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Research Article
Phenotypic and Molecular Characterization of some Virulence Factors in Multidrug Resistance *Escherichia coli* Isolated from Different Clinical Infections in Iraq

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Abstract

Objective: The aim of this study was to detect 10 virulence genes in 50 *Escherichia coli* strains isolated from patients with different infections. **Methodology:** Different phenotypic assays and multiplex PCR were used to detect virulence genes. Antimicrobial susceptibility testing was performed to 14 antimicrobials types by disk diffusion method. **Results:** The most common virulence gene was *feoB* (98%) and at lower prevalence were genes *eaeA* and *stx1* (4%). *Escherichia coli* strains were highly resistant to most antimicrobials. **Conclusion:** *Escherichia coli* strains isolated from patients with bacteremia were highly virulence and more resistance to antimicrobials than those isolated from other sites.

Key words: *Escherichia coli*, multidrug resistance, phenotypic, molecular, virulence genes

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Escherichia coli is one of the most important opportunistic Gram negative bacteria. It resist many type of antimicrobials and cause different infections because it has many type of virulence genes encoding to many important virulence factors. It's known that *Escherichia coli* is a commensal bacterium, found as normal flora in intestines of human and animals[1]. *Escherichia coli* strains are classified into three different groups according to site of infection and pathogenicity; extraintestinal pathogenic *E. coli* (ExPEC) strains, commensal strains and intestinal pathogenic strains[2]. One of the most important group it is ExPEC, found as normal flora in the intestine but also can infect extraintestinal sites and causing many infections such as, Urinary Tract Infection (UTI), acute and chronic diarrhea and bacteremia[3,4].

Escherichia coli is the most common cause of many infections in the worlds wide, such as; Urinary Tract Infection (UTI), heavy diarrhea and bacteremia[5,6]. *Escherichia coli* can cause these infections due to its ability to the acquisition of mobile genetic factors carrying many virulence genes[7,8]. Adhesions, iron uptake, toxins and capsules are the most common virulence factors responsible for attachment, adherence and invasion in the host and then lead to infection[9].

Pathogenicity of *E. coli* is due to the presence of many virulence genes, located on chromosomes or plasmids or both that encodes important virulence factors, if present on the chromosome, these genes are typically found in specific regions called pathogenicity islands[10].

Because of several strains of *E. coli* have plasmids that code for antimicrobial enzymes, which promote resistance to third generation cephalosporins, β-lactam antibiotics and other antimicrobials and are associated with the difficult of treatments and high morbidity and mortality rates, therefore, the aim of this study was to characterize 50 clinical isolates of *E. coli* isolated from urine, heavy diarrhea and bloodstream based on their resistance to antibiotics and the presence of ten virulence genes associated with their pathogenicity.

MATERIALS AND METHODS

Isolation and identification of *Escherichia coli*: A total of 390 clinical samples were collected from patients infected with urinary tract infection, heavy diarrhea and blood infection in Al-kufa hospital during the period of October, 2015 to April, 2016. All samples (urine and diarrhea) were collected in sterile cup and streaked immediately onto the surface of blood agar (Oxoid, UK), MacConkey agar (Oxoid, UK) and Chrome agar medium (Orientation company, France) by sterile loop and incubated overnight at 37°C. By a sterile syringe, 5 mL of blood were collected from adult patients and suspended with 45 mL of brain heart infusion broth (Oxoid, UK) and incubated at 37°C for 5-7 days and streaked onto the surface of blood (Oxoid, UK) and MacConkey agar (Oxoid, UK) by sterile loop and incubated overnight at 37°C[11]. All bacterial isolates were diagnosed by standard morphological and biochemical tests according to Faddin[12]. Finally, Vitek2® system (BioMerieux® -France) was used as a final identification step for all suspected *E. coli* isolates.

Detection of virulence factors by phenotypic methods

Capsule stain: This method was carried out according to Sorensen[13] as follows: Fresh and pure of each bacterial colony was transferred on a slide, mixed with nigrosin stain and stained with methylen blue for 2 min and under light microscope the nigrosin stain provides a dark background to unstained capsule and metylene blue stain provides blue color to the cells.

Blood hemolysis production: Blood hemolysis production was detected with 5% sheep's blood agar (Oxoid, UK). Strains producing a clear or semi clear zone of lysis after incubation for 24 h at 37°C were considered positives[12].

Detection of siderophores production: In this method, nutrient agar (Oxoid, UK) supplemented with 200 mM of 2,2'-dipyridyl was used as iron-restricted agar medium according to Schwyn and Neilands[14]. All *E. coli* isolates streaked onto the surface of iron-restricted agar medium and incubated overnight at 37°C. Appearance of any bacterial growth considered as a positive results for ability of bacteria to siderophores production.

Biofilm formation testing: Fresh and pure colony of *E. coli* streaked on congo red agar plate and incubated at 37°C for 24-48 h. Formation of black or red colonies considered as a positive result for ability of bacterium to biofilm formation[15].

Adhesion test: This test was carried out according to Svanborg[16], as follows: A fresh morning urine samples of healthy female were collected and centrifuged at 3000 rpm for 20 min. The pellet was washed three times with phosphate buffered saline. One milliliter of bacterial culture of each isolates (Compared with McFarland standard tube) was added to a same volume of epithelial cells suspension, mixed well

Table 1: Sequence of primers used in multiplex polymerase chain reaction for 10 virulence genes of *Escherichia coli*

Activity	Gene	Oligo sequence (3'→5')	Product size (bp)	References
Adhesins	*fimH*	F: TGCAGAACGGATAAGCCGTG	508	Yun *et al.*[9]
		R: GCAGTCACCTGCCCTCCGGTA		
	papA	F: ATGGCAGTGGTGTTTTGGTG	720	
		R: CGTCCCACCATACGTGCTCTC		
	eaeA	F: GACCCGGCACAAGCATAAGC	384	Chapman *et al.*[18]
		R: CCACCTGCAGCAACAAGAGG		
Siderophores	*feoB*	F: AATTGGCGTGCATGAAGATAACTG	470	Yun *et al.*[9]
		R: AGCTGGCGACCTGATAGAACAATG		
	iutA	F: ATGAGCATATCTCCGGACG	587	Moulin-Schouleur *et al.*[19]
		R: CAGGTCGAAGAACATCTGG		
Toxins	*stx1*	F: ATAAATCGCCTATCGTTGACTAC	180	Chapman *et al.*[18]
		R: AGAACGCCCACTGAGATCATC		
	stx2	F: GGCACTGTCTGAAACTGCTCC	225	
		R: TCGCCAGTTATCTGACATTCTG		
	hlyF	F: TCGTTTAGGGTGCTTACCTTCAAC	444	Moulin-Schouleur *et al.*[19]
		R: TTTGGCGGTTTAGGCATTCC		
Invasins	*ibeA*	F: TGAACGTTTCGGTTGTTTTG	814	
		R: TGTTCAAATCCTGGCTGGAA		
Capsule synthesis	*kpsMTIII*	F: GCGCATTTGCTGATACTGTTG	272	Yun *et al.*[9]
		R: CATCCAGACGATAAGCATGAGCA		

Table 2: Thermo cycling conditions of multiplex polymerase chain reactions for 10 virulence genes of *Escherichia coli*

Gene	Initial denaturation time (°C)	Denaturation time (°C)	Annealing time (°C)	Extension time (°C)	No. of cycles	Final extension time (°C)	References
fimH *papA* *feoB* *kpsMTII*	94°C for 5 min	94°C for 60 sec	55°C for 60 sec	72°C for 2 min	35	72°C for 10 min	Yun *et al.*[9]
iutA *hlyF* *ibeA*	94°C for 3 min	94°C for 1 min	51°C for 60 sec	72°C for 1 min	30	72°C for 10 min	Moulin-Schouleur *et al.*[19]
eaeA *stx1* *stx2*	95°C for 3 min	95°C for 20 sec	58°C for 40 sec	72°C for 90 sec	35	72°C for 5 min	Chapman *et al.*[18]

and incubated at 37°C for 1 h with shaking. The tubes were centrifuged and washed three times with phosphate buffer saline to discard unattached bacteria, the supernatant was decanted and the sediment cells were mounted on glass microscope slide and separated using the margin of other slide. The slides were dried by air and fixed with methanol for 10 min. The slides were stained with giemsa stain for 1.5 h to color uroepithelial cells. The excess stain removed by water and the slides were dried and examined by light microscope under oil immersion to calculate the number of bacteria adhered to cells. Adhesion of bacterial cells on 50 of epithelial cells is considered as a positive result for ability of bacterium to adhesion.

Multiplex PCR for detection of virulence genes

Extraction of DNA: This method was carried out according to Yang *et al.*[17], as follows: Five pure and fresh colonies were suspended in 200 µL of dry weight and the bacterial cells were lysed by boiling at 100°C for 20 min (in water bath). The bacterial cells were placed in ice for 40 min and the other cellular components were removed by centrifugation at 9000 rpm for 10 min. Finally the supernatant was used as the DNA template[17].

PCR detection of virulence-associated genes: A multiplex PCR was used to detect 10 genes encoding: Adhesins (*fimH*, *papA* and *eaeA*), siderophores production (*feoB* and *iutA*), capsule synthesis (*kpsMTII*), heamolysine (*hlyF*), toxin production (*stx1* and *stx2*) and invasion (*ibeA*) (Table 1, 2).

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing for the *E. coli* isolates was performed using a Kirby-Bauer method according to the Clinical Laboratory Standards Institute CSLI[20]. The following 14 antimicrobials were tested provided from (Bioanalyse, Turkey): amoxicillin+clavulanic acid (AMC) 30 µg, amoxicillin (AX) 25 µg, cefotaxime (CTX) 30 µg, ceftriaxone (CRO) 30 µg, ceftazidime (CAZ) 30 µg, imipenem (IMP) 10 µg, gentamicin (CN) 10 µg, tetracycline (TE) 30 µg, amikacin (AK) 30 µg, tobramycin (TM) 10 µg, doxycycline (DO) 30 µg,

ciprofloxacin (CIP) 5 µg, chloramphenicol (C) 30 µg and nitrofurantoin (F) 300 µg. A fresh and pure colony from each *E. coli* isolate was grown overnight in Mueller Hinton broth (Oxoid, UK) at 37°C. Bacterial growth were adjusted to 0.5 on the MacFarland nephelometer scale (1.5×10^8 CFU mL^{-1}) and plated on Mueller Hinton agar (Oxoid, UK) by the streaking method using a sterile swab. Antibiotics susceptibility and resistance was determined by growth zone diameter of isolate according to CLSI[21]. *Escherichia coli* ATCC 25922 strain was used as control.

Statistical analysis: Graphpad prism software (version 7.0) was used to statistically analyze the data.

RESULTS

Total isolates: Out of the 390 total specimens there were 190 specimens (48.717% stained with Gram negative) while there were 172 specimens (44.104% stained with Gram positive) and 28 specimens (7.179% without growth) (Table 3).

Also the results proved that, a total of 190 g negative bacterial isolates were collected from different infections (90 from urine, 75 from heavy diarrhea, 25 from blood) there were 50 *E. coli* isolate, 25 isolates (50%) from urine, 18 isolates (36%) from heavy diarrhea and 7 isolates (14%) from blood (Table 4).

Virulence factors: Different phenotypic procedures were used to investigate virulence factors, siderophores production, biofilm formation and adhesion were observed in 50 isolates (100%). The capsule productions and hemolysis were observed in 18 isolates (36%) and 8 isolates (18%), respectively (Fig. 1-3, Table 5).

Also, out of total 50 isolates, there were 45 isolates (90%) were positive for *fimh* gene, 18 isolates (36%) were positive

Table 3: Total specimens isolated from patients in the current study (N = 390)

Gram stain	No.	Percentage
Gram negative	190	48.717
Gram positive	172	44.104
No growth	28	7.179
Total	390	100.00

Table 4: Prevalence of *Escherichia coli* strains according to source of infections (N = 190)

Source of isolate	Total of gram negative bacteria	*E. coli*	Percentage
Urine	90	25	50
Stool	75	18	36
Blood	25	7	14
Total	190	50	100

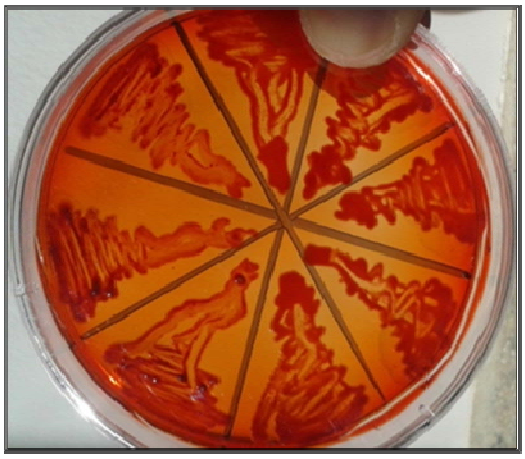

Fig. 1: *Escherichia coli* on congo red agar plate, showing red-black colonies as a positive result for biofilm formation

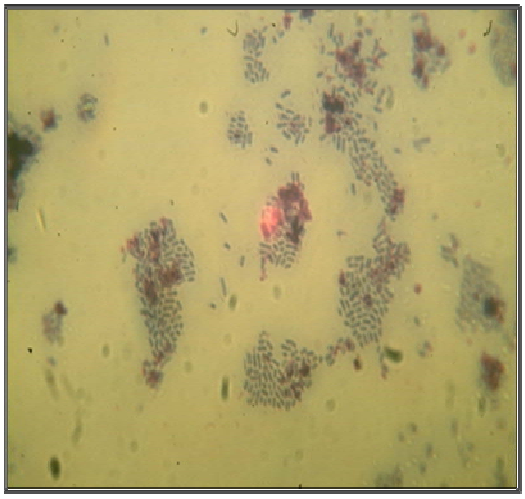

Fig. 2: Aggregation of *Escherichia coli* around epithelial cells as a positive result for ability to adhesion

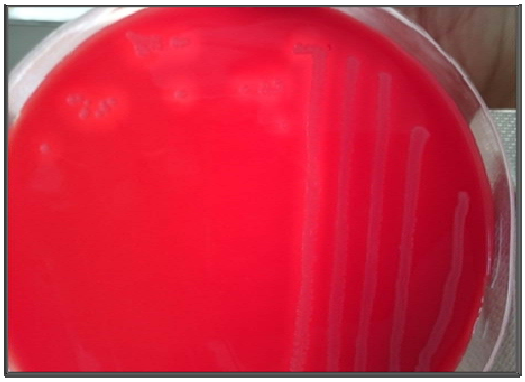

Fig. 3: *Escherichia coli* strain on blood agar plate surface after 24 h showing hemolysis phenomenon

Table 5: Prevalence of virulence factors among *Escherichia coli* by phenotypic characterization according to source of infections (n = 50)

Source of sample	Capsule	Hemolysis	Siderophores	Biofilm formation	Adhesion
Urine (25)	8 (32%)	1 (4%)	25 (100%)	25 (100%)	25 (100%)
Stool (18)	6 (33.33%)	0 (0%)	18 (100%)	18 (100%)	18 (100%)
Blood (7)	4 (57.14%)	7 (100%)	7 (100%)	7 (100%)	7 (100%)
Total	18	8	50	50	50

Table 6: Prevalence of virulence genes among 50 *Escherichia coli* strains according to sample source

Gene	Urine isolates 25 (%)	Stool isolates 18 (%)	Blood isolates 7 (%)	Total 50 (%)
feoB	24 (96%)	18 (100%)	7 (100%)	49 (98%)
iutA	12 (48%)	6 (33.33%)	7 (100%)	25 (50%)
fimH	23 (92%)	15 (83.33%)	7 (100%)	45 (90%)
papA	8 (32%)	6 (33.33%)	4 (57.14%)	18 (36%)
eaeA	0 (0%)	2 (11.11%)	0 (0%)	2 (4%)
kpsMTII	11 (44%)	11 (61.11%)	4 (57.14%)	26 (52%)
stx1	0 (0%)	2 (11.11%)	0 (0%)	2 (4%)
stx2	0 (0%)	4 (22.22%)	0 (0%)	4 (8%)
hlyF	1 (4%)	0 (0%)	7 (100%)	8 (16%)
ibeA	2 (8%)	3 (16.66%)	0 (0%)	5 (10%)

Table 7: Profile of virulence genes among 50 *Escherichia coli* strains according to sample source

No. of		Virulence genes										No. of		Virulence genes									
isolates	Source	*feoB*	*iutA*	*fimH*	*papA*	*eaeA*	*kpsMTII*	*stx1*	*stx2*	*hlyF*	*ibeA*	isolates	Source	*feoB*	*iutA*	*fimH*	*papA*	*eaeA*	*kpsMTII*	*stx1*	*stx2*	*hlyF*	*ibeA*
1	Blood	+	+	+	+	-	+	-	-	+	-	26	Blood	+	+	+	-	-	-	-	-	+	-
2	Blood	+	+	+	+	-	+	-	-	+	-	27	Stool	+	+	+	-	-	-	-	-	-	-
3	Urine	+	-	+	+	-	+	-	-	-	-	28	Stool	+	-	+	-	-	+	-	-	-	-
4	Urine	+	+	+	-	-	+	-	-	-	-	29	Stool	+	-	-	-	-	+	-	-	-	-
5	Urine	+	-	+	-	-	+	-	-	-	-	30	Stool	+	-	-	-	-	+	-	-	-	-
6	Urine	+	-	+	+	-	+	-	-	-	-	31	Stool	+	-	-	-	-	+	-	-	-	+
7	Blood	+	+	+	+	-	+	-	-	+	-	32	Stool	+	+	+	-	-	+	-	-	-	-
8	Stool	+	-	+	+	-	+	-	-	-	-	33	Stool	+	+	+	-	-	-	-	-	-	-
9	Stool	+	-	+	+	-	+	-	-	-	+	34	Stool	+	+	+	-	+	-	+	+	-	-
10	Stool	+	+	+	-	-	+	-	-	-	-	35	Stool	+	-	+	+	-	-	-	-	-	-
11	Stool	+	-	+	+	-	+	-	-	-	-	36	Stool	+	-	+	-	-	-	-	+	-	+
12	Stool	+	+	+	+	-	+	-	-	-	-	37	Blood	+	+	+	-	-	-	-	-	+	-
13	Stool	+	-	+	+	-	+	-	-	-	-	38	Stool	+	-	+	-	+	-	+	+	-	-
14	Urine	+	+	+	-	-	-	-	-	-	-	39	Stool	+	-	+	-	-	-	-	+	-	-
15	Urine	+	+	+	-	-	-	-	-	-	-	40	Urine	+	+	+	-	-	-	-	-	-	-
16	Urine	+	+	+	+	-	-	-	-	-	-	41	Urine	+	+	+	-	-	-	-	-	-	+
17	Urine	+	+	+	+	-	+	-	-	-	-	42	Urine	+	+	+	-	-	-	-	-	-	-
18	Urine	+	-	+	+	-	-	-	-	-	+	43	Urine	+	+	+	-	-	-	-	-	-	-
19	Urine	+	-	+	-	-	-	-	-	-	-	44	Urine	+	+	+	-	-	-	-	-	+	-
20	Blood	+	+	+	+	-	+	-	-	-	-	45	Urine	+	-	+	-	-	+	-	-	-	-
21	Urine	+	-	+	+	-	-	-	-	-	-	46	Urine	+	-	+	-	-	+	-	-	-	-
22	Urine	+	-	+	-	-	+	-	-	-	-	47	Urine	+	-	-	+	-	+	-	-	-	-
23	Urine	+	-	+	+	-	-	-	-	-	-	48	Urine	-	+	-	-	-	-	-	-	-	-
24	Urine	+	-	+	-	-	+	-	-	-	-	49	Blood	+	+	+	-	-	-	-	-	+	-
25	Urine	+	+	+	-	-	+	-	-	-	-	50	Urine	+	-	+	-	-	-	-	-	-	-

for *papA* gene, 49 isolates (98%) were positive for *feoB* gene, 26 isolates (53%) were positive for *kpsMTI* (Fig. 4), 5 isolates (10%) were positive for *ibeA* gene, 25 isolates (50%) were positive for *iutA* gene and 8 isolates (16%) were positive for *hlyF* gene (Fig. 5), 2 isolates (4%) were positive for *stx1* and *eaeA* gene and 4 isolates (8%) were positive for *stx2* gene (Fig. 6). The prevalence of virulence factors and virulence genes were given in Table 6 and 7.

Antimicrobial susceptibility test: Antimicrobial susceptibility test was conducted for 50 strains of *E. coli*. This study indicated that the antimicrobial resistance rates of the 50 isolates to amoxicillin, amoxicillin+clavulanic acid, cefotaxime, ceftriaxone and ceftazidime were all high 90-100%. The lowest resistance rate was observed for imipenem (6%). Resistance test to 14 antibiotics of *E. coli* strains were given in Table 8 and 9.

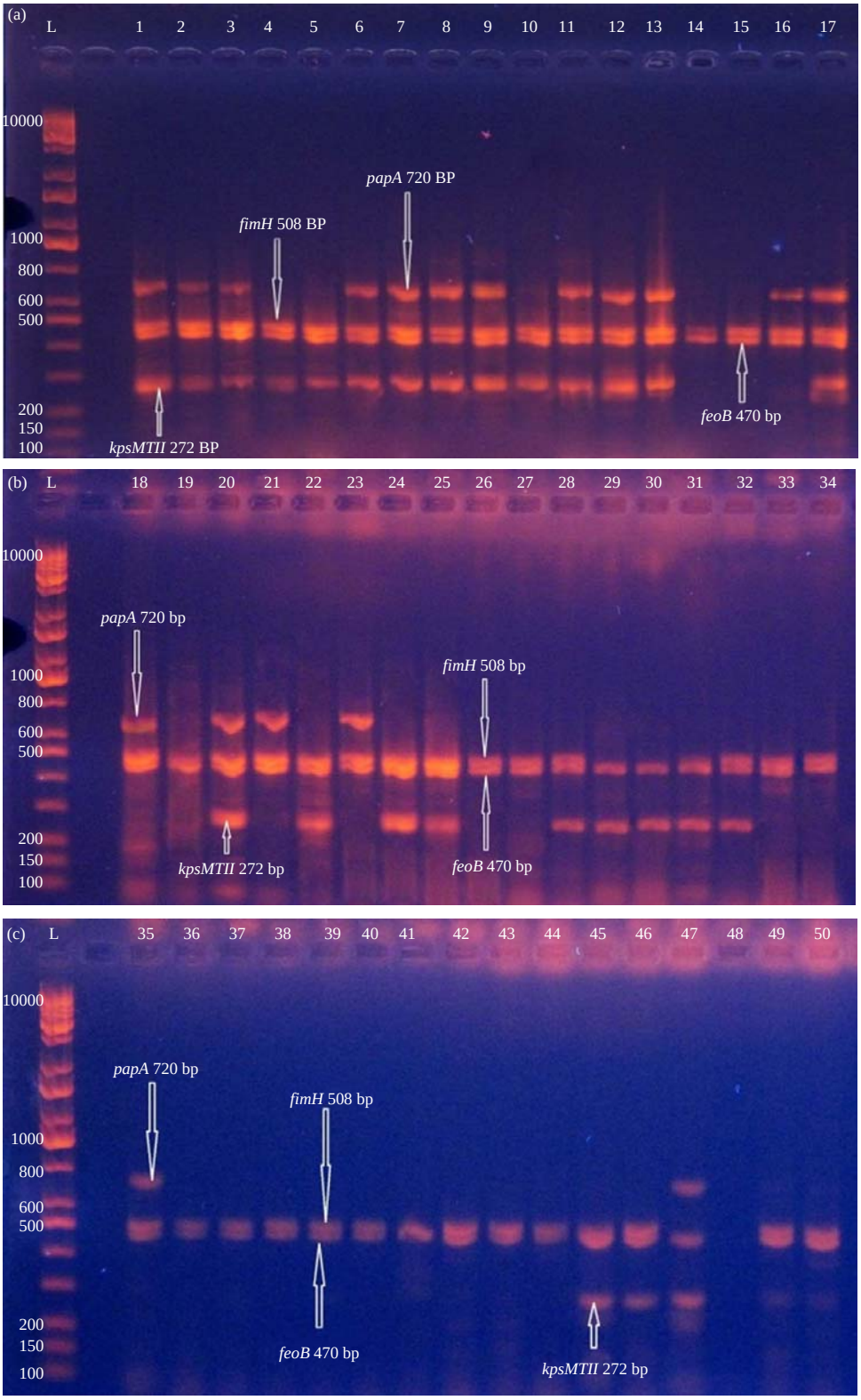

Fig. 4(a-c): Multiplex PCR amplified products from extracted DNA of 50 *Escherichia coli* isolates amplified with diagnostic genes; *fimH*, *papA*, *kpsMTII* and *feoB* show positive results at 508, 720, 272 and 470 bp, respectively, L: DNA molecular size marker, (a) Strain No. 1-17, (b) Strain No. 18-34 and (c) Strain No. 35-50

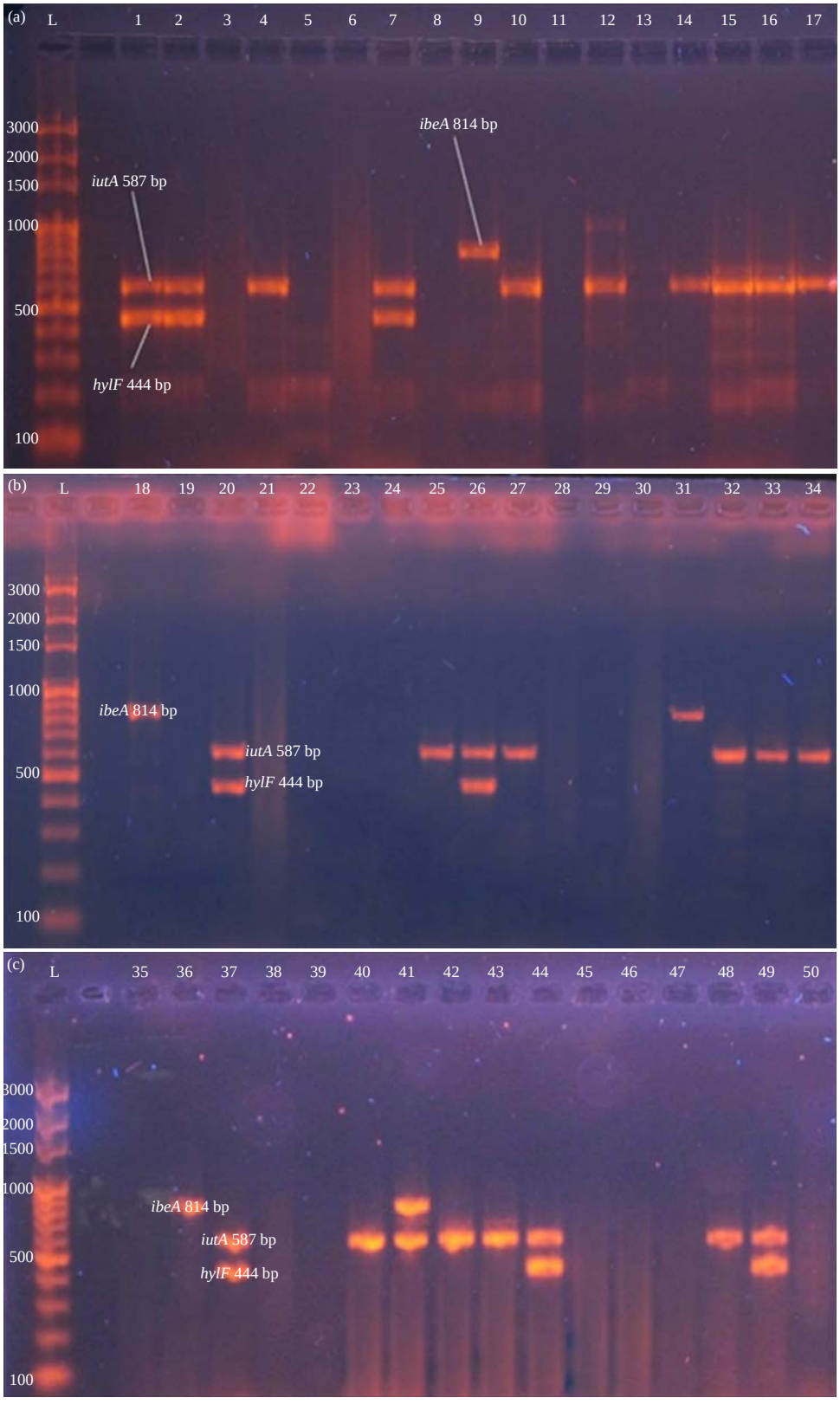

Fig. 5(a-c): Multiplex PCR amplified products from extracted DNA of 50 *Escherichia coli* strains amplified with diagnostic genes; *iutA*, *ibeA* and *hlyF* show positive results at 587, 814 and 444 bp, respectively, L: DNA molecular size marker, (a) Strain No. 1-17, (b) Strain No. 18-34 and (c) Strain No. 35-50

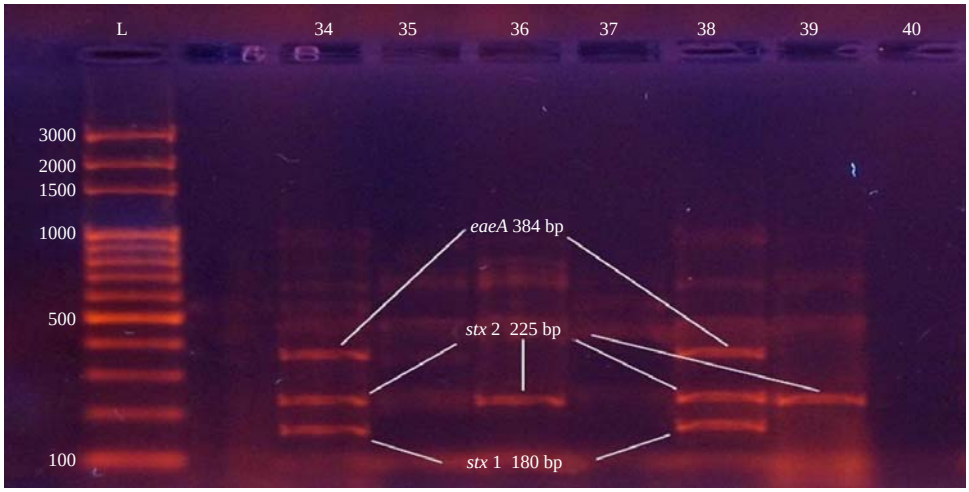

Fig. 6: Multiplex PCR amplified products from extracted DNA of *Escherichia coli* amplified with diagnostic genes; *eaeA, stx2* and *stx1* show positive results at 384, 225 and 180 bp, respectively, L: DNA molecular size marker

Table 8: Antimicrobial sensitivity test to 14 antimicrobials for 50 *Escherichia coli* strains isolated from patients with different infections

Antimicrobials	Concentration	Sensitive	Intermediate	Resistant	Percentage of resistance (%)	*E. coli* 35218 ATCC
Amoxicillin	25 µg	0	0	50	100	R
Amoxicillin+clavulanic acid	20+10 µg	4	0	46	92	R
Cefotaxime	30 µg	4	0	46	92	R
Ceftriaxone	30 µg	5	0	45	90	R
Ceftazidime	30 µg	2	0	48	96	R
Imipenem	10 µg	45	2	3	6	S
Gentamicin	15 µg	20	7	23	46	R
Amikacin	30 µg	19	9	22	44	R
Tobramycin	10 µg	15	1	34	68	R
Tetracycline	30 UL	23	2	25	50	R
Doxycycline	30 µg	16	5	29	58	S
Ciprofloxacin	5 µg	24	3	23	46	S
Chloramphenicol	30 µg	36	2	12	24	R
Nitrofurantoin	30 µg	10	0	40	80	R

Table 9: Profile of 50 clinical isolates of *Escherichia coli* according to antimicrobials resistance and prevalence of virulence genes

Source	Phenotypic resistance profile 100 (%)	Virulence genes
Blood	AX, AMC, CTX, CRO, CAZ, IMP, CN, TM, TE, DO, CIP, C, F 13 (92.8%)	*feoB, iutA, fimH, kpsMTII, hlyF, papA*
Blood	AX, AMC, CTX, CRO, CAZ, CN, AK, TM, TE, DO, CIP, C, F 13 (92.8%)	*feoB, iutA, fimH, kpsMTII, hlyF, papA*
Urine	AX, AMC, CTX, CRO, CAZ, IMP, CN, TM, TE, DO, CIP, C, F 13 (92.8%)	*feoB, fimH, kpsMTII, papA*
Urine	AX, AMC, CTX, CRO, CAZ, TM, TE, C, F 9 (64.28%)	*feoB, iutA, fimH, kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, TM, DO, CIP, F 9 (64.28%)	*feoB, fimH, kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, AK, TM, DO, CIP, C, F 11 (78.57%)	*feoB, fimH, papA, kpsMTII*
Blood	AX, AMC, CTX, CRO, CAZ, IMP, CN, TM, TE, DO, CIP, F 12 (85.71%)	*feoB, iutA, fimH, kpsMTII, hlyF, papA*
Stool	AX, F 2 (14.28%)	*feoB, fimH, papA, kpsMTII*
Stool	AX, AMC, CTX, CRO, CAZ, CN, F 7 (50%)	*feoB, fimH, papA, kpsMTII, ibeA*
Stool	AX, CAZ, AK, F 4 (8%)	*feoB, iutA, fimH, kpsMTII*
Stool	AX, AMC, CTX, AK 4 (8%)	*feoB, fimH, papA, kpsMTII*
Stool	AX, AMC, CTX, CRO, CAZ, AK 6 (42.85%)	*feoB, iutA, fimH, papA, kpsMTII*
Stool	AX, AMC, CTX, CRO, CAZ, AK, F 7 (50%)	*feoB, fimH, papA, kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, CN, AK, TM 9 (64.28%)	*feoB, iutA, fimH*
Urine	AX, AMC, CTX, CRO, CAZ, CN, AK, TM, DO, CIP 10 (71.42%)	*feoB, iutA, fimH*
Urine	AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, F 9 (64.28%)	*feoB, iutA, fimH, papA*
Urine	AX, AMC, CTX, CRO, CAZ, CN, AK, TM, CIP 9 (64.28%)	*feoB, iutA, fimH, papA, kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, CN, AK, TM, TE, DO, CIP, F 12 (85.71%)	*feoB, fimH, papA, ibeA*
Urine	AX, AMC, CTX, CRO, CAZ, CN, TM, TE, DO, CIP, C 11 (78.57%)	*feoB, fimH*
Blood	AX, AMC, CTX, CRO, CAZ, AK, TM, TE, DO, CIP, F 11 (78.57%)	*feoB, iutA, fimH, papA, kpsMTII*

Table 9: Continue

Source	Phenotypic resistance profile 100 (%)	Virulence genes
Urine	AX, CAZ, CN, AK, TM, TE, DO, F 8 (57.14%)	*feoB*, *fimH*, *papA*
Urine	AX, AMC, CTX, CRO, CAZ, CN, AK, TM, TE, DO, CIP 11 (78.57%)	*feoB*, *fimH*, *kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, AK, TM, TE, DO, F 10 (71.42%)	*feoB*, *fimH*, *papA*
Urine	AX, AMC, CTX, CRO, CAZ, AK, TM, TE, DO, CIP, F 11 (78.57%)	*feoB*, *fimH*, *kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, TM, TE, DO, C, F 10 (71.42%)	*feoB*, *iutA*, *fimH*, *kpsMTII*
Blood	AX, AMC, CTX, CRO, CAZ, CN, TM, TE, DO, CIP 10 (71.42%)	*feoB*, *iutA*, *fimH*, *hlyF*
Stool	AX, AMC, CTX, CRO, CAZ, TE, C 7 (50%)	*feoB*, *iutA*, *fimH*
Stool	AX, CAZ, TM 3 (21.12%)	*feoB*, *fimH*, *kpsMTII*
Stool	AX, AMC, CTX, CRO, CAZ, C 5 (35.71%)	*feoB*, *kpsMTII*
Stool	AX, AMC, CTX, CRO, CAZ, F 6 (42.85%)	*feoB*, *kpsMTII*
Stool	AX, AMC, CTX, CRO, CAZ, C, F 7 (50%)	*feoB*, *kpsMTII*, *ibeA*
Stool	AX, AMC, CTX, CRO, CAZ, F 6 (42.85%)	*feoB*, *iutA*, *fimH*, *kpsMTII*
Stool	AX, AMC, CTX, CRO, CAZ, C, F 7 (50%)	*feoB*, *iutA*, *fimH*
Stool	AX, AMC, CTX, CRO, CAZ, CN, TM, TE, DO, CIP, C, F 12 (85.71%)	*feoB*, *iutA*, *fimH*, *eaeA*, *stx1*, *stx2*
Stool	AX, AMC, CTX, CRO, CAZ, F 6 (42.85%)	*feoB*, *fimH*, *papA*
Stool	AX, AMC, CTX, CRO, CAZ, TM, F 7 (50%)	*feoB*, *fimH*, *stx2*, *ibeA*
Blood	AX, AMC, CTX, CRO, CAZ, TM, F 7 (50%)	*feoB*, *iutA*, *fimH*, *hlyF*
Stool	AX, AMC, CTX, CRO, CAZ, AK, TM, F 8 (57.14%)	*feoB*, *fimH*, *eaeA*, *stx1*, *stx2*
Stool	AX, AMC, CTX, CRO, CAZ, CN, TM, TE, DO, F 10 (71.42%)	*feoB*, *fimH*, *stx2*
Urine	AX, AMC, CTX, CRO, CAZ, CN, TM, TE, DO, CIP,F 11 (78.57%)	*feoB*, *iutA*, *fimH*
Urine	AX, AMC, CTX, CRO, CAZ, CN, TM, TE, DO, CIP, F 11 (78.57%)	*feoB*, *iutA*, *fimH*, *ibeA*
Urine	AX, AMC, CTX, CRO, CAZ, CN, TM, TE, DO, CIP, F 11 (78.57%)	*feoB*, *iutA*, *fimH*
Urine	AX, AMC, CTX, CRO, CAZ, CN, AK, TM, TE, DO, CIP, F 12 (85.71%)	*feoB*, *iutA*, *fimH*
Urine	AX, AMC, CTX, CRO, CAZ, AK, F 7(50%)	*feoB*, *iutA*, *fimH*, *hlyF*
Urine	AX, AMC, CTX, CRO, CAZ, AK, TM, DO, F 9 (64.28%)	*feoB*, *fimH*, *kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, AK, TM, DO, CIP, F 10(71.42%)	*feoB*, *fimH*, *kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, AK, TM, DO, F 9 (64.28%)	*feoB*, *papA*, *kpsMTII*
Urine	AX, AMC, CTX, CRO, CAZ, AK, TM, DO, F 9 (64.28%)	*iutA*
Blood	AX, AMC, CTX, CRO, CAZ, AK, TM, DO, F 9 (64.28%)	*feoB*, *iutA*, *fimH*, *hlyF*
Urine	AX, AMC, CTX, CRO, CAZ, AK, TM, TE, DO, F 10 (71.42%)	*feoB*, *fimH*

AMC: Amoxicillin+clavulanic acid, AX: Amoxicillin, CTX: Cefotaxime, CRO: Ceftriaxone, CAZ: Ceftazidime, IMP: Imipenem, CN: Gentamicin, AK: Amikacin, TM: Tobramycin, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, C: Chloramphenicol, F: Nitrofurantoin

DISCUSSION

Escherichia coli is one of the most important Gram negative bacteria causing urinary tract infections, acute diarrhea and bloodstream infections worldwide. *Escherichia coli* have many important virulence factors are associated with adhesion, colonization and survival in different human tissues[9,22]. These virulence factors are fimbriae, proteins, toxins, siderophores and capsular polysaccharides[23].

In this study, the results indicated that the *fimH* adhesion gene was present in 45 of all isolates (90%), 23 isolates (92%) from urine, 15 isolates (83.33%) from heavy diarrhea and 7 isolates (100%) from blood. The *papA* was the second prevalent adhesion gene (36%) of all isolates; 8 isolates (32%) from urine, 6 isolates (33.33%) from heavy diarrhea and 4 isolates (57.14%) from blood. The third prevalent gene in all our isolates was *eaeA* (4%), it was prevalent in two isolates from heavy diarrheal samples only. Some previous studies indicated that *fimH*, *papA* and *eaeA* were most important virulence genes in *E. coil* strains isolated from urine and fecal samples[9,24-26].

The most important step to development of UTI is adherence to urinary epithelia, allowing the bacteria to stay and persist in the urinary tract against flushing by urine flow and activation of host signaling pathways[27,28].

The *feoB* and *iutA* genes which are two of the bacterial iron acquisition systems. In this study, the *feoB* gene was present in 49 of all isolates (98%), 18 and 7 isolates (100%) from heavy diarrhea and blood. respectively and 24 isolates (96%) from urine. The *iutA* gene was present in 25 of all isolates (50%); 7 isolates (100%) from blood, 12 isolates (48%) from urine and 6 isolates (33.33%) from heavy diarrhea. These findings concerning the frequency of the *iutA* gene in *E. coli* isolates are in accordance with previous reports of 30% Moreno *et al.*[29] for faecal isolates. Because of there was low concentrations of free iron (in human host) for multiplication of pathogenic bacteria. Therefore, many bacteria such as *E. coli* have several means for iron uptake from the host through the expression of iron-acquisition systems[30].

Siderophore production is important for each pathogen especially in pathogenic bacteria for persisting and survival in the blood stream and other human tissues such as epithelial

tissues. When iron availability is become limited in the host, siderophore system play an important role as a virulence factor that contributes to bacterial growth in fluids and host tissues[31,32]. The prevalence of the *iutA* gene found in our *E. coli* isolates correlates with results published by Moulin-Schouleur *et al.*[19], it is noted that the *iutA* gene, which encodes the aerobactin receptor was present in a high rate of strains isolated from patients with bloodstream infection.

The *iutA* gene is commonly associated with ExPEC and those isolated from blood infection in human[33,34]. The relationship between bacterial iron acquisition systems and many types of infections in human had been suspected in previous studies[35,36]. Zhao *et al.*[37] and Yun *et al.*[9] reported that *feoB* and *fimH* were the most prevalent virulence genes in *E. coli* strains isolated from human with UTI. The *feoB* and *iutA* genes might have a role in iron acquisition in bloodstream infection and UTI. The *feoB* gene seems to be work differently and has an additional mechanism in bloodstream infection strains in addition to in UTI.

In this study, genes related to hemolysin and toxins production (*hlyF*, *stx1* and *stx2*) were researched. The *hlyF* gene is frequently detected in all blood isolates (7 isolates 100%) and detected in only one isolate from urine (4%). While the *stx1* and *stx2* genes were present in diarrheal samples only, 2 isolates (11.11%) and 3 isolates (16.66%), respectively. Mohsin *et al.*[38] showed that 11 (78.5%), 11 (78.5%) and 6 (42.8%) were positive for *stx1*, *stx2* and *eaeA* genes, respectively.

The utilization of many toxins secreted by some strains of *E. coli* is not fully understood. Among these toxins, putative hemolysin encoded by *hlyF* gene is an extracellular cytolytic protein toxin that is produced by more than 30% of total strains of *E. coli*. This toxin has been associated with both clinical severity in UTI patients and bloodstream infection[39]. Haemolytic activity in *E. coli* has been attributed to many types of haemolysin genes. A new class of haemolysin represents a novel bacterial gene designated *hlyF* gene[40].

These results suggest that this gene (*hlyF*) is strongly associated with *E. coli* isolates from bloodstream infection. Each enterohemorrhagic *Escherichia coli* (EHEC) strains cause dangerous infections in humans and have at least one Shiga like toxin (*stx1* or *stx2*) gene. Shiga like toxins are also is the major virulence factors of Shiga toxin-producing *Escherichia coli* (STEC) strains, among these toxins, *stx1* and *stx2* encoded by *stx1* and *stx2* genes which have an important role in human pathogens linked to non-blood and bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome[41].

The *stx1* genotype is one of the most important factors of clinical outcome of shiga toxin producing *Escherichia coli* (STEC) infection and that pathogenicity for humans was higher in the *stx2* genotype strains[42].

The *eaeA* gene which has been shown to be necessary for attaching and effacing activity, encodes a protein which is called intimin. Numerous investigators have underlined the strong association between the carriage of *eaeA* gene and the capacity of STEC strains to cause severe human illnesses, such as blood stream infection and haemolytic uraemic syndrome. But the production of this virulence factor is not a very essential for pathogenesis, because of many cases of bloodstream infection in human have been caused by STEC were negative for *eaeA* gene[26,43].

This current study revealed that the *ibeA* gene was present in 10% of total isolates, 2 isolates (8%) from urine and 3 isolates (16.66%) from stool. The percentage of *E. coli* isolates with the *ibeA* gene was not similar to that found for strains isolated from faeces by Watt *et al.*[44] (4%) and Kaczmarek *et al.*[45] (4.5%). These differences in percentage might be that according to the differences between types of virulent strains associated with their different phylogenetic backgrounds.

The *ibeA* is one of the most important virulence gene encoded for invasion protein has been identified as an important virulence factor involved in the invasion of brain micro-vascular endothelial cell that enables penetration across the blood brain barrier[45,46]. Soto *et al.*[47] suggested that *ibeA* gene may has an important role in the early steps of *E. coli* sepsis and meningitis in neonatal because of he reported that the *ibeA* gene is significantly more prevalent in *E. coli* strains causing early infection than in those involved in late infection.

Capsular polysaccharides is another important virulence factor in bacteria, which protects this pathogen from phagocytosis, bactericidal effects of serum factors and antimicrobials effect.

Escherichia coli have many capsular virulence genes responsible for more than 80 chemically and serologically distinct capsules, called K antigens[48,49]. The majority of ExPEC strains of *E. coli* associated with invasive disease express group 2 capsules[50]. The *kpsMTII* gene is one of the most important virulence genes that encodes proteins required for translocation of *E. coli* group II capsular polysaccharide across the inner membrane[51]. In these results the *kpsMTII*gene was prevalence in 26 of total isolates (52%), 11 isolates (44%) in urine, 11 isolates (61.11%) in heavy diarrhea and 26 isolates (52%) were detected in blood samples.

Bacterial capsule is well known as a virulence determinants that contribute to resistance against different human host defenses. Capsular polysaccharides (type K) is associated with ExPEC and uropathogenic *E. coli* (UPEC) infection, often to different degrees depending on the serotype of the strain[52]. These results suggest that the *kpsMTII* gene is particularly associated with pathogenicity of *E. coli* strains.

In recent years, antibiotic resistance has increased and became a major problem in many countries especially in developing countries[23,53-55]. Antimicrobial resistant *E. coli* strains cause more severe diseases for longer periods, therefore, the treatment of diseases caused by this bacterium often require many classes of antimicrobial therapy.

In this study, most *E. coli* strains isolated from bloodstream were resistant to most antimicrobials particularly β-lactam antibiotics and third generation cephalosporins. It might be that long term exposure to these antimicrobials by patients infected with bacteremia lead to horizontal transfer of plasmid resist antimicrobial genes between different strains of bacteria.

The results of this study demonstrated that there was positive relationship between the ability of *E. coli* strains to antimicrobial resistance and the prevalence of virulence genes and source of isolates. Almost all *E. coli* strains isolated from bloodstream were resistant to most antimicrobials, in the same time they have most of virulence genes. The AX, AMC, CTX, CRO, CAZ, IMP, CN, TM, TE, DO, CIP, C and F resistance has been associated with a higher prevalence of *feoB*, *iutA*, *fimH*, *kpsMTII*, *hlyF* and *papA* genes.

There was strong relationship between resistance of antimicrobials and prevalence of some virulence factors in bacteria. Virulence factors such as capsular polysaccharides, outer membrane lipoproteins and biofilms play an important role in protecting bacteria from antimicrobials exposure when compared with those do not have these virulence factors[56]. Bacteria with biofilm forming are generally more resistant to many antimicrobials[57]. Capsular polysaccharides, outer membrane lipoproteins and biofilms act as biodegradable effect on β-antimicrobials. The β-lactamase enzymes are secreted and maintain their activity inside of biofilm matrix and decompose β-lactam antibiotics before reach to the bacterial cells[57]. Antimicrobials penetration inside of capsular polysaccharides, outer membrane and biofilms could be blocked by other factors, such as the presence of surfaces with negatively-charged, particularly for large polar molecules such as aminoglycosides with positively charged. Also, trace amount of metabolic rates and limited oxygen are may be important factors contributing to increasing resistance to aminoglycosides, β-lactamase, fluoroquinolones and cephalosporins[58,59].

Many investigations have been reported that *E. coli* strains have multidrug resistance and virulence factors[10,60,61]. Also, Ibrahim *et al.*[62] proved that the clinical *E. coli* isolates had the highest resistance to β-lactam antibiotics.

Multiple resistances of *E. coli* strains to β-lactams antibiotics including sulphonamides, fluoroquinolones, expanded spectrum aminoglycosides, cephalosporins and tetracycline have been reported previously by Bradford *et al.*[63]. While, De Verdier *et al.*[64] reported that 61% of all *E. coli* strains were resistant to more than one antimicrobial. These differences in virulence genes and antibiotic resistance properties in the clinical samples could be related to types of bacterial strains, differences in geographical area, type of samples tested and antibiotic prescription preference among clinicians.

CONCLUSION

In conclusion, these results revealed that *E. coli* isolates from bloodstream showed higher level of antibiotic resistance and have many virulence factors than those isolates from urine and heavy diarrhea. The *feoB*, *iutA*, *fimH*, *kpsMTII*, *hlyF* and *papA* genes were implicated as important virulence genes for development of bloodstream infection in human.

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