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RESEARCH ARTICLE

Molecular characterization of virulence genes Shiga-like, Heat-labile toxins and antibiotics resistance in multidrug-resistant *Escherichia coli*

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ABSTRACT

Escherichia coli is a part of human intestinal flora and considered the second most common death factor between children younger than five years. *Escherichia coli* Shiga-like, Heat-labile toxins and multidrug-resistant are important virulence factors. Therefore, the main targets of this work were molecular diagnosis and characterization of toxine genes Shiga-like and Heat-labile and resistance genes of ciprofloxacin and gentamicin on molecular basis for *Escherichia coli* isolated from children stool sample in Egypt. The gene detection was carried out using two different approaches which were using chromosome and plasmid. *Escherichia coli* isolates were resistant to 24 antibiotics including four categories based on their functions. For example, ciprofloxacin, gentamicin, novobiocin and vancomycin. All of tested strains showed band near 300 bp that represents of shiga-like toxin (SLT) gene in their genomic DNA. Heat-labile toxin (LT) fragment ~ 200 bp was detected in plasmid of strain 8H and 8G. Moreover, gentamycin resistance fragment (aac C2) was detected in chromosomal DNA of all strains as a single robust fragment of molecular weight ~ 856 bp. In the case of ciprofloxacin-resistance gene a slight amplification was detected in strain 8G as well as 8H at about 1 kb and 800 bp in genomic, while it was absent in plasmids of tested strains.

Keywords: *Escherichia coli*, Shiga-like toxin, Heat-labile toxin, multidrug-resistant, ciprofloxacin, gentamicin.

INTRODUCTION

Escherichia coli a part of human intestinal flora, and *Escherichia coli* Shiga-like toxin, (STEC) are importance virulence factor which cause severe intestinal diseases like diarrhea. Diarrhea is the most common causes of mortality and morbidity among children[1,2]. Multiresistant *Escherichia coli* have become a worldwide public health trouble caused by inappropriate use of antibiotics to treat *Escherichia coli* infection[3,4,5].

The multidrug-resistant *Escherichia coli* have increased as causative agent in urinary tract infections, in young children and common the infection is repeated[6, 7, 8]. In addition to, *Escherichia coli* is strong important factor of uropathogen linked with uncomplicated cystitis[9, 10, 11].

The resistance to the fluoroquinolones class in *Escherichia coli* isolated from uropathogenic was thought to be transferred from animal sources food through the gut transfer of virulence and antibiotic resistance genes[12, 13, 14]. In addition to, the animal origin food suggested a reservoir of Escherichia coli resistance to aminoglycoside class[15, 16].

The targets of this research was to diagnosis and characterization of the two virulence toxin genes Shiga-like toxin and Heat-labile toxin and two resistance genes ciprofloxacin resistance gene and gentamicin the resistant gene on molecular basis specially of *Escherichia coli* from children stool sample in Egypt.

MATERIALS and METHODS

Escherichia coli isolation, identification and growth conditions

Three *Escherichia coli* strains were obtained from children younger than five years in Egypt. Patient suffered from serious diarrhea infection. The *Escherichia coli* were propagated at 37 °C in enrichment broth followed by sub culturing on MacConkey agar under aerobic conditions[17]. Further confirmation of E. coli was carried out using PCR to detect the 16S rRNA gene[18].

Antibiotic susceptibility test

The antibiotic resistance test of the *Escherichia coli* strains was carried according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) were examined using a disc diffusion method[19]. The following 24 antibiotics covered the main four categories based on their targets cell wall, protein synthesis, DNA synthesis and nucleic acid targeting were tested: ciprofloxacin, gentamicin kanamycin, vancomycin, novobiocin, cefadroxil, oxacillin, azithromycin, linezolid, ertapenem, tobramycin, cefuroxime, linomycin, rifamycin, cefotaxime, cephradine ceftazidime, streptomycin, ceftriaxone, amoxicillin, cefadroxil, erythromycin, cephalothin and cephalexin. Tested plates were incubated at 37°C for 24 h, and the results were read and reported based on the inhibition zone diameter as sensitive or resistant[20].

Molecular diagnosis and characterization

Extraction of nucleic acids

Genomic DNA was extracted using GeneJet genomic DNA purification kit (ThermoFisher Scientific , USA) according to the user manual. Briefly, 2×10^9 bacterial cells were harvested by centrifugation for 10 min at 5000 xg, and the cell pellet of each strain was resuspended in 180 µL of Digestion Solution (provided in the kit) and 20 µL of Proteinase K were added and one thoroughly mixed until a uniform suspension was obtained and incubated at 56°C shaking water bath for 30 min for lysis. 20 µL of RNase a Solution were then added and mixed by vortexing and the mixture was further incubated for 10 min at room temperature. 200 µL of lysis solution was added to the mixture and mixed and then 400 µL of 50% ethanol was added and mixed by vortexing. The cell lysate was then transferred to the purification column and centrifuged for 1 min at 6000 xg. The column was washed with 500 µL of each washing buffer (I and II) and centrifuged at maximum speed for 2 min to remove ethanol remnants. Purified DNA was eluted with 100-200 µL elution buffer and stored at -20°C till use.

For plasmid preparation, cells from 5 ml of overnight cultures were spinned down and plasmids were extracted using GeneJet plasmid DNA miniprep kit (Thermofisher Scientific, USA). According to the user manual; overnight grown culture (1-3ml) was shifted into microcentrifuge tubes (1.5ml) and centrifuged at 12,000xg for 2 min. The supernatant was decanted and the pellet was gently re-suspended in 250µl of ice cold resuspension buffer supplied with the kit. To the resuspended culture, 250µl of lysis buffer (supplied with the kit) were added and mixed by inverting the tube 5-6 times. Tubes were allowed to incubate at room temperature for 5 min. After that 350µl of ice-cold neutralization buffer (supplied with the kit) was added to lysate and mixed by inverting 6-8 times. Tubes were centrifuged at 14,000xg for 5 min in microcentrifuge at room temperature. Supernatant was transferred to the spin column/collection tube and centrifuged at 10,000xg for 30 sec. Flow through was discarded and 500µl of wash buffer (supplied with the kit) were added twice to miniprep column/collection tube and again centrifuged at 10,000xg for 30 sec, and once at maximum speed for 1 min to dry the resin. Plasmid DNA bound to the resin of column was eluted by adding 50µl of pre-warmed ddH2O and incubated at room temperature for 3 min and centrifuged at maximum speed (14,000xg) for 30 sec.

Gene amplification

PCR reactions were carried out in thermal cycler (BIO-RAD, USA) using GoTaq DNA Polymerase (PROMEGA, USA). The target gene amplification was performed with 1µl of the purified genetic materials (genomic DNA/ plasmid preps) as template, 5µl buffer, 2.5µl MgCl2, 1µl of each primer (listed in table 1 below), 0.5µl of dNTPs mix, 0.25µl of Taq Polymerase enzyme and the reaction volume was completed to 25µl with free-nuclease water. The cycling conditions included one cycle of pre-denaturation for 5 min at 95°C, followed by 35 cycles of denaturing at 95°C for 30 sec, annealing for 30 sec according to the primers used and extension for 30 sec at 72°C and a final extention cycle for 5 min at 72°C. PCR products were resolved on agarose gel (1%) premixed with 0.5µg/ml ethidium bromide, the size of the resolved products was determined

either with 100bp or 1kb DNA ladder. Gel was run at 80V for 50 min followed by visualization on a gel documentation system (Biometra, Goettingen, Germany).

Table 1. List of Primers Used in Gene Amplification

Target Gene	Primer ID	Primer sequence
Shiga-like toxin (SLT)	**SLT F:**	5'-AAGAAGATGTTTATGGCGGTTT-3'
	SLT R:	3'-GTCATTATTAAACTGCACTTCAGCA-5'
Heat-labile toxin (LT)	**LT F:**	5'-ATTGACATCATGTTGCATATAGGTTAG-3'
	LT R:	3'-ACATTTTACTTTATTCATAATTCATCCCG-5'
Ciprofloxacin resistance gene	**aac (6′)-F:**	5'-TTTATTATTTTTAAGCGTGCATAATAAGCC-3'
	aac (6′)-R:	3'-TTAAGACCCTTAATTGTTGGGATTT-5'
Gentamycin resistance gene	**aac C2-F:**	5'-CATACGCGGAAGGCAATAAC-3'
	aac C2-R:	3'-ACCTGAAGGCTCGCAAGA-5'

RESULTS and DISCUSSION

Shiga-Like Toxin (SLT) detection

Molecular techniques used for diagnosis and molecular characterization of *Escherichia coli* revealed three model isolates. Strains were typed as 8G, 8H and 8L recovered strains from children younger than five years suffering from serious diarrhea infection. All of tested strains showed a homogenous band that represents the subunit B of shiga-like toxin (SLT) gene in their genomic DNA. The amplified fragment has nearly 300 bp molecular weight. The first responsible for infections is multidrug-resistant *Escherichia coli*, especially more than 80% recorded for beta-lactamase (ESBL). Several studies revealed that genetic similarity between the resistant *Escherichia coli* isolates indicated that strains from same origins and transferred to the human being causing serious infections[6].

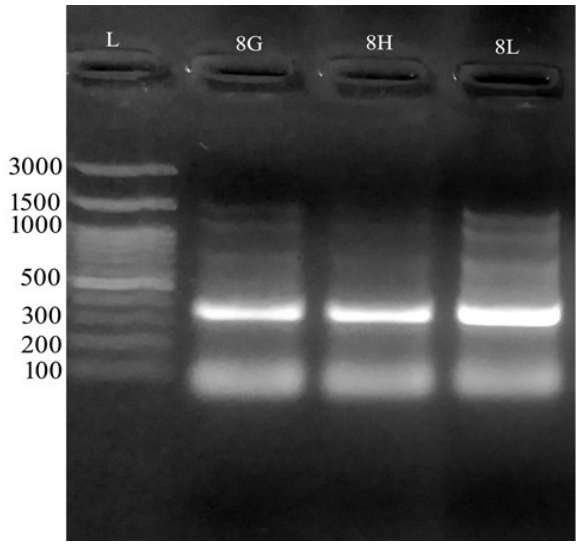

Figure 1. Shiga-like toxin (SLT) in genomic DNA at a molecular weight of ~300 bp, Lane (L) a standard DNA ladder with 100-3000 bp range.

Heat-Labile Toxin (LT) Screening

Heat-labile toxin (LT) gene was screened in both genomic DNA and plasmid preps in all of the tested strains. A fragment of ~ 200 bp was detected in strains. It was absent in the strains 8G, 8H and 8L. In contrast, the target gene was detected in plasmid preparations of strain 8H and to a lower extent in the control strain 8G, while it was absent in other tested strains which would reflect the variability of the LT gene type present in tested strains. Escherichia coli from clinical source harbored two copies of the heat-labile toxin (LT) -encoding gene (elt) on a plasmid[21]. Serious human diseases are caused by bacteria producing Shiga toxin such as ashemorrhagic colitis, severe inflammations of ileocolonic regions of gastrointestinal tract, thrombocytopenia, septicemia, malignant disorders in urinary ducts, hemolytic uremicsyndrome. Shiga toxin 1 (stx1), shiga toxin 2 (stx2), or a combination of both are responsible for most clinical symptoms of these diseases[22, 23].

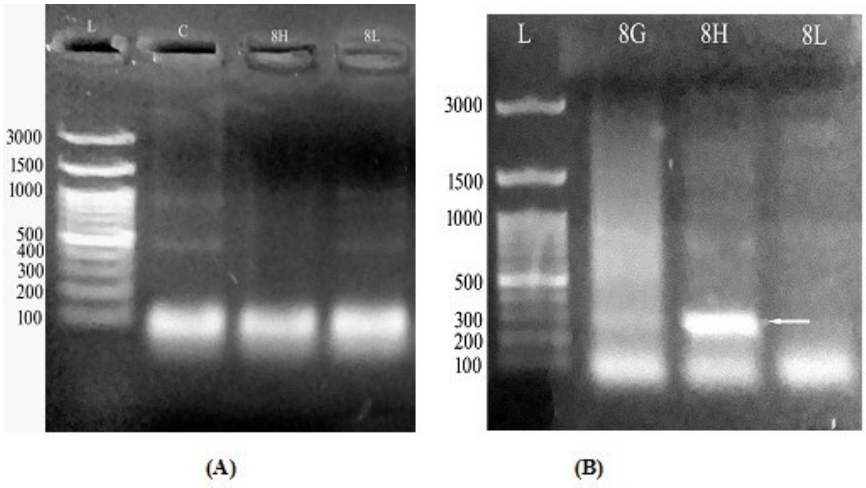

Figure 2. Heat-labile toxin (LT) gene in genomic DNA (A) and plasmid preparations (B) at a molecular weight of ~ 200 bp.

Gentamycin resistance profile

In our research, the antibiotic resistance evaluation for *E. coli* isolates indicates that strains have resistance genes responsible for major antibiotic groups. Gentamycin resistance gene (aac C2) fragment was detected in all strains 8G as well as 8H and 8L strains as a single robust fragment of molecular weight ~ 856 bp.

Mainly E. coli has Multidrug-resistant profile obtained from both animal and human clinical samples[24]. The resistance levels variable in *E. coli* and common reported against antimicrobial agents, like ampicillin, penicillin, streptomycin, amoxicillin, tylosin, erythromycin oxytetracycline, and neomycin[25]. Various studies mention the variation in sensitivity test, laboratory culture protocol and conditions, identification of bacteria methods, sampling tools, and sample origin, and the treatment with types of antimicrobials all may lead to variation in sensitivity results. Generally, the resistant to antibiotics is not genetic in all cases of bacterial strains; other mechanisms may be responsible of presence of resistance in isolates[26]. Other study revealed that a large number of *E. coli* isolated from bovine mastitis were resistant to some of the major antibiotic groups, irrespective of the resistance genes that were present[27].

In this study, all *Escherichia coli* were tested from children younger than five years suffering from serious diarrhea infection were tested using PCR for detection of the gentamycin resistance genes. Amplification results observed specific fragments for aaC2 gene at 856 bp in all the tested strains as shown in Fig. 3. The obtained results indicated the harboring of the gentamicin-resistant strains to the aaC2 gene responsible for aminoglycoside resistance[28].

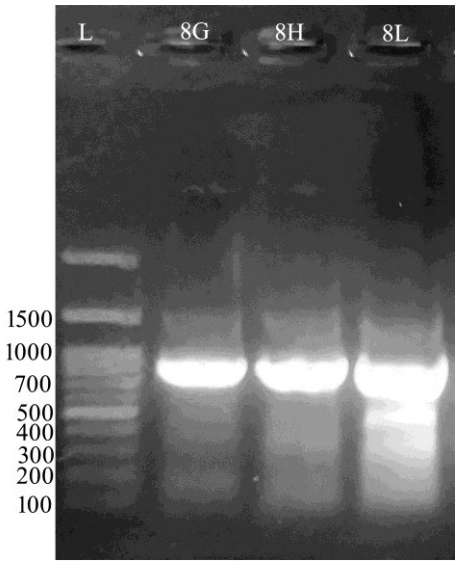

Figure 3. Gentamycin-resistance gene at molecular weight ~ 856 bp.

Ciprofloxacin resistance profile

Ciprofloxacin-resistance gene was also screened in both genomic and plasmid preparations in all strains under test. In control strain 8G as well as 8H strain there was a slight amplification detected at about 1 kb and 800 bp respectively (Fig. 4.A), while it was absent in the 8L. In plasmid preps, no amplification of the target gene could be detected in all strains tested (Fig. 4.B). From our study, focused on resistant strains in patients but should controlling include the environment, acquired infections decline the classification of hospital and community.

Other study summarized the significant causative agent for diarrheal and a foodborne outbreak is *E. coli*, responsible of severe economic losses. The virulotyping indicated that the plurality of *E. coli* strains were carried for eae, stx1, nleA, stx2, ehxA, ureC, and iha but subA was positive in littel isolates[29]. Finally, but very importantly, we reported that *Escherichia coli* strains a high level of virulence factors in addition to high Multidrug-resistant.

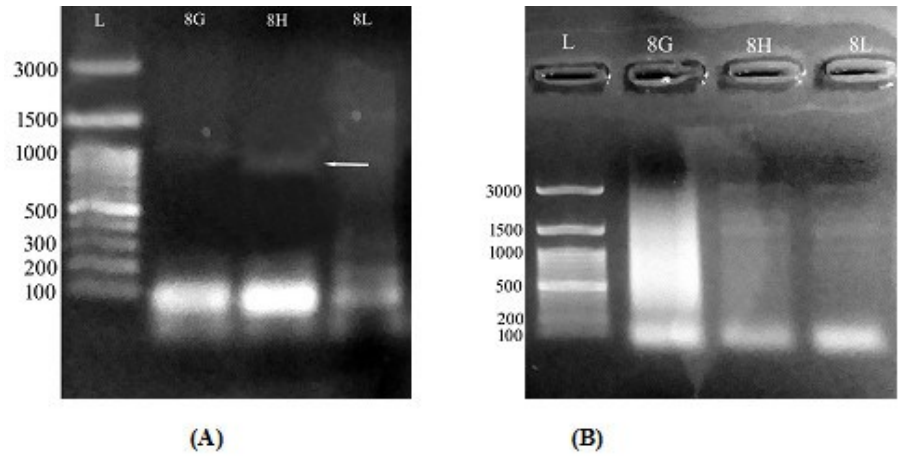

Figure 4. Ciprofloxacin-resistance gene at 1kb size. (B) Plasmids examined for target genes.

CONCLUSION

In our study, the multidrug-resistant *Escherichia coli* loaded with Shiga-like, Heat-labile toxins and two resistance genes of ciprofloxacin and gentamicin considered high risk factor on children. The bad use of antibiotics is very dangerous in future. *Escherichia coli* considered the second death factor between children younger than five years. Virulence factors are importance trend in *Escherichia coli*.

Conflict of interest

The authors declare no conflict of interest**.**

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