

Determination of Antibiotic Resistance Profile and Virulence Genes in *Escherichia coli* Isolates from Palestinian Patients

ABSTRACT

INTRODUCTION: *Escherichia coli* (*E. coli*) is considered one of the most frequent intestinal and extraintestinal pathogen. **Methods:** A total of 49 isolates of *E. coli* were collected from different clinical samples, from different hospitals in Northern West Bank-Palestine, during January-March 2019. Aims: to detect the distribution of Type III secretion system (T3SS) genes, class 1, 2 and 3 integrons, virulence factors (*fyuA*, *papGIII*, *iutA* and *sfa/foc*) using multiplex PCR and antibiotic resistance using disc diffusion method. **Results:** In this study, *E. coli* isolates showed high resistant against different types of antibiotics and 71.4% of the isolates were multidrug resistant (MDR). Only class 1 integron was detected in these isolates with prevalence 57%, and the results showed that 65.7% of MDR isolates carried class 1 integron. The prevalence of T3SS genes was 0.0%. In addition, results of this study showed that that virulence genes ranged from 4.1% to 79.6% in *E. coli* strain tested. The prevalence of virulence genes *papGIII*, *sfa/foc*, *fyuA* and *iutA* had the following prevalence 4.1%, 40%, 64%, and 79.6%, respectively. **Conclusions:** The isolates of *E. coli* showed high resistant against different types of antibiotics. The co-occurrence of class 1 integrons and antimicrobial resistance genes in current study is an additional threat for spread of the antimicrobial resistance traits which may further complicate future strategies for treatment the infections caused by this pathogen. In addition, *E. coli* isolated from Palestinian patients showed one or more virulence factors that could increase their pathogenesis.

Keywords: *Escherichia coli*, class 1 integrons, virulence factors, antibiotic resistant, multidrug resistance.

1. INTRODUCTION

Most *E. coli* strains are harmless commensals of the human and animal intestine, but some strains are capable of causing a variety of different diseases. There are several pathotypes of *E. coli* which include: Enteropathogenic *E. coli* (EPEC), Atypical Enteropathogenic *E. coli* (A-EPEC), Enterotoxigenic *E. coli* (ETEC), nterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Diffusely Adherent *E. coli* (DAEC), Enteroaggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli* (STEC) and Extraintestinal Pathogenic *Escherichia coli* (ExPEC) which includes Uropathogenic *E. coli* strains [1].

Type III secretion system is considered one of these vital virulence factors, which is present in a several pathogenic microorganisms particularly Gram-negative bacilli. The clinical spectrum of disease caused by T3SS-containing pathogens is remarkably broad. This complex has capable of injecting certain effector secretion proteins (toxins) into the host cell cytoplasm that then modulate its functions [2]. The effector secretion toxins vary among different T3SS pathogens. The T3SS is composed of three separate protein complexes: the secretion apparatus, the translocation or targeting apparatus, and the secreted toxins (effector proteins) and their cognate chaperones [3]. The most prominent feature of EPEC- and EHEC-induced intestinal pathology is the attaching and effacing (A/E) lesion. The formation of A/E lesion depends on a T3SS encoded within the loci of enterocyte effacement (LEE). There are 5 major operons (LEE1 to LEE5), which are responsible for encoding a T3SS secreted proteins, chaperones, and regulators. The secreted

51 proteins consist of effectors that are translocated into the host cell by the T3SS, and translocators
52 required for delivering the effectors. *LEE4* encodes the translocators EspA, EspB and EspD, a
53 chaperone for EspD (CesD2) [4]. The A/E lesion is characterized by intimate attachment of
54 bacteria to the enterocyte membrane, transfer of secretion effector molecules into the host
55 enterocytes, disruption of tight junctions and brush border microvilli and disrupt the host
56 cytoskeleton system in these cells, that includes accumulation of polymerized actin beneath the
57 site of attachment, forming pedestal-like structures. In addition, mislocalization of aquaporin water
58 channels which are an important factors contributing to diarrhea [5,6]. The prevalence of *espA*,
59 *espD* and *espB* genes in clinical *E. coli* isolates have been previously studied. It was found that
60 the prevalence of *espA*, *espD* and *espB* among STEC was 63%, 67% and 61%, respectively.
61 The prevalence among EPEC were 55%, 100% and 100% for *espA*, *espD* and *espB*,
62 respectively, the prevalence in EIEC strains for these genes was 50% , while the these genes
63 were not detected in ETEC and EAEC strains [2]. In a study carried out in India on 67 STEC, 5
64 EPEC and 22 ETEC strains were isolated from 256 rectal swabs from yaks. the *aspB* gene was
65 detected in two EPEC isolates (10%) only, while not detected in the STEC or ETEC isolates [7].

66
67 Five classes of integrons are known to play a role in the dissemination of antibiotic determinants
68 to aminoglycosides and β -lactams among Gram negative species, and the most extensively
69 studied was class 1 integrons [8,9]. Due to the importance of antibiotic resistance in *E. coli* and
70 the possible role of integrons in creating of resistance. The prevalence of integrons has been
71 reported by several authors These studies showed that the prevalence of integrons ranged from
72 22%-80% [10-13].

73
74 The pathogenicity of a given *E. coli* strain is mainly determined by specific virulence factors which
75 include adhesion (fimbriae or pili), invasions, toxins (exotoxins and endotoxins), siderophores,
76 capsule and flagella. Iron is an essential factor for pathogenicity of *E. coli* strains in the human
77 body, and these strains must have advanced strategies for acquiring iron from the host to
78 produce disease. Products of yersiniabactin (*fyuA*) and aerobactin (*iutA*) genes are among the
79 most common iron acquiring systems of pathogenic and nonpathogenic *E. coli*. P-fimbrial
80 adhesins in *Escherichia coli* enable the colonization of host tissues. By mediating attachment to
81 P-blood group antigens on uroepithelial cells. P fimbriae play a critical role in the development of
82 urinary tract infections (UTIs). The P-fimbrial-tip adhesin, which is encoded by *papG*, attaches
83 directly to host cells [14]. The class II G adhesin is associated with pyelonephritis and bacteremia,
84 while the class III G adhesin sequence is associated with cystitis, although they have been found
85 in pyelonephritis and bacteremia. The *PapGI* strains might have a larger prevalence among fecal
86 isolates [15]. The two genetic determinants *sfa* and *foc*, coding for the S fimbrial adhesin and for
87 *F1C* fimbriae, respectively, and belong to the same general group of fimbrial gene clusters. These
88 factors represent functionally distinct adhesins in their receptor specificities but enable bacteria to
89 attach to eukaryotic cells [16]. The ability of uropathogenic *Escherichia coli* (UPEC) to cause
90 symptomatic urinary tract infections (UTIs) is enhanced by adhesins, e.g. S fimbriae and F1C
91 fimbriae. Tissue surfaces in the urinary tract are submitted to strong hydrodynamic shear forces.
92 Adherence to the urinary tract epithelium enables the bacteria to resist the hydrodynamic forces
93 of urine flow and to establish infection [17] . There are many studies carried out to detect the
94 prevalence of virulence factors in *E. coli*. The prevalence of *fyuA* gene had a range from 72.2%-
95 96% [18-21], *iutA* 39.8% -84% [18-20], *papGIII* virulence factor 10%-19.3 [15,18,22,23] and
96 *sfa/foc* 34%-36% [18,24].

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98 This study aimed to detect the distribution of T3SS, class 1, 2 and 3 integrons, some virulence
99 factors (*fyuA*, *papGIII*, *iutA* and *sfa/foc*) and antibiotic resistance among *E. coli* isolates recovered
100 from different clinical samples in Palestine.

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107 MATERIALS and METHODS

108

109 2.1 Sample Collection

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111 A total of 49 isolates of *E. coli* were collected from different clinical samples (Table 1). These
112 isolates were obtained from inpatients and outpatients from different hospitals at Northern West
113 Bank-Palestine, during January-March 2019. These hospitals were An-Najah National University
114 Hospital-Nablus, Alwatany Hospital-Nablus, Rafidia Hospital-Nablus, Turkey Hospital-Tubas,
115 Thabet Hospital-Tulkarm and Darweesh Nazzal Hospital-Qalqilia. All the clinical isolates were
116 identified using the API 20E system at the respective hospital laboratories and then confirmed
117 using conventional methods in our microbiology research laboratory.

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122

123 **Table 1.** A sample source of 49 of *E. coli* isolates collected from different hospitals at Northern
124 West Bank-Palestine.

Hospital	Isolate source and No. of samples						Total
	urine	Wound swab	blood	stool	vagina	Rectal swab	
N	5	3	0	0	0	0	8
A	8	0	1	2	0	0	11
T	5	0	0	0	0	1	6
TH	4	0	1	0	0	0	5
D	4	3	0	0	2	0	9
R	10	0	0	0	0	0	10
Total	36	6	2	2	2	1	49

125 N: An-Najah National University Hospital-Nablus; A: Alwatany Hospital-Nablus; T: Turkey
126 Hospital-Tubas; TH: Thabet Hospital-Tulkarm; D: Darweesh Nazzal Hospital-Qalqilia; R: Rafidia
127 Hospital-Nablus.

128

129 2.2 Antibiotic Resistance

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131 Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standard
132 Institute (CLSI) using the disk diffusion method [25]. All *E. coli* isolates were examined for
133 resistance to Levofloxacin (LEV) 5µg, Ciprofloxacin (CIP) 5µg, Aztreonam (ATM) 30µg,
134 Tetracycline (TE) 30µg, Imipenem(IPM) 10µg, Ceftriaxone (CFX) 30µg,
135 Trimethoprim/Sulfamethoxazole (SXT) 25µg, Ceftazidime (CAZ) 30µg, and Amoxicillin/clavulanic
136 acid (AMC) 30 µg. Normal saline suspensions of all *E. coli* isolates were adjusted to the
137 McFarland 0.5 standard and used to inoculate Mueller Hinton agar plates. The plates were
138 incubated at 37°C for 14-16 hrs. The zone of inhibition was measured in millimetres. Isolates
139 were classified as resistant, intermediate or susceptible according to the criteria recommended by
140 CLSI guidelines [25]. Strains were considered multidrug-resistant (MDR) when they showed
141 resistant to 3 drugs or more belonged to different classes.

142

143 2.3 Polymerase Chain Reaction and DNA Extraction

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145 2.3.1 DNA extraction

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147 *E. coli* DNA genome was prepared for PCR according to the method described previously [26].
148 Briefly, cells will be scraped off an overnight nutrient agar plate with a sterile loop, washed with 1
149 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), then the pellet was

150 resuspended in 0.5 ml of sterile distilled water, and boiled for 10-15 min. The cells then were
151 incubated on ice for 10 min. The debris was pelleted by centrifugation at 11,500 X g for 5 min.
152 DNA concentration was determined using nanodrop spectrophotometer (Genova Nano, Jenway)
153 and the samples were stored at -20°C until use for further DNA analysis.
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155 **2.3.2 Detection of virulence genes by multiplex PCR**

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157 Detection of gene sequences encoding for T3SS proteins (*espA espB* and *espD*) were performed
158 by the multiplex PCR using specific oligonucleotide primer sets as described previously [2]. All *E.*
159 *coli* isolates were screened for the presence of integrase genes *intl1*, *intl2* and *intl3* using primers
160 previously described [27]. The presence of *sfa/foc*, *papGIII*, *iutA*, *fyuA* virulence genes was
161 investigated by multiplex PCR using primers described previously [28]. Primer sequences and
162 size of amplicons are presented in Table 2. Master mix was performed according to primer mix
163 described in Table 2. Each PCR reaction mix (25 µL) was performed using 12.5 µL of PCR
164 premix with MgCl₂ (ReadyMix™ Taq PCR Reaction Mix with MgCl₂, Sigma), 0.4 µM of each
165 primer, and 3 µL (100-200 ng) DNA template. A negative and positive controls were also
166 included. The DNA amplification was carried out using the thermal cycler (Mastercycler personal,
167 Eppendorf, Germany). The cycling conditions for primer mix 1 were: initial denaturation at 94 °C
168 for 3 min; followed by 40 cycles of denaturation at 94°C for 50 s, annealing at 50°C for 40 s and
169 extension at 72°C for 1 min; were followed by a single final extension step at 72 °C for 5min, for
170 primer mix 2 were: denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension
171 at 72°C for 1 min for 30 cycles, with a final extension step at 72°C for 2 min and for primer mix 3
172 were: initial denaturation for 4 min at 94°C followed by 25 cycles of denaturation at 94°C for 30 s,
173 annealing at 60°C for 30 s and extension at 72°C for 1 min, ending with a final extension step at
174 72°C for 5 min. The PCR products were then detected by electrophoresis (Tis-acetate-EDTA
175 (TAE), 40 mM Tris-acetate and 1 mM EDTA. pH 8) through 1.8% (w/v) agarose gels to determine
176 the size of amplified fragment after staining with a final concentration 0.5 µg/ml of ethidium
177 bromide dye. The sizes of the amplicons were determined by comparing them with a 100-bp DNA
178 ladder.

179 **Table 2.** Target genes for PCR amplification, amplicon size, annealing temperature, primer sequences and primer mix that were
 180 used in this study.

Target gene	Primer sequence (5'→3')	Amplicon size (bp)	T _a	Primer mix	Reference
Type III secretion protein (<i>espA</i>)	espA F GTT TTT CAG GCT GCG ATT CT espA R AGT TTG GCT TTC GCA TTC TT	187	50 °C	1	[2]
Type III secretion protein (<i>espD</i>)	espD F AAA AAG CAG CTC GAA GAA CA espD R CCA ATG GCA ACA ACA GCC CA	145	50 °C	1	[2]
Type III secretion protein (<i>espB</i>)	espB F GCC GTT TTT GAG AGC CAG AA espB R AAA GAA CCT AAG ATC CCC A	106	50 °C	1	[2]
Integrase 1 (<i>intl1</i>)	intl1 F GCA TCC TCG GTT TTC TG G intl1 R GGT GTG GCG GGC TTC GTG	457bp	58°C	2	[27]
Integrase 2 (<i>intl2</i>)	intl2 F CAC GGA TAT GCGAC AAA AAG G T intl2 R GTA GCA AAC GAG TGA CGA AAT G	789bp	58°C	2	[27]
Integrase 3 (<i>intl3</i>)	intl3 F ATT GCC AAA CCT GAC TG intl3 R CGA ATG CCC CAA CAA CTC	922bp	58°C	2	[27]
<i>sfa/foc</i>	<i>sfa/foc</i> F CTC CGG AGA ACT GGG TGC ATC TTA C <i>sfa/foc</i> R CGG AGG AGT AAT TAC AAA CCT GGC A	410	60°C	3	[28]
<i>iutA</i>	<i>iutA</i> F GGC TGG ACA TCA TGG GAA CTG G <i>iutA</i> R CGT CGG GAA CGG GTA GAA TCG	300	60°C	3	[28]
<i>papGIII</i>	<i>papGIII</i> F GGC CTG CAA TGG ATT TAC CTG G <i>papGIII</i> R CCA CCA AAT GAC CAT GCC AGA C	258	60°C	3	[28]
<i>fyuA</i>	<i>fyuA</i> F TGA TTA ACC CCG CGA CGG GAA <i>fyuA</i> R CGC AGT AGG CAC GAT GTT GTA	880	60°C	3	[28]

181 *T_a: Annealing temperature

182 **RESULTS**

183

184 The isolates of *E. coli* in this research showed high resistance against Amoxicillin/Clavulanic acid
 185 (98%), while showed resistance between 45% to 65% against other tested antibiotics. Antibiotic
 186 resistance profile of these *E. coli* isolates is presented in Table 3. In addition, 71.4% (35/49) of
 187 the isolates were multidrug resistant.
 188

189 **Table 3.** Antibiotic resistance profile of 49 *E. coli* isolates recovered from different clinical
 190 samples.

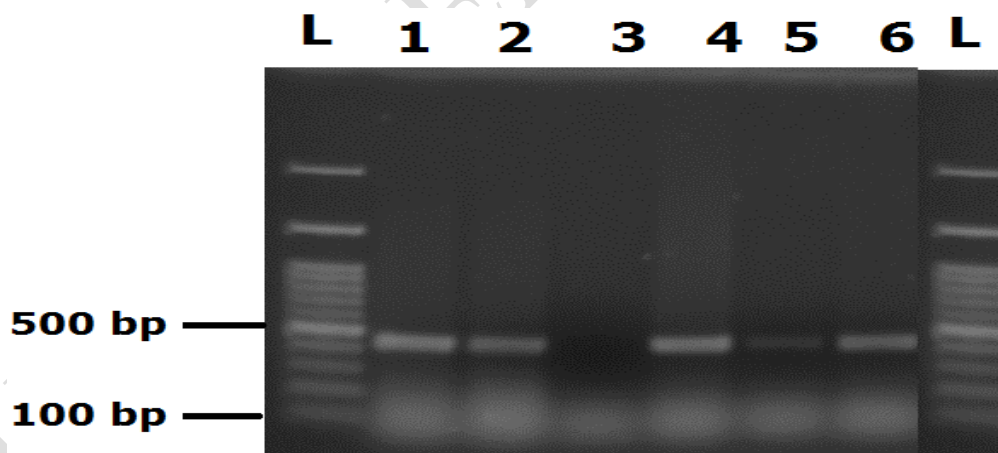
Antibiotic	Antibiotic resistance n (%)		
	S	I	R
Ciprofloxacin (5 µg)	23 (47.0)	1 (2.0)	25 (51.0)
Levofloxacin (10 µg)	25 (51.0)	0 (0.0)	24 (49.0)
Aztreonam (30µg)	21 (43.0)	2 (4.0)	26 (53.0)
Tetracycline (30µg)	23 (47.0)	4 (8.0)	22 (45.0)
Ceftazidime (30µg)	20 (41.0)	5 (10.0)	24 (49.0)
Imipenem (10 µg)	14 (29.0)	8 (16.0)	27 (55.0)
Ceftriaxone (30µg)	19 (38.78)	3 (6.12)	27 (55.0)
Amoxicillin/Clavulanic acid (20/10 µg)	1 (2.0)	0 (0.0)	48 (98.0)
Trimethoprim/Sulphamethoxazole (1.25/23.75 µg)	17 (35.0)	0 (0.0)	32 (65.0)

191

n: number of isolates; S: Susceptible; I: Intermediate; R: Resistant

192

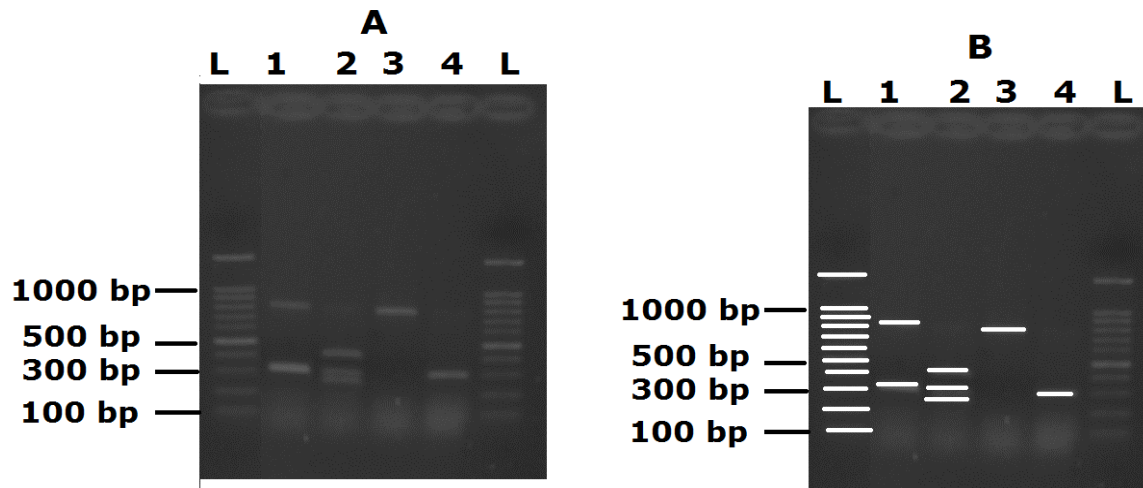
193 Results of this study showed that the prevalence of T3SS genes was 0.0%. In addition, only class
 194 1 integron was detected in *E. coli* isolates with prevalence (28/49) 57%. Results are presented in
 195 Fig. 1. Also, results showed that 65.7% (23/35) of MDR isolates carried class 1 integron.
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197

198 **Fig. 1.** Multiplex PCR profile specific for integrons detected from *E. coli*. Lanes L for the ladder;
 199 Lanes 1, 2, 4, 5 and 6 for class I integron and Lane 3 for negative control.
 200

201 The current study showed that the prevalence of *papGIII*, *sfa/foc*, *fyuA* and *iutA* genes was 4.1%,
 202 40%, 64%, 67.3% and 79.6%, respectively. Results of these virulence factors are presented in
 203 Fig. 2.



204
 205 **Fig. 2.** Multiplex PCR profile specific for virulence genes *papGIII*, *sfa /foc*, *fyuA* and *iutA* detected
 206 from *E. coli*. Lanes L for ladder, lane 1 for *fyuA* gene 880 bp and *iutA* gene 300 bp; lane 2 for
 207 *Saf/foc* gene 410 bp; lane 3 for *fyuA* gene 880 bp, *iutA* gene 300 bp, *papGIII* gene 258 bp and
 208 lane 4 for *iutA* gene 300 bp. A1: It is the same as B but bands are demarcated to be obvious.
 209

210 DISCUSSION

211
 212 Results of this study showed that *E. coli* isolates had high prevalence of antibiotic resistance. In
 213 addition, results showed that 71.4% (35/49) of the isolates were multidrug resistant which is
 214 higher than reported previously [29]. This high prevalence of antibiotic resistance in Palestine
 215 may be due to selective pressure of antibiotic imposed by the high rate and misuse of
 216 antimicrobial agents could be the only major cause [29,30].
 217

218 In current study, 57% of *E. coli* isolates carried class 1 integrons and none carried other tested
 219 classes of integrons. In addition, 65.7% of the multidrug resistant isolates had class 1 integrons.
 220 These results are in contrast to other reports previously published, which showed that the class 1
 221 and 2 integrons detected in *E. coli* isolates [11,31-33]. This result is in agreement with other
 222 report previously published, which showed that the class 1 integrons was the only type detected
 223 among clinical *E. coli* isolates [10]. These results showed that 65.7% of the multidrug resistant
 224 isolates had class 1 integrons, this result is in contrast with these previously published [11], which
 225 showed that 30% of the multidrug resistant isolates harbor integrons, while in other study [12],
 226 reported that all MDR isolates were carried integrons. Presence of class 1 integrons among multi-
 227 drug resistant isolates might be responsible for dissemination of antibiotic resistance gene
 228 [12,27]. In this study, the prevalence of integron genes among *E. coli* isolates is in contrast to
 229 other study previously reported [33], which showed that 80% diarrheagenic *E. coli* isolates carried
 230 integron genes. Other studies showed lower prevalence (22%-43.56%) [10-13]. Differences in
 231 prevalence of integrons might be due to differences in source of isolates.
 232

233 The prevalence of *espA*, *espD* and *espB* genes in clinical *E. coli* isolates have been previously
 234 studied in few reports. Our results showed that the prevalence of T3SS (*espA*, *espD* and *espB*)
 235 genes was 0.0%. This may due to these genes are detected only in Shiga-toxigenic *E. coli*
 236 (STEC), enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC) strains [2]. This may
 237 be true because most of our isolate were extraintestinal pathogenic strains. In previous published
 238 report, it was found that the prevalence of *espA*, *espD* and *espB* among STEC was 63%, 67%
 239 and 61%, respectively. The prevalence among EPEC was 55%, 100% and 100% for *espA*, *espD*
 240 and *espB*, respectively, and the prevalence in EIEC strains for these genes was 50% for each
 241 gene, while the these genes were not detected in ETEC and EAEC strains [2]. In other study
 242 carried out in India on 67 STEC, 5 EPEC and 22 ETEC strains were isolated from 256 rectal

243 swabs from yaks. the *aspB* gene was detected in two EPEC isolates (10%) only, while not
244 detected in the STEC or ETEC isolates [7].

245
246 Virulence genes papGIII, *sfa/foc*, *fyuA* and *iutA* have the following prevalence 4.1%, 40%, 64%,
247 and 79.6%, respectively. The high prevalence of *fyuA* observed in the present study among the
248 pathogenic *E. coli* was in agreement with other reports, which showed that the prevalence had a
249 range from 72.2%-96% [18-21]. It was found that *fyuA* gene was detected in about 92% of EAEC
250 strains and 13% among EIEC strains[34]. Also it was found that the prevalence of *fyuA* gene was
251 80.3% and 39.5% among pathogenic and commensal strains, respectively. The presence of *fyuA*
252 gene within a high pathogenicity island (HPI) seemed to be associated with the pathogenesis of
253 these strains as its presence increased the growth of the strains in site of infection [21,35]. In
254 addition, the *fyuA* gene was found to be highly important for biofilm formation in iron-poor
255 environments such as human urine [35]. The high prevalence of *iutA* observed in the present
256 study among the pathogenic *E. coli* was in agreement with other report [18,19], which showed
257 that the prevalence had a range from 67%-84% among *E. coli* isolated from patients with UTIs.
258 The same result is in contrast to other report [21], which showed that the prevalence was 39.8%
259 among diarrheagenic *E. coli* strains. It was found that *iutA* gene was detected in about 12% fro *E.*
260 *coli* isolated from fecal samples, while 67% from patients with Cystitis [19]. Also it was found that
261 the prevalence of *iutA* gene was 65.8% and 33.7% among pathogenic and commensal strains,
262 respectively [36]. The prevalence papGIII virulence factor in our *E. coli* isolates was 4.1%. In
263 other studies it was reported higher than Palestine with a range 10%-19.3 [15,18,22,23]. In other
264 study, the prevalence was 26% and 8% in *E. coli* isolated from cystitis and fecal samples [19].
265 The prevalence of *sfa/foc* virulence factor among *E. coli* isolated from North Palestine was 40%.
266 This result is in agreement with other reports which showed that prevalence had a range from
267 34%-36% [18,24]. It was found that *sfa/foc* gene was detected in about 11% from *E. coli* isolated
268 from fecal samples, while 26% from patients with Cystitis [19]. Although the exact role of S-
269 fimbriae is not identified; however, the dissemination of bacterium within the host tissue is
270 suggested for this adhesin [37]. The prevalence of papGIII, *sfa/foc*, *fyuA* and *iutA* genes may
271 depend on sample source.

272 In conclusion, *E. coli* isolates showed high resistant against different types of antibiotics. The co-
273 occurrence of class 1 integrons and antimicrobial resistance genes in current study is an
274 additional threat for spread of the antimicrobial resistance traits which may further complicate
275 future strategies for treatment the infections caused by this pathogen. These results reinforce
276 international knowledge on antimicrobial resistance and the high rate of multidrug resistance
277 found invites us to encourage population awareness of the proper use of antimicrobials. In
278 addition, *E. coli* isolated from Palestinian patients showed one or more virulence factors that
279 could increase their pathogenesis.

280 281 **COMPETING INTERESTS**

282
283 Authors have declared that no competing interests exist.

284 285 **REFERENCES**

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