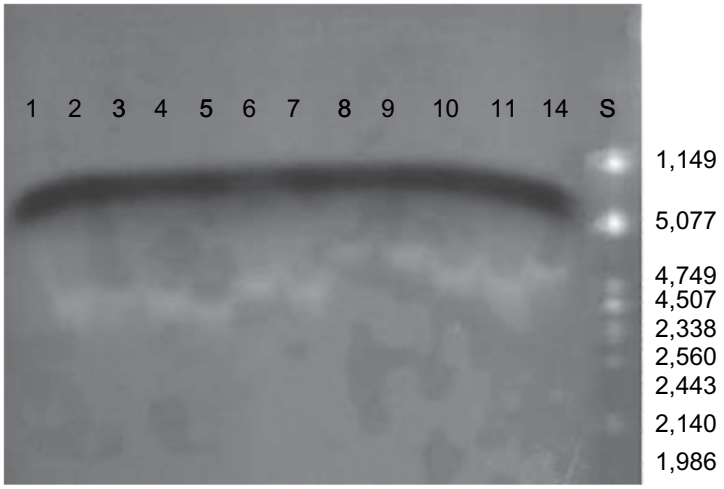

Figure 5 Agarose gel electrophoresis of plasmids extracted from various *E. coli* isolates.
Notes: Numbers 1–11 water isolates, 14 clinical isolates. S-ladder λ DNA (Pst1) Kb; (1% agarose, 50 vol, 1.5 hours).

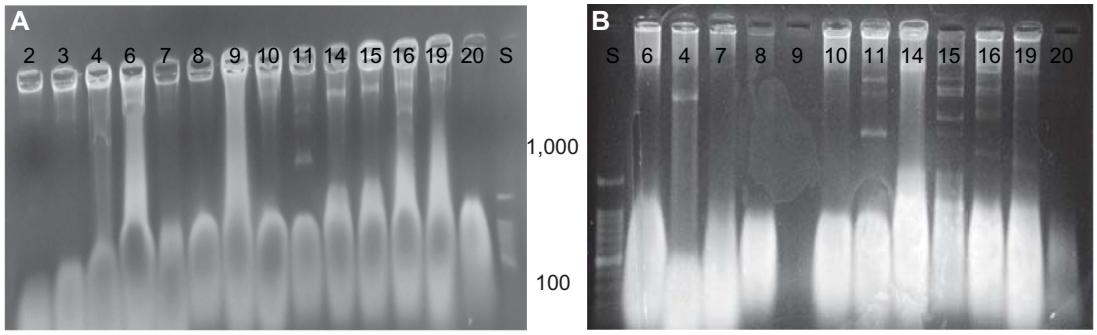

Figure 6 Plasmids comparative results.
Notes: (**A**) Agarose 1%, 50 vol, 1 hour. (**B**) Agarose (1% agarose, 50 vol, 1.5 hours). Gel electrophoresis of plasmids extracted from various *E. coli* isolates. Numbers 1–11 water isolates, 14 and 21 clinical isolates. S-DNA ladder (100–1,000 bp).

to the main β-lactamase production and MDR to plasmids range 4.507–5.07 kbp.

Clinical isolates (Number 11–20 isolates) showed multiple plasmids more than 1,000 bp (Figure 6). These multiple copies of plasmid bands might have resulted from covalently close circular, open circular, or linear forms of the same plasmid. There are several types of *E. coli* virulence plasmids, including those essential for the virulence of enterotoxigenic *E. coli*, enteroinvasive *E. coli*, EPEC, EHEC, enteroaggregative *E. coli*, and extraintestinal pathogenic *E. coli*.[40] It has been suggested that special pathogenicity is due to small and large plasmids; some researcher revealed that 212.8 kbp plasmid was significantly associated with enteroinvasiveness[41] and EHEC carry a large plasmid (91.2 kbp) that is associated with the presence of fimbriae.[8]

Disclosure

The authors report no conflicts of interest in this work.

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