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MOLECULAR CHARACTERIZATION OF MULTI DRUG RESISTANCE UROPATHOGENIC ESCHERICHIA COLI ISOLATES FROM IRAQI CHILDREN WITH URINARY TRACT INFECTION

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ABSTRACT : Urinary tract infections (UTI) are common causes of morbidity and sometimes lead to significant mortality. *Escherichia coli* was the most common etiological agent of UTI especially in infant and children. The current study aimed to isolation, identification in addition molecular identification for Urooathogenic *E.coli* from hospitalized children under 12 years, detect antibiotic susceptibility pattern, and resistance genes. Collection of 200 of urine samples from infant and children, then cultured in selective and differential media, final identification was done using Vitek 2 compact system. Molecular identification for identify gene *pap*E, antibiotic resistance genes *tet*A, *tet*B, CITM, *sul*1, *sul*2 and *sul*3 were done using convention polymerase chain reaction. The results were shown 40/200 (20%) of isolates belong to *E.coli* (32 female and 8 male), The result for identification gene *pap*E for Urooathogenic *E.coli* that percentage (37.5%) harbors antibiotic resistance was varies, highly resistance was for ampicillin and sulfamethoxazole and less resistance for tigecycline while most isolates were multi drug resistance, from the other hand the percentage for ability biofilm formation using Congo-red agar methods was (55%) in congo-red and (26.6%) using microtiter- plate methods. Percentage of resistance genes *tet*A, *tet*B, *sul*1 and *sul*2 (60%), (6%), (53.3%) and (40%) respectively, while CITM and *sul*3 gene not present in any isolates under study.

Key words : UPEC, children, resistance genes, *pap*E gene.

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INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC) is the most abundant causative bacteria of urinary tract infections (UTIs), worldwide accounting for 80–90% of all infections (Dhahi, 2020). UPEC are strains of *Escherichia coli* that divert from their commensal status as intestinal flora, grow and persist in the urinary tract and exhibit diverse array of virulence factors and strategies, which allow them to infect and cause diseases in the urinary tract. These strains of *E. coli* are consistently associated with uropathogenicity and are called as UPEC (Shah *et al*, 2019). The increase in antimicrobial resistance has been driven by a diverse set of factors, including inappropriate antibiotic prescribing and sales, use of antibiotics outside of the health care sector, and genetic factors intrinsic to bacteria especially in developing countries making that problem health and reduces choices in treating UPEC such aminoglycosides, fluoroquinolones and cephalosporins (Marston *et al*, 2016; Homles *et al*, 2016). Biofilm formation seems to be an important consideration for pathogenesis and the reason of therapeutic failure, especially in some of the device-associated infections such as long-term catheterized patients with urinary tract infections, Biofilms consider as assemblages of microorganisms attached to a surface (Crémet *et al*, 2013; Zainab *et al*, 2021). It has become obvious that sessile bacterial cells in the biofilms express properties distinct from planktonic cells, for example, the higher resistance to antibiotics and antibacterial agents, which leads to survival in hostile environments (Poursina *et al*, 2018). The study aimed to detect the incidence of UPEC in children and the presence of resistance genes.

METHODS

Specimens collection

200 specimens generated by Iraqi children under 12 years of age under specialized medical supervision for the period July to December 2020 were collected from the Central Child Hospital in Baghdad, which included patients who were resuscitated and reviewed by the

hospital. They were implanted on macConkry agar, EMB agar and blood agar.

Identification of bacteria using VITEK 2 compact system

Bacteria were subjected for identification by VITEK 2 compact system according to the instruction provided by the company. The turbidity was adjusted to 0.5 MacFarland turbidity range and measured using visible spectrophotometer DensiChekTM Plus. The bacterial suspension was used to inoculate the Vitek2 system (bioMérieux, France). Interpretation of results was performed according to VITEK 2 compact system special software to identify bacterial species and strains**.**

Determination of antibiotic susceptibility using VITEK® 2 compact

Susceptibility to the following antimicrobial agents (depending on the bacterial genus) was determined using VITEK 2 compact system: antibiotic included:Ampicillin, Piperacillin/Tazobactam, Cefazolin, Imipenem, Meropenem, Amikacin, Trimethoprim/sulfamethoxazole, Gentamicin, Tobramycin, Ciprofloxacin, Ceftriaxone, Ceftazidime, Cefoxitin, Cefepime, Nitrofurantoin, Ertapenem, Gentamicin, Levofloxacin. The break point for each antimicrobial used was determined according to CLSI (2019).

Antibiotic susceptibility using disk diffusion methods

Antibiotic susceptibility test was done using Kirby-Bauer Disk Diffusion method for additional antibiotic including: Ticarcillin/ clavulanate, Aztreonam, Tobramycin, Azithromycin, Doxycycline, Sulfonamides, Chloramphenicol and Cefpodoxime. The diameter of inhibition zone was matured and the results were recorded as sensitive, resist or intermediate according to CLSI (2019).

Identification of uropathic *E.coli* by detection of *pap E* using Conventional PCR

Uropathogenic *E. coli* was identified by detection of *pap E* using conventional PCR. Extraction of DNA was done using XIT™ Genomic DNA Purification Kit following manufacturer instructions. PCR was performed using a specific primer set for the detection of *pap E* in bacterial extracted DNA. PCR products were electrophoresed in 1.5% agarose gel. The appearance of a band with a molecular size 321 bp referred to the amplification of *papE.*

Biofilm assay using congo-red agar methods

Qualitative indicator of the detection of microorganisms produced, the Congo medium is prepared by mixing (0.8) Congo congo-red dye, (36) Scrooge gum and (37) gum from the middle of the solid heart-brain mixture, sterilizing with autocliff, and infusing with dishes after inoculation of the implant medium, the incubator dishes are placed with Co37 for 24 hours. Colonies with shimmering black mean strong production and red is unproductive (Sahra, 2019).

Biofilm assay using micro-titer plate method

The biofilm formation ability of Escherichia coli isolates was determined by 96-well microtiter plate assay based on the crystal violet staining method. Briefly, each 96-well flat-bottomed sterile polystyrene microplate well containing199µL of Mueller–Hinton broth supplemented with 1% glucose were inoculated with 1µL from suspended bacterium of 0.5-0.7 McFarland (1.108cfu/ml). 4). Microplates are incubated 24 h at 37°C. The liquid media was discarded, and the adherent cells were

Table 1 :

Genes	Primer	Annealing	Product size	References
CITM	F- TGGCCAGAACTGACAGGCAAA R- TTTCTCCTGAACGTGGCTGGC	56^{0}C for 30 sec	462	Sa´enz *et al* (2004)
*tet*A	F-GGTTCACTCGAACGACGTCA R-CTGTCCGACAAGTTGCATGA	57^{0}C for 30 sec	577	Momtaz *et al* (2012)
*tet*B	F- CCTCAGCTTCTCAACGCGTG R- GCACCTTGCTGATGACTCTT	56^{0}C for 30 sec	634	
SUL1	F- TGGTGACGGTGTTCGGCATTC R- GCGAGGGTTTCCGAGAAGGTG	63^{0}C for 30 sec	789	Sa´enz *et al* (2004)
SUL2	F- CGGCATCGTCAACATAACC R- GTGTGCGGATGAAGTCAG	50^{0}C for 30 sec	722	
SUL3	F- CATTCTAGAAAACAGTCGTAGTTCG R-CATCTGCAGCTAACCTAGGGCTTTGGA	51^{0}C for 30 sec	763	

washed twice with phosphate buffered saline (PBS) and wells are dried at 60°C for1 h or less. After that it was stained with 150µL of 2% of crystal violet for 15 min. Then the crystal violet -stained wells of microplates were washed twice with PBS to discharge crystal violet stain. After air drying process of wells of microplate, dye of biofilms that lined the walls of the microplate is re-solubilized by 150ìL of 95% ethanol. After 5-10min microplate is measured spectrophotometrically at 570 nm by a microplate reader. The assay was done at least three times using fresh samples each time. The optical density cut-of value (ODc) was established as three standard deviations (SD) according to Kerkeni *et al* (2016).

PCR assay for resistance genes

Extraction of DNA was done using XIT™ Genomic DNA Purification Kit following manufacturer instructions. PCR was performed using a specific primer set for CITM, *tet*A, *tet*B, *sul*1, *sul*2 and *sul*3 in bacterial extracted DNA. PCR products were electrophoresed in 1.5% agarose gel. The appearance of a band with a molecular size specific for each genes.

RESULTS

Identification of *Escherichia coli* from urine specimens

Identification of bacteria in 200 urine specimens (107 female and 93 male) using culturing on MacConkey agar, blood agar, EMB agar and Vitek2 system were showed only 62 specimens positive for bacterial growth which as the following: 40/62(64%) isolates of E.coli, 15/62(24%) isolates of proteus sp , 7/62(11%) isolates of *Klebsiella pneumonie*. UTI is the most common bacterial infection in childhood (O'Brien *et al*, 2011) and up to 30% of infants and children experience recurrent infections during the first 6–12 months after initial UTI [15,16]. In very young infants, symptoms of UTI differ in many ways from those in older infants and children. The prevalence is higher in the first age group with a male predominance. Most infections are caused by *Escherichia coli*, although in the first year of life *Klebsiella pneumoniae*, *Enterobacter* spp, *Enterococcus* spp and *Pseudomonas* are more frequent than later in life and there is a higher risk of urosepsis compared with adulthood (Stein *et al*, 2015). UTI infection in children less than two years may related to congenital defects in urinary system or kidney (Okarska *et al*, 2019). Iraqi study done by Abdul-Mohammed *et al* (2012) for determination of the incidence of urinary infaction included 100 children under 12 years indicated higher incidence among males than females in the category, The younger age of 3 and the main cause is E. coli . Another study in Erbil Iraq in 2015 to investigate the prevalence of UTI in infants and children, including 300 children with symptoms, only 130 of them were for bacterial growth, infection rate for females was 91/130 (70%) and for males 39/130 (30%). The main cause of infection is bacteria *E. coli* by 48/130 (36.9%) (Saeed *et al*, 2015).

Detection of pap EF

Only 15/40 (37.5%%) E. coli isolates from urine were positive to pap EF and considered as UPEC.The result of agarose gel electrophoresis of PCR amplified products of papEF shown in Fig. 1.

The adhesive systems are the most common virulence factors in UPECs. They play an important role in colonization and invasion of the bladder epithelial cells by UPECs (Yazidi *et al*, 2018). Egyption study by Khairyi *et al* (2019) showing results on 173 isolation from two different strains of bacteria (UPEC and DEC) The UPEC was carrying the papE gene while the DEC strain was not carrying the gene, and this confirms that the gene is diagnostic for UPEC. In another study in Iraq 2020, 45 samples was investigated UPEC Using pap E gene, 29/45 (64%) harbor that gene (Obeed and Dhahi, 2020). That differences between current and other study may related to the nature and size of the sample, geographical area, time of sample collection and other surrounding conditions.

Antibiotic susceptibility test

Antibiotic susceptibility of 15 isolates of UPEC was tested for 26 antibiotics (Table 2). The results showed that all isolates were highly resistant to ampicillin (93.5%) the lower percentage of resistance was for imipenem, Etrapenem, Ticarcillin/Clavulanic acid, Amikacin, Chloramphenicol and Tigecycline (93.3%) isolates. The resistance may related to production of ESBLs enzymes or carrying resistance genes.

The results of the current study were nearly to local studies such a study in Zacho by Polse *et al* (2016), which included 106 bacterial isolation of UPEC, showed that the resistance ratio for Penicillin, Empicillin and Aztreonam (100%) was sensitive to Imipenem. Another study in Erbil by Mohammad *et al* (2018), involving 60 isolation from UPEC bacteria was multi-drug resistant (100%), being resistant to Amikacin, Amoxicillin, Chloramphenicol, Ampicillin, Ciprofloxacin, Erythromycin, Nalidixic acid, Penicillin, Tetracycline and Trimethoprim in percentage between (28-965%). In the current study, the percentage of MDR was (93%), which resist at least for 3 antibiotic. MDR means the ability of bacteria to resist one or more antibiotic in three group of antibiotics, antibiotic resistance is a global problem, so scientists'

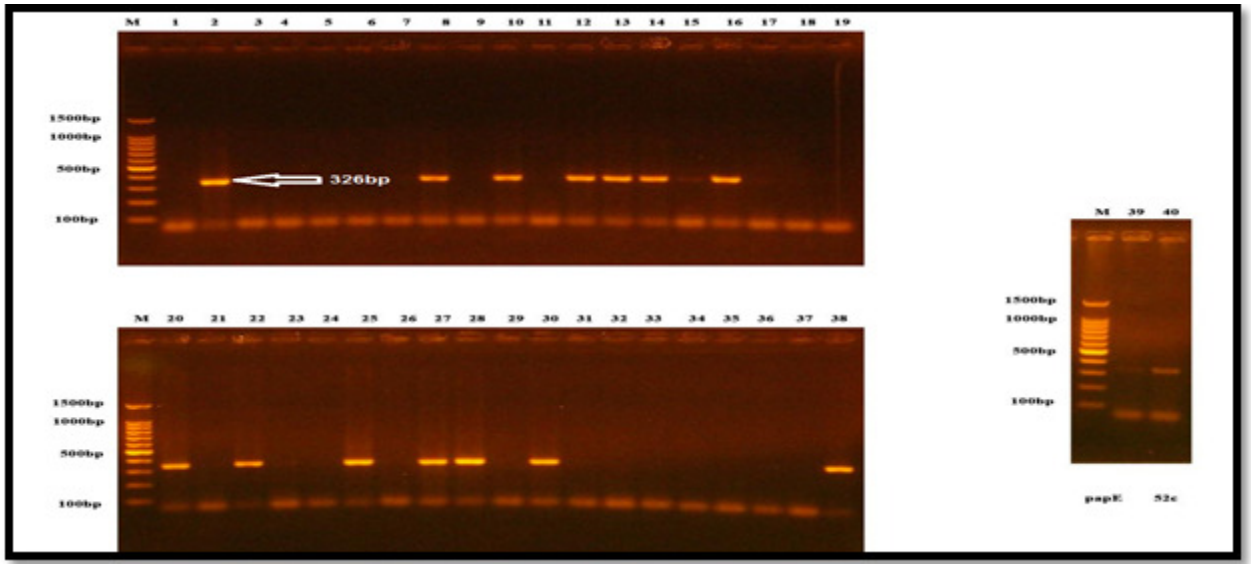

Fig. 1 : Agarose gel electrophoresis of PCR amplified products of papE (326 bp empty arrow).MW: molecular weight ladder of 100bp. Electrophoresis was done in 1.5% agarose gel at (5V/cm) for 60 min.

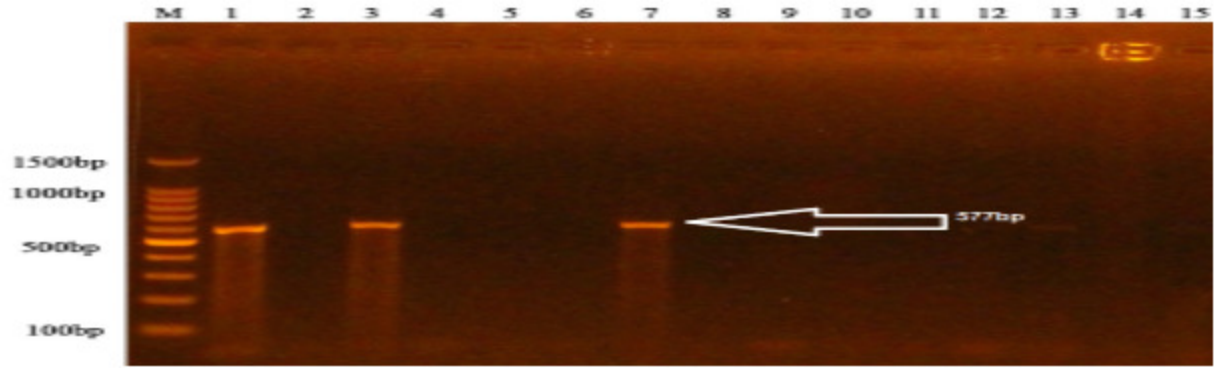

Fig. 2 : Agarose gel electrophoresis of PCR amplified products of *tet*A (577 bp empty arrow).MW: molecular weight ladder of 100bp. Electrophoresis was done in 1.5% agarose gel at (5V/cm) for 60 min.

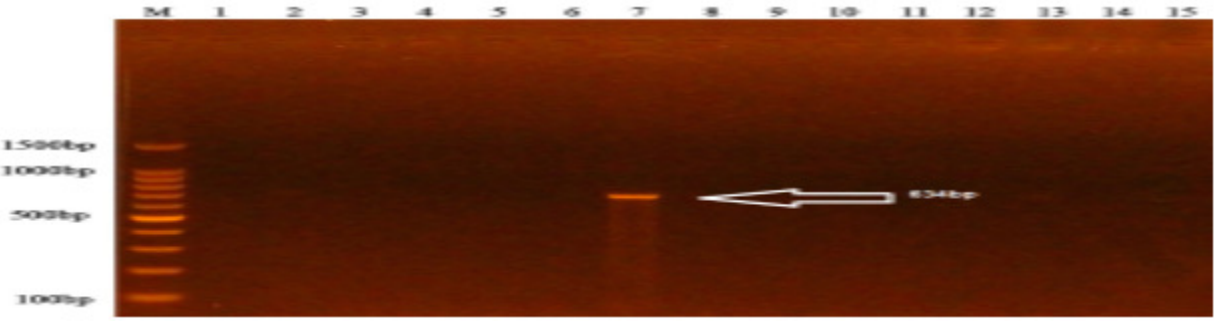

Fig. 3 : Agarose gel electrophoresis of PCR amplified products of *tet*B(634 bp empty arrow). MW: molecular weight ladder of 100bp. Electrophoresis was done in 1.5% agarose gel at (5V/cm) for 60 min.

efforts are aimed at developing New antibiotics or modification of existing antibiotics to combat resistant pathogens (Breijyeh *et al*, 2020). The misuse and inappropriate consumption of antibiotics has led to an increase in microbial-resistant bacteria around the world. The most serious consequence, therefore, is resistance to antimicrobials in the case of UPEC has led to a significant increase in mortality due to failure of response to treatment caused by antibiotic resistance. The resistance genes are either on chromosome or plasmid. UPEC, on the other hand, acquires resistance genes through DNA mutations or horizontal gene transfer between UPEC strains (Jahandeh *et al*, 2015). A study in Thailand by Tewawong *et al* (2020) to detect UPEC's multi-antigen resistance, results on 208 isolation from UPEC showed that 129/208 (62%) were multi-drug resistance. Treatment of urinary system infections was a challenge due to the prevalence of multi-drug resistance, according to a study by Kot (2019), in Poland, the rate of resistance to trimethoprim-sulfamethoxazole,

Table 2 :

Antibiotic class according to mode of action	Antibiotic	Percentage		
		S	I	R
Inhibit cell wall synthesis	Ampicillin	6.7%	-	93.3%
	Piperacillin/Tazobactam	80%	13.3%	6.7%
	Cefazolin	26.6%	6.7%	66.7%
	Ceftazidime	26.6%	-	73.4%
	Cefpodoxime	33.3%	-	66.7%
	Cefoxitin	73.4%	13.3%	13.3%
	Ceftriaxone	33.3%	-	66.7%
	Cefepime	33.3%	-	66.7%
	Etrapenem	93.3%	-	6.7%
	Imipenem	93.3%	-	6.7%
	Azteronam	46.7%	-	53.3%
	Ticarcillin/Clavulanic acid	93.3%	-	6.7%
Inhibit protein synthesis	Amikacin	93.3%	-	6.7%
	Gentamycin	75%	2.5%	22.5%
	Tobramycin	25%	-	75%
	Tetracycline	42.5%	-	57.5%
	Azithromycin	46.7%	-	53.3%
	Doxycycline	46.7%	-	53.3%
	Chloramphenicol	93.3%	-	6.7%
	Tigecycline	93.3%	-	6.7%
Inhibit DNA synthesis	Ciprofloxacin	53.3%	-	46.7%
	Levofloxacin	46.7%	-	53.3%
	Nitrofurantoin	86.7%	-	13.3%
	Sulfamethoxazole	10%	-	80%
Inhibit folic acid synthesis	Trimethoprim/Sulfamethoxazole	40%	-	60%

ciprofloxacin and amoxicillin-clavulant in UPEC insulation in developing countries ranges from (50-80%) while the proportion varies in developed countries, between (3-40%), this rise in antibiotic resistance was due to the unauthorized availability and uncontrolled use of antibiotic, especially in developing countries.

Molecular identification of resistance genes

The percentage of isolates harbor *tet*A and *tet*B gene was 9l15 (60%) and 1l15 (6%), respectively (Figs. 2, 3).

The results of the current study correspond to those of Rubab and Oh (2021) in Switzerland, where the study included 51 isolation from E. coli was the presence of the *tet* A gene (35). In another study by Nouri and Hamid (2020) investigated the prevalence of tetracycline resistance genes in 50 isolation of *E. coli* from the urinary system, finding that the presence of the *tet*A gene was 32% while the *tet*B gene was 38%. Another study by Bonyadian *et al* (2019) involved 114 isolates of E. coli to detect the presence of resistance genes the percentage of isolates carrying *tet*A gene was. (9.6%). A comprehensive study conducted in Mexico by researchers Pe ìrez *et al* (2020), to detect the presence of different resistance genes in 200 isolates of E. coli, results showed the percentage of a tetA gene was (56%). The reason for the difference in ratios is a difference in the number of isolates as well as a difference in the age of the patients.

The percentage of isolates harbor *sul*1 and *sul*2 gene was 8l15 (53.3%) and 6l15 (40%), respectively (Figs. 4, 5).

In the current study, CITM genes and *sul*3 not present in UPEC. There are many genes responsible for resistance to Beta- lactam such blaTEM, blaSHV, blaOXA, blaPSE and blaCTXM genes had been detected in clinical isolates of *E. coli*. The results of this study showed a high rate of resistance. (% 93.5) in epithelial detection, while resistance gene CITM not present, that may be due to the presence of another gene or a number of genes encoded to resist this antibiotic, as well as other

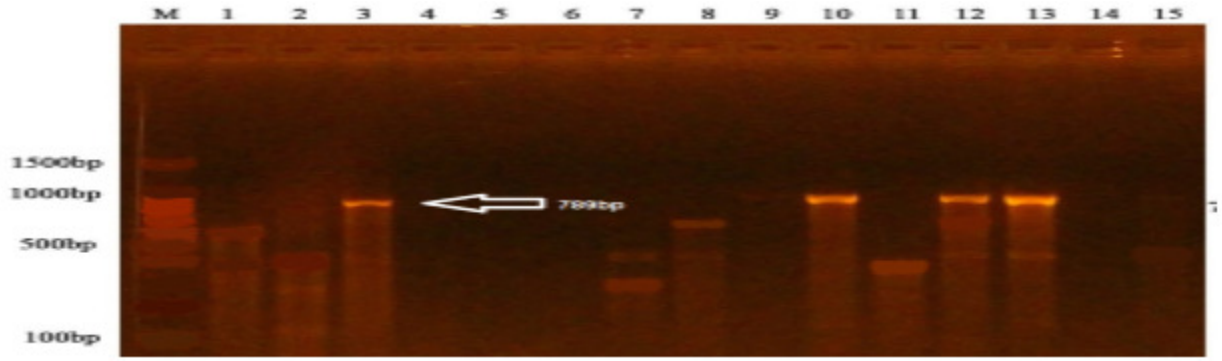

Fig. 4 : Agarose gel electrophoresis of PCR amplified products of *sul1* (789 bp empty arrow). MW: molecular weight ladder of 100bp. Electrophoresis was done in 1.5% agarose gel at (5V/cm) for 60 min.

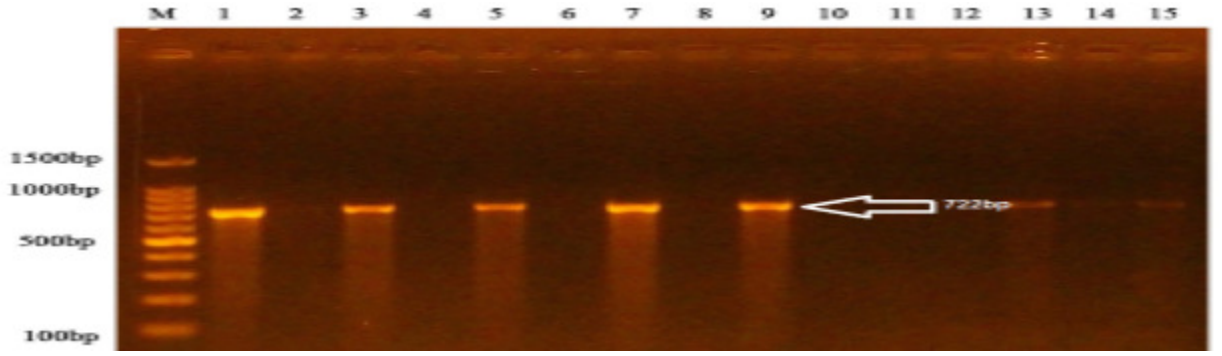

Fig. 5 : Agarose gel electrophoresis of PCR amplified products of *sul2* (722 bp empty arrow). MW: molecular weight ladder of 100bp. Electrophoresis was done in 1.5% agarose gel at (5V/cm) for 60 min.

mechanisms used by bacteria to resist that antibiotic (Abrar *et al*, 2019). Resistance to sulfonamides occurs principally through the acquisition of the alternative dihydropteroate synthase (DHPS) gene *sul*, the product of which has a low affinity for sulfonamides. Unlike resistance genes to other classes of antimicrobials such as tetracycline, which are encoded by many different genes, only three known sulfonamide resistance (*sul*) genes (*sul1*, *sul2* and *sul3*) have been identified. Based on alignments using MegAlign software, the nucleotide sequences of *sul1*, *sul2* and *sul3* are approximately 50% similar (Jiang *et al*, 2019). The results of the current study nearly to study in Iran by researchers Torkan and Yousefi (2017), studying the prevalence of resistance genes in 200 isolation of UPEC bacteria, showed that the sul1 gene was present in (40%) of isolated. Another study by Bonyadian *et al* (2019) involved 114 isolated E. coli indicated that (7%) of isolates harbor *sul1*, another study by Dehkordi *et al* (2020), included 65 isolates of *E. coli* to detect the spread of resistance genes indicating that (72.72%), carrying *sul*1 gene, the cause of different ratios is a difference in the number of isolates studied as well as in the age of patients.

CONCLUSION

E. coli isolated from children was highly resistance to antibiotic and had the ability for biofilm formation in addition presence of resistance gene which possible transmitted among bacteria and spread antibiotic resistance.

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