Original Research Article

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 resistance rate 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 65.7% of MDR isolates carried integron genes. The prevalence of T3SS genes was 0.0%. In addition, results of this study showed that the prevalence of virulence genes *papGIII*, *sfa/foc*, *fyuA* and *iutA* was 4.1%, 40%, 64%, and 79.6%, respectively. **Conclusions:** The isolates of *E. coli* showed high resistance rate against different types of antibiotics. The co-occurrence of class 1 integrons and antimicrobial resistance genes in these isolates 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 pathogenic potential.

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), Enterohemorragic *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 *E. coli* (ExPEC) which includes Uropathogenic *E. coli* strains [1].

Type III secretion system (T3SS) is considered to be a one of these vital virulence factors, which is present in several pathogenic microorganisms particularly Gram-negative bacilli. The clinical spectrum of disease caused by T3SS-containing pathogens is remarkably broad. This complex is capable of injecting certain effector secretion proteins (toxins) into the host cell cytoplasm that then modulate its functions [2]. 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]. In EPEC and EHEC, 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 *LEE4* encodes the translocators EspA, EspB and EspD, a chaperone for EspD (CesD2) [4]. The prevalence of *espA*, *espD* and *espB* genes in clinical *E. coli* isolates have been previously studied. It was found that

the prevalence of *espA*, *espD* and *espB* among STEC was 63%, 67% and 61%, respectively, among EPEC were 55%, 100% and 100% for *espA*, *espD* and *espB*, respectively. The prevalence in EIEC strains for these genes was 50%. However, these genes were not detected in ETEC and EAEC strains [2]. In a study carried out in India on 67 STEC, 5 EPEC and 22 ETEC strains were isolated from 256 rectal swabs from yaks. the *aspB* gene was detected in two EPEC isolates (10%) only, while not detected in the STEC or ETEC isolates [5].

Five classes of integrons are known to play a role in the dissemination of antibiotic determinants to aminoglycosides and β-lactams among Gram negative species, and the most extensively studied was class 1 integrons [6,7]. In *E. coli*, the prevalence of integrons has been reported by several authors. These studies showed that the prevalence of integrons ranged from 22%-80% [8-11].

The pathogenicity of a given *E. coli* strain is mainly determined by specific virulence factors which include adhesion (fimbriae or pili), invasions, toxins (exotoxins and endotoxins), siderophores, capsule and flagella. Iron is an essential factor for pathogenicity of *E. coli* strains. Products of yersiniabactin (*fyuA*) and aerobactin (*iutA*) genes are among the most common iron acquiring systems of pathogenic and nonpathogenic *E. coli*. P-fimbrial adhesins in *E. coli* enable the colonization of host tissues. P fimbriae play a critical role in the development of urinary tract infections (UTIs). The P-fimbrial-tip adhesin, which is encoded by *papG*, attaches directly to host cells [12]. The class II G adhesin is associated with pyelonephritis and bacteremia, while the class III G adhesin sequence is associated with cystitis, although they have been found in pyelonephritis and bacteremia. The *PapGI* strains might have a larger prevalence among fecal isolates [13]. The two genetic determinants *sfa* and *foc*, coding for the S fimbrial adhesin and F1C fimbriae, respectively, and belong to the same general group of fimbrial gene clusters. These factors represent functionally distinct adhesins in their receptor specificities but enable bacteria to attach to eukaryotic cells [14]. The ability of uropathogenic *Escherichia coli* (UPEC) to cause symptomatic UTIs is enhanced by adhesins, e.g. S fimbriae and F1C fimbriae. Adherence to the urinary tract epithelium enables the bacteria to resist the hydrodynamic forces of urine flow and to establish infection [15]. There are many studies carried out to detect the prevalence of virulence factors in *E. coli*. The prevalence of *fyuA* gene had a range from 72.2%-96% [16-19], *iutA* 39.8% -84% [16-18], papGIII virulence factor 10%-19.3% [13,16,20,21] and *sfa/foc* 34%-36% [16,22].

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

MATERIALS and METHODS

2.1 Sample Collection

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

Table 1. A sample source of 49 *E. coli* isolates collected from different hospitals at Northern 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

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

2.2 Antibiotic Resistance

Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method [23]. All *E. coli* isolates were examined for resistance to Levofloxacin (LEV) 5µg, Ciprofloxacin (CIP) 5µg, Aztreonam (ATM) 30µg, Tetracycline (TE) 30µg, Imipenem(IPM) 10µg, Ceftriaxone (CFX) 30µg, Trimethoprim/Sulfamethoxazole (SXT) 25µg, Ceftazidime (CAZ) 30µg, and Amoxycillin/clavulanic acid (AMC) 30 µg. Normal saline suspensions of all *E. coli* isolates were adjusted to the McFarland 0.5 standard and used to inoculate Mueller Hinton agar plates. The plates were incubated at 37°C for 14-16 hrs. The zone of inhibition was measured in millimeters. Isolates were classified as resistant, intermediate or susceptible according to the criteria recommended by CLSI guidelines [23]. Strains were considered multidrug-resistant (MDR) if they were resistant to at least three different classes of the antimicrobial agents tested.

2.3 Polymerase Chain Reaction and DNA Extraction

2.3.1 DNA extraction

E. coli DNA genome was prepared for PCR according to the method described previously [24]. Briefly, cells were scraped off an overnight nutrient agar plate with a sterile loop, washed with 1 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), then the pellet was resuspended in 0.5 ml of sterile distilled water, and boiled for 10-15 min. The cells then were incubated on ice for 10 min. The debris was pelleted by centrifugation at 11,500 X *g* for 5 min. DNA concentration was determined using nanodrop spectrophotometer (Genova Nano, Jenway) and the samples were stored at -20ºC until use for further DNA analysis.

2.3.2 Detection of virulence genes by multiplex PCR

Detection of gene sequences encoding for T3SS proteins (*espA espB* and *espD*) was performed by the multiplex PCR using specific oligonucleotide primer sets as described previously [2]. All *E. coli* isolates were screened for the presence of integrase genes *intI1*, *intI2* and *intI3* using primers previously described [27]. The presence of *sfa/foc*, *papGIII*, *iutA*, *fyuA* virulence genes was investigated by multiplex PCR using primers described previously [28]. Primer sequences used in this study and size of amplicons are presented in Table 2. Master mix was performed according to primer mix described in Table 2. Each PCR reaction mix (25 µL) was performed using 12.5 µL of PCR premix with $MgCl_2$ (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4 µM of each primer, and 3 µL (100-200 ng) DNA template. A negative and positive controls were also included. The DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany). The cycling conditions for primer mix 1 were: initial denaturation at 94 ºC

for 3 min; followed by 40 cycles of denaturation at 94ºC for 50 s, annealing at 50ºC for 40 s and extension at 72ºC for 1 min; followed by a single final extension step at 72 ºC for 5min, for primer mix 2 were: denaturation at 94ºC for 30 sec, annealing at 58ºC for 30 sec, and extension at 72ºC for 1 min for 30 cycles; followed by a single final extension step at 72ºC for 2 min and for primer mix 3 were: initial denaturation for 4 min at 94ºC followed by 25 cycles of denaturation at 94ºC for 30 s, annealing at 60ºC for 30 s and extension at 72ºC for 1 min; followed by a single final extension step at 72ºC for 5 min. The PCR products were then detected by agarose gel electrophoresis on 1.8% (w/v) agarose gel to determine the size of amplified fragments after staining with a final concentration 0.5 µg/ml of ethidium bromide dye. The sizes of the amplicons were determined by comparing them with a 100-bp DNA ladder.

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

Target gene	Primer sequence (5'→3')	Amplicon size (bp)	T_a	Primer mix	Reference
Type III secretion protein (*espA*)	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 (*espD*)	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 (*espB*)	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 (*intI1*)	intI1 F GCA TCC TCG GTT TTC TG G intI1 R GGT GTG GCG GGC TTC GTG	457bp	58°C	2	[25]
Integrase 2 (*intI2*)	intI2 F CAC GGA TAT GCGAC AAA AAG G T intI2 R GTA GCA AAC GAG TGA CGA AAT G	789bp	58°C	2	[25]
Integrase 3 (*intI3*)	intI3 F ATT GCC AAA CCT GAC TG intI3 R CGA ATG CCC CAA CAA CTC	922bp	58°C	2	[25]
sfa/foc	sfa/foc F CTC CGG AGA ACT GGG TGC ATC TTA C sfa/foc R CGG AGG AGT AAT TAC AAA CCT GGC A	410	60°C	3	[26]
iutA	iutA F GGC TGG ACA TCA TGG GAA CTG G iutA R CGT CGG GAA CGG GTA GAA TCG	300	60°C	3	[26]
papGIII	papGIII F GGC CTG CAA TGG ATT TAC CTG G papGIII R CCA CCA AAT GAC CAT GCC AGA C	258	60°C	3	[26]
fyuA	fyuA F TGA TTA ACC CCG CGA CGG GAA fyuA R CGC AGT AGG CAC GAT GTT GTA	880	60°C	3	[26]

162 *Ta: Annealing temperature

RESULTS

The isolates of *E. coli* in this research showed high resistance rate against Amoxicillin/Clavulanic acid (98%). However, resistance rate against other tested antibiotics has ranged from 45% to 65%. Antibiotic resistance profile of these *E. coli* isolates is presented in Table 3. In addition, 71.4% (35/49) of the isolates were multidrug resistant.

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

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

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

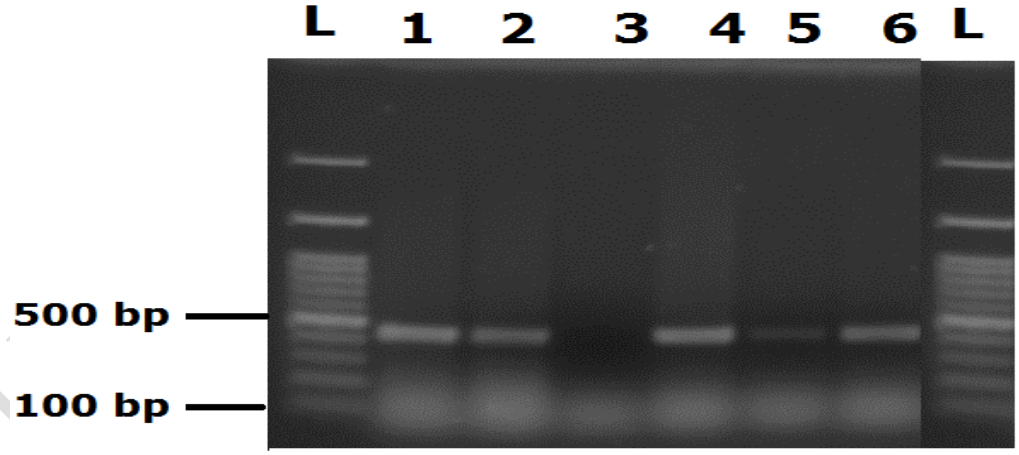

Fig. 1. Multiplex PCR profile specific for integrons detected from *E. coli*. Lanes L for the ladder; Lanes 1, 2, 4, 5 and 6 for class I integron and Lane 3 for negative control.

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

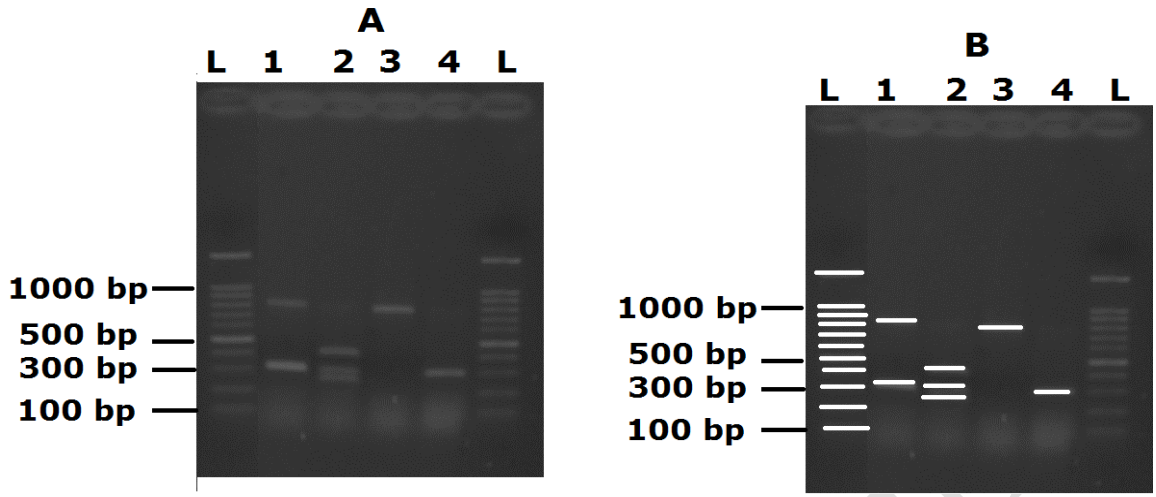

Fig. 2. Multiplex PCR profile specific for virulence genes papGIII, *sfa /foc*, *fyuA* and *iutA* detected from *E. coli*. Lanes L for ladder, lane 1 for *fyuA* gene 880 bp and *iutA* gene 300 bp; lane 2 for Saf\foc gene 410 bp; lane 3 for *fyuA* gene 880 bp, *iutA* gene 300 bp, papGIII gene 258 bp and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

DISCUSSION

Results of this study showed that *E. coli* isolates had high prevalence of antibiotic resistance. In addition, results showed that 71.4% (35/49) of the isolates were multidrug resistant which is higher than reported previously [27]. This high prevalence of antibiotic resistance in Palestine may be due to selective pressure of antibiotic imposed by the high rate and misuse of antimicrobial agents could be the only major cause [27,28].

In current study, all *E. coli* isolates that had integron genes were carried only class 1 integron and none other tested classes. This result is in contrast to other reports previously published, which showed that the class 1 and 2 integrons were detected in *E. coli* isolates [9,29-31]. However, this result is in agreement with other report previously published, which showed that the class 1 integron was the only type detected among clinical *E. coli* isolates [8]. In addition, results of this study showed that 65.7% (23/35) of the multidrug resistant isolates had integron genes. This result is in contrast to previously published report [9], which showed that 30% of the multidrug resistant isolates harbored integrons. However, in other study [10], reported that all MDR isolates were carried integrons. Presence of integrons among multi-drug resistant isolates might be responsible for dissemination of antibiotic resistance genes [10,25]. In this study, the prevalence of integron genes among *E. coli* isolates was 57%. This result is in contrast to other study previously published [31], which showed that 80% diarrheagenic *E. col* isolates carried integron genes. However, other studies showed lower prevalence (22%-43.56%) [8-11]. Differences in prevalence of interons might be due to differences in source of isolates.

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

The *aspB* gene was detected in two EPEC isolates (10%) only, while not detected in the STEC or ETEC isolates [5].

The prevalence of virulence genes *papGIII*, *sfa/foc*, *fyuA* and *iutA* was 4.1%, 40%, 64%, and 79.6%, respectively. The high prevalence of *fyuA* observed in the present study among the pathogenic *E. coli* was in agreement with other reports, which showed that the prevalence had a range from 72.2%-96% [16-19]. It was found that *fyuA* gene was detected in about 92% of EAEC strains and 13% among EIEC strains[32]. Also it was found that the prevalence of *fyuA* gene was 80.3% and 39.5% among pathogenic and commensal strains, respectively. The presence of *fyuA* gene within a high pathogenicity island (HPI) seemed to be associated with the pathogenesis of these strains as its presence increased the growth of the strains in site of infection [19,33]. In addition, the *fyuA* gene was found to be highly important for biofilm formation in iron-poor environments such as human urine [33]. The high prevalence of *iutA* observed in the present study among the pathogenic *E. coli* was in agreement with other reports [16,17,34], which showed that the prevalence had a range from 67%-84% among *E. coli* isolated from patients with UTIs. However, this result is in contrast to other report [19], which showed that the prevalence was 39.8% among diarrheagenic *E. coli* strains. It was found that *iutA* gene was detected in about 12% for *E. coli* isolated from fecal samples, while 67% from patients with Cystitis [17]. Also it was found that the prevalence of *iutA* gene was 65.8% and 33.7% among pathogenic and commensal strains, respectively [35]. The prevalence *papGIII* virulence factor in our *E. coli* isolates was 4.1%. However, in other studies it was reported higher than in Palestine with a range 10%-19.3% [13,16,20,21]. In other study, the prevalence was 26% and 8% in *E. coli* isolated from cystitis and fecal samples [17]. The prevalence of *sfa/foc* virulence factor among *E. coli* isolated from North Palestine was 40%. This result is in agreement with other reports which showed that prevalence had a range from 34%-36% [16,22]. It was found that sfa/foc gene was detected in about 11% from *E. coli* isolated from fecal samples, while 26% from patients with Cystitis [17]. Although the exact role of S-fimbriae is not identified; however, the dissemination of bacterium within the host tissue is suggested for this adhesin [36]. The prevalence of *papGIII*, *sfa/foc*, *fyuA* and *iutA* genes may depend on sample source.

CONCLUSION

Results of this study showed that *E. coli* isolates had high level of resistance rate 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. These results reinforce international knowledge on antimicrobial resistance and the high rate of multidrug resistance found invites us to encourage population awareness of the proper use of antimicrobials. In addition, *E. coli* isolated from Palestinian patients showed one or more virulence factors that could increase their pathogenic potential.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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