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Vol. 10(33), pp. 1363-1370, 7 September, 2016
DOI: 10.5897/AJMR2016.8087
Article Number: 27C435160360
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Resistance genes to sulphonamide in commensal *Escherichia coli* isolated from stool of patients in Mansoura University Children Hospital

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Received 30 April 2016, Accepted 9 August, 2016.

Commensal bacteria have a great impact on the emergence and spread of antibiotic resistance. This emphasizes a great need to underscore the magnitude of this problem in our locality, and children are taken as a sector in this research because they are usually subjected to heavy load of antibiotic usage. This study aimed at determining sulphonamide resistance genes presence among fecal isolates of commensal *Escherichia coli* detected in patients attending Mansoura University Children Hospital (MUCH) and to check the value of these commensals in the appearance and transmission of antimicrobial resistance. Forty five (45) co-trimoxazole resistant *E. coli* were haphazardly chosen for detection of resistant determinant to sulphonamide. The methods used were antibiotic sensitivity tests by disc diffusion, detection of *sul* and *int1* genes by PCR and conjugation assay. Co-trimoxazole resistance was found in 80.3% of the examined fecal commensal *E. coli*. *sul2* gene recorded the highest prevalence in the examined co-trimoxazole resistant *E. coli* strains (73%). *int1* gene was found in 62% of those isolates. 35.5% of the studied isolates had the ability to transmit genes of resistance to the recipient susceptible isolates by conjugation experiment. The recorded great prevalence of resistance genes to sulphonamide in commensal isolates of *E. coli* among children seems to be alarming which may indicate the future increase in the prevalence of those resistant genes in our community. This problem underlines the necessity of limitation of antibiotic usage, particularly among children.

Key words: Sulphonamide resistance, *Escherichia coli*, *sul* genes, integrons.

INTRODUCTION

The dissemination of antimicrobial resistance was found to be an important problem that worsens the outcome of antibiotic therapy and leads to more duration of the diseases periods, high mortality rates in addition to increased hospital related payment (WHO, 2015). In developing localities, a great effect of this problem was

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found. Actually, a great prevalence of decreased response to antibiotics is usually recorded in screening assays of different bacterial strains (Shears, 2001), and in researches that examined normal bacterial flora as an important index for distribution of genes that are responsible for decreased response to antibiotics (Alves et al., 2014). In addition, developing localities usually suffer from the bad impact of antibiotic resistance on disease outcome and rates of mortality which is due to recurrent infections by bacteria and the great value of antibiotics in fighting them (Adefisoye and Okoh, 2016). Treatment of many human diseases was greatly dependent on sulphonamid drugs, but, sulphonamides were usually added to trimethoprim in order to decrease the appearance of resistance, this usually limits the prescription of these drugs that have the advantage of being low cost. Sulfamethoxazole plus trimethoprim (co-trimoxazole) was still found to be one of the major antibiotics used in dealing with many diseases caused by bacterial infection and WHO reports this antimicrobial as the only one that should be used in management of certain serious diseases (Perreten and Boerlin, 2003).

The decreased response to sulphonamides specially by *Escherichia coli*, is usually due to genetic alteration of dihydropteroate synthase gene (*folP*) in the chromosome, that limit the binding ability of this enzyme with the inhibitory agents, also it may be due to gaining of *sul* type determinants encoding enzymes with lower sulphonamides binding ability (Sk¨old, 2000).

Three genetic determinants have been described, *sul1*, *sul2* and *sul3*. *sul1* is commonly found in association with integrons of class 1 (Deng et al., 2015). *sul2* gene is usually found to be controlled by various plasmids, on the other hand, *sul3* is a new sulphonamide genetic determinant, that carry several enzyme variants (Singha et al., 2015). All of these genetic determinants have been found in the isolates of *E. coli* recovered from human sources (Perreten and Boerlin, 2003).

The important value of normal bacterial flora in the appearance and dissemination of antibiotic unresponsiveness is globally observed (APUA, 2008). Certain strains of the normal bacterial flora, like *E. coli* of stool were studied as an important determinant in the assays of antibiotic limited response (Osterblad et al., 2000). So, this research aimed at checking the presence of *sul*-type genes in the commensal *E. coli* recovered from stool samples of patients attending MUCH, and also determined the value of these commensal pathogens in emergence and transmission of antimicrobial unresponsiveness.

MATERIALS AND METHODS

Design of the study

Cross sectional descriptive study was conducted on 173 patients between 6 month and 6 years of age attending the outpatient clinics of MUCH. The duration of the study was six months, starting from first of May to the end of October, 2015. The protocol of this study was approved by the ethical committee in the Faculty of Medicine, Mansoura University.

Clinical samples

Stool samples were collected from all the children under complete aseptic condition.

Microbiologic studies

Stool samples were processed in Microbiology Diagnostic and Infection Control Unit in the Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University. The collected specimens were cultivated on MacConkey's agar and Eosin Methylene Blue (EMB) agar media.

Strains identification

E. coli bacterial isolates were identified by Gram stained films, appearing as Gram-Negative rods. They produced deep red colonies on MacConkey's agar and gave characteristic greenish metallic sheen on EMB agar. Further identification was done by conventional biochemical IMViC (Indole, Methyl red, Voges Proskauer and Citrate) tests. As they were Indole and Methyl red positive, Voges Proskauer and Citrate negative (Cheesbrough, 2002) identification was confirmed by (API) 20 E analytical profile index (Bio-merieux SA, Montalieu Vercica and France). *E. coli* ATCC 25922 was used as an organism for quality control. The isolated strains were stored on fresh Nutrient agar slopes for antimicrobial sensitivity testing.

Testing for antimicrobial sensitivity

Antimicrobial susceptibility tests were carried out on the identified *E. coli* as recorded by the recommendations of CLSI. Disc diffusion on the agar of Mueller-Hinton (MHA; Bio -Rad, Marnes –La -Coquette, France) was done to determine the sensitivity to co-trimoxazole (SXT) (25 µg) [sulfamethoxazole in combination with trimethoprim], ampicilline (AMP) (10 µg), amoxicilline/clavulinic acid (AMC) (30 µg), azteronam (ATM) (30 µg), cefotaxime (CTX) (30 µg), imipenem (IPM) (10 µg), netilmicin (NET) (30 µg), chloramphenicol (C) (30 µg), kanamycin (K) (30 µg), amikacin (AK) (30 µg), gentamicin (CN) (30 µg), (Oxoid AB) (Koneman et al., 1997). The inhibitory zone limits of the tested antimicrobials were referred to CLSI (2014).

Co-trimoxazole resistant strains were determined in 139 specimens among the studied 173 subjects (80.3%). Forty five isolates were selected randomly for detection of *sul* and *int1* genes and conduction of conjugation assay.

Detection of *sul* and *int1* genes by polymerase chain reaction

PCR was used to detect *sul1, sul2, sul3* and *int1* genes. Freshly isolated colony of each bacterial isolate was added to distilled sterile water (100 mL) and boiled for 10 min at a temperature of 100°C. Centrifugation was done, PCR assays were performed with the supernatant using primers shown in Table 1. A total volume of 50 µL reaction mixture had these reagents: Primers (1 µM), DNA

Table 1. The primers used for polymerase chain reaction detection of *sul1, sul2, sul3* and *int1* genes.

Gene	Primer	Size (bp)	Annealing temperature (°C)	Reference
sul1	F: 5'-CGGCGTGGGCTACCTGAACG-3' R: 5'-GCCGATCGCGTGAAGTTCCG-3'	432	55	Arabi et al., 2015
sul2	F: 5'-GCGCTCAAGGCAGATGGCATT-3' R: 5'-GCGTTTGATACCGGCACCCGT-3'	293	53	Arabi et al., 2015
sul3	F: 5'-CAGATAAGGCAATTGAGCATGCTCTGC-3' R: 5'–GATTTCCGTGACACTGCAATCATT-3'	569	55	Arabi et al., 2015
int1	F: 5'-GCCTGTTCGGTTCGTAAGCT-3' R: 5'-CGGATGTTGCGATTACTTCG-3'	585	56	Ma et al., 2009

(100 ng), Tris-HCl (10 mM; pH 8.3), KCl (50 mM), dNTP (200 µM), 1 U of Taq DNA polymerase and MgCl2 (1.5 mM) (Frank et al., 2007). DNA amplification was done in DNA Thermal cycler (peltier-Effect cycling- MJ Researches, INC.). PCR temperature conditions and genes band size are shown in Table 1. Agarose gel (1.5%) was used to electrophorese the PCR products. Bands were detected in comparison with DNA standard marker: #SMO323 marker (Fermentas) and visualized under UV light (Van Tongeren et al., 2011).

Conjugation experiment (Sunde and Sørum, 2001)

For detection of transferability of *sul* genes, conjugation experiment was done as follows: co-trimoxazole resistant *E. coli* isolates were used as donor for mating experiment, and *E. coli* BM21 (resistant to nalidixic acid, positive for lactose fermentation, and free of plasmide) was used as the recipient for conjugation experiment with co-trimoxazole resistant *E. coli* isolates (Vacsera, Cairo, Egypt). The recipient and donor isolates were cultured in broth of brain heart infusion (BHI) for 5 h at 37°C, then the recipient (50 µL) and donor (25 µL) were mixed in fresh BHI broth (3 mL), after that it was kept in the incubator overnight at 37°C. The transconjugants were detected on agar plates of Mueller-Hinton that contained 40 mg/L nalidixic acid and 256 mg/L sulfamethoxazole.

Confirmation of resistance features in the conjugated isolates

PCR assays with the same previous techniques were carried out to confirm the existence of resistant genetic determinant in the conjugated isolates, and this was done using DNA of those isolates as a template.

Also, confirmatory antibiotic sensitivity testing was done to the transconjugants to phenotypically check the transmission of the antibiotic resistance pattern in those isolates.

Analysis of data

The data were entered and analyzed statistically with Statistical Package of Social Science (SPSS) using software version 17. Qualitative data was described as numbers and percentages. Inter-group comparison of categorical data was done using Chi-square test (X^2-value). *P*-value <0.05 was considered to be statistically significant.

RESULTS

Forty five (45) isolates, haphazardly chosen from 139 co-trimoxazole resistant *E. coli* strains isolated from the studied stool samples of children attending the outpatient clinics of MUCH, were examined as regarding antibiotic sensitivity, PCR for detection of *sul1, sul2, sul3* and *int1* genes and conjugation assay.

Antibiotic sensitivity tests revealed that all the examined strains except three were nonresponsive to two distinct family of the tested antibiotics. Fourteen (14) isolates exhibited identical pattern of resistance, as they showed antibiotic non responsiveness to ampicilline (AMP), amoxicilline/clavulinic (AMC) and chloramphenicol (C) in addition to co-trimoxazole. The examined isolates showed a great resistance to augmentin (32 isolates, 71%), ampicillin (26 isolates, 58%), and chloramphenicol (21 isolates, 47%). The pattern of antimicrobial resistance exhibited by the examined 45 strains is shown in Table 2.

PCR checking for *sul1, sul2* and *sul3* genes showed the existence of one form of *sul*-genes at least in 41 of the studied 45 isolates (91%). Only one form of *sul*-gene was detected in 33 strains (*sul2* in 25 strains and *sul1* in 8 strains), on the other hand, 8 strains were found to harbor 2 distinct forms of *sul* genes (*sul2* with *sul1* in 6 strains, and *sul3* with *sul2* in 2 strains). Totally, the percentages of different *sul* genes types in the examined strains were 73% for *sul2*, 31% for *sul1* and 4% for *sul3*. Regarding PCR results for *int1* gene, it was revealed that 28 isolates (62%) were positive for this gene by PCR (Figures 1 and 2).

Regarding the distribution of *sul* genes in relation to *int1* gene (Table 3), it was found that *sul1* gene was more frequently associated with the presence of *int1* gene than *sul2* and *sul3*, as 79% of strains that harbor *sul1* gene were found to be positive for *int1*, whereas 61 and 50% of strains that harbor *sul2* and *sul3* genes respectively, were positive for that gene.

The conjugation experiments showed that 16 (35.5%)

Table 2. Resistance phenotypes of the examined 45 Co-trimoxazole resistant *E. coli* strains isolated from stool samples.

Isolate	Other resistance	Isolate	Other resistance	Isolate	Other resistance
1	AMP, AMC,C	16	AMC, C	31	AMC, NET
2	AMC, NET	17	AMP, AMC	32	AMP, AMC, C
3	AMP, C	18	AMP, AMC, C	33	AMC, C
4	AMP,AMC,C	19	AMP, K	34	_
5	AMC, CN	20	AMP, AMC, C	35	AMC
6	_	21	AMC, C, CN	36	AMP, AMC, C
7	AMC	22	AMP	37	AMP, AMC, C
8	AMP	23	AMC	38	AMC, CTX
9	AMC, C, CN	24	AMC, C	39	AMP, AMC, C
10	AMP, AMC, C	25	AMP, AMC, C	40	AMP, AMC, C
11	AMP, AMC, C	26	AMP, K	41	AMP, K
12	AMC	27	AMP	42	AMP
13	AMP, AMC, C	28	AMC, CN	43	AMP, AMC, C
14	AMC, NET	29	AMP, AMC	44	_
15	AMP	30	AMP	45	AMC,C, CN

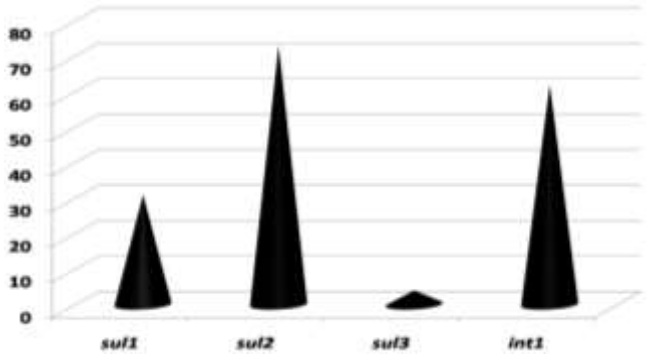

Figure 1. PCR results revealing the percentage of *sul1*, *sul2, sul3* and *int1* genes in the examined isolates.

of the studied isolates had the ability to transmit the detected resistant genes to the receiver isolates as revealed by the confirmatory PCR. Nearly all the strains had the ability to transmit their pattern of antibiotic resistance to the receiver isolates, except 2 strains that could not transmit all their antibiotic resistance profile as revealed by the confirmatory antibiotic sensitivity testing (one isolate not to ampicillin and the other not to gentamicin).

Transfer of resistance features was found to be significantly associated with the existence of *int1* gene in the examined strains ($P < 0.05$), as 15 (94%) of the strains that were positive by conjugation experiment were found to harbor class 1 integrase gene.

DISCUSSION

The persistent increased resistance to sulphonamide compounds among different infectious agents is aggravating. Novel resistant genetic determinant that are responsible for non-responsiveness to 'outdated' antimicrobials, like those compounds, are continuously being detected (Arabi et al., 2015).

Previous studies reported a great incidence of *E. coli* in stool samples with gained resistant determinant to different antibiotics, particularly the outdated anti-microbials (for example, penicillin and co-trimoxazole) especially in young age (Niaz et al., 2016).

Commensal bacteria are similar to pathogenic ones in being subject to heavy load of antibiotics. Normal bacterial flora like *E. coli* is usually used as an index of transmission and spread of the gained resistant determinant (Adefisoye and Okoh, 2016).

To the authors' knowledge, this study is the first study in Egypt that assessed the presence of sulphonamide resistant determinant among commensal *E. coli* in children.

This study revealed that the randomly selected 45 co-trimoxazole resistant faecal *E. coli* strains showed great resistance to augmentin, ampicillin and chloramphenicol (71, 58 and 47%, respectively). This antibiotic resistance profile was previously detected in healthy individuals by Bartoloni et al. (1998) in Bolivia, van de Mortel et al. (1998) in Venezuela and Okeke et al. (2000) in Ile-ife, Nigeria. These data are also consistent with the finding of further studies that showed a great antibiotic resistance pattern recorded by the commensal *E. coli* from low-resource settings (Bailey et al., 2010).

Ampicillin and chloramphenicol are among the older generations of antibiotics which are used in children and high resistance observed in them may be due to selective pressure from their inappropriate and excessive uses in our locality which by cross-resistance affected

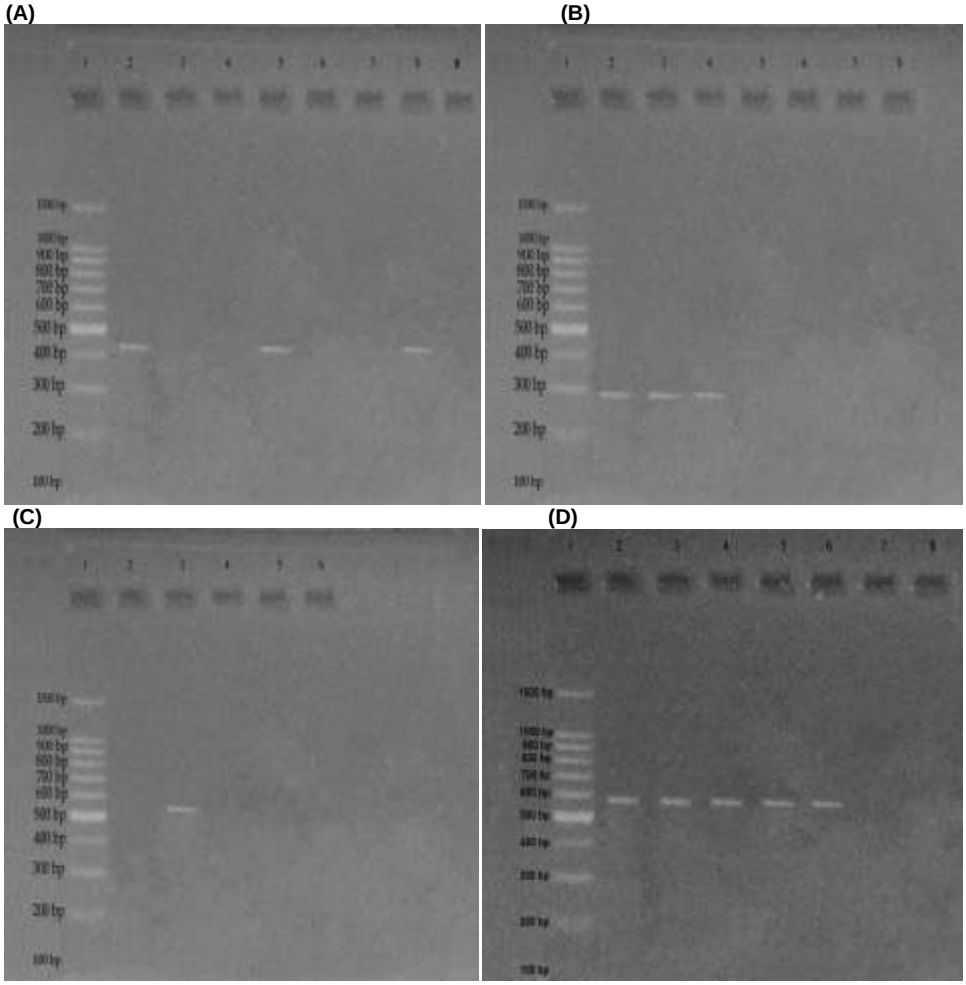

Figure 2. PCR results of *sul1*, *sul2, sul3* and *int1* genes of the studied isolates. Lane 1 shows molecular size marker #SMO323. (A): lanes 2, 5 and 8 show bands of 432 bp from positive strains that carry the *sul1* genes; (B): lanes 2, 3 and 4 show bands of 293 bp from positive strains that carry the *sul2* genes; (C): lane 3 shows band of 569 bp from positive strain that carry the *sul3* gene; (D): lane 2,3,4,5 and 6 show bands of 585 bp from positive strains that carry the *int1* genes.

Augmentin. Based on the PCR results, the *sul2* gene has the highest prevalence in the examined co-trimoxazole resistant *E. coli* strains. Frequency of *sul2* (73%) was higher than that of *sul1* (31%) and *sul3* (4%), which is in accordance with other studies conducted by Grape et al. (2003), Infante et al. (2005) and Wu et al. (2010).

This study is in parallel with earlier researches that determine the prevalence of sulphonamide resistance genes *sul2* and *sul1* among the commensal isolates of *E. coli* in different localities and recorded a great incidence of *sul* genes resistant determinant in the majority of the studied isolates mainly *sul2* or *sul1* alone or the two genes together (Rådström et al., 1991). Although, Frank et al. (2007) demonstrated an elevated prevalence of *sul1* than *sul2* gene among their studied isolates, the study is still in agreement with recent studies that recorded *sul2* gene with a higher existence rate than *sul1* and *sul3* in commensal isolates of *E. coli* from studied individuals in Denmark and other localities (Trobos et al., 2008); also in the UK, non-responsiveness to sulfonamide in *E. coli* of human source remains high and *sul2* is still the most prevalent one although the prescription of sulphonamide drugs has been ended many years ago (Bean et al., 2009).

sul3 is a new genetic resistance determinant to sulphonamides. It has genetic relatedness to *sul2* and *sul1*, it was firstly detected in pigs in 2003 (Perreten and Boerlin, 2003). It has been commonly observed in *E. coli* isolated from pigs in Switzerland. In the same year, Grape et al. (2003) detected this gene in *E. coli* recovered

Table 3. Distribution of *sul1*, *sul2* and *sul3* in the examined isolates and its relation to *int1* gene presence.

Isolate	Type of gene			
	sul1	***sul2***	***sul3***	***int1***
1	+	+		+
2		+		+
3		+		
4	+			+
5		+		+
6				
7	+			+
8		+		+
9	+	+		+
10		+		
11		+		+
12	+			+
13		+		+
14		+	+	+
15		+		
16	+			+
17		+		+
18	+	+		+
19		+		
20		+		+
21	+	+		
22				+
23		+		+
24		+		+
25		+		
26		+		+
27	+			
28		+		
29		+		+
30	+			+
31		+		
32	+	+		
33		+		
34				
35		+		+
36		+		
37	+			+
38		+		
39	+	+		+
40		+		+
41		+		+
42				
43		+	+	
44	+			+
45		+		+

from human samples in Sweden. *sul3* has an amino acid identity of about 40% relatedness to the already present genes (*sul2* and *sul1*). It was first detected in conjugative plasmid of 54 kb weight, also it could be carried by another huge plasmid in addition to the first one (Perreten and Boerlin, 2003). According to the studies of Wu et al. (2010) and Ziembińska-Buczyńska et al. (2015), *sul3* gene is the least prevalent one in *E. coli* strains recovered from human and animal resources, this data is in parallel with the present results. Although, *sul3* gene was rarely detected in the studied isolates (4% only), this small percentage should be considered as it may be a warning sign revealing that its existence can be widespread in the locality.

Resistance to various antibiotic is usually caused by integrons which are harbored by bacterial chromosome or carried by plasmids (Tajbakhsh et al., 2015). These antibiotic resistance determinants are able to hold antibiotic resistant genes by site-specific recombination system. Also, they gain novel genes in various types of bacteria (El-Sokkary and Abdelmegeed, 2015). Integrons of class 1 are movable elements which were found to be effective in transmission of antimicrobial resistant genes due to presence of mobile gene cassettes (Ammar et al., 2016). As approved, transmission of *sul* genetic determinant among different bacterial strains is usually accompanied by integration of genetic cassettes into the integrons (Sobia et al., 2016). This study assessed the presence of *int1* gene among the studied isolates, and detected the gene in 28 (62%) of them. These results approximates the finding of Infante et al. (2005), who found *int1* gene in 9 (45%) of their studied 20 isolates and Lavakhamseh et al. (2016) who recorded the presence of the same gene in 47% of their studied isolates. On the other hand, higher percentage of *int1* gene (95%) was recorded by Frank et al. (2007).

The discrepancy among different studies could be attributed to different localities where each one has its own pattern of pathogens resistance genes. Also, the studied isolates were different, as Frank et al. (2007) conducted study on different strains of *Enterobacteriaceae* not *E. coli* alone. The remaining isolates in this research that were found to be negative for *int1* gene may carry other mobile genetic elements, that could act as sources of *sul* genes.

In this study, it was reported that 79, 61 and 50% of the isolates which harbor *sul1, sul2* and *sul3* genes, respectively, were found to be positive for *int1* gene, with *sul1* being the most frequent one found in association with that gene. These findings are in harmony with that of Antunes et al. (2005), who observed great association between *sul1* and *int1* gene. Similar to this study results, Shehabi et al. (2006), recorded that, *sul1* was more frequently associated with *int1* gene than *sul2*, also Khamesipour and Tajbakhsh (2016) recorded the association of *sul1* genes with class 1 integrons in 66.66%

of their examined strains which was more than *sul2* and *sul3.* However, Infante et al. (2005) and Wu et al. (2010) observed *sul3* gene, as the most frequent one that was found in association with *int1* gene. These different studies observations suggest the necessity of spending more efforts to do future researches on the new *sul3* gene in relation to *int1*.

The conjugation testing used in this research demonstrated that the resistant genes were mostly present in conjugative plasmids, as it was successfully transferred in 35.5% of the studied isolates approximating the results of Antunes et al. (2005), who stated that, sulfonamide resistance was transferred in 43% of their studied isolates. The significant association that was found between *int1* gene occurrence and transfer of resistant features, as 94% of strains that were positive by conjugation experiment were found to harbor *int1* gene indicates the high prevalence of conjugative plasmids carrying *int1* gene, this significant association was also confirmed by Sunde and Norstro¨m (2006) and Ravi et al. (2015). In those studied isolates, this may be due to presence of certain powerful plasmid harboring *int1* gene and it has a high transmission ability or it may be that *int1* gene represents a portion of an 'antimicrobial resistance island' which has the ability of incorporation in different types of conjugative plasmids. Briefly, resistance determinant can be transmitted by conjugal transfer, indicating the association of the responsible resistant genes with mobile elements like plasmids.

Furthermore studies seem to be necessary to describe the plasmids and the genetic characters of *int1* gene which they harbor.

Conclusion

Commensal isolates of *E. coli* that shows resistance to co-trimoxazole were proved to be prevalent among children in this study locality. The three *sul*-genetic variants (*sul1, sul2* and *sul3*) were detected in those isolates, indicating the high prevalence of such resistant elements. *sul2* gene was higher in prevalence than *sul1 and sul3*. The heavy presence of sulphonamide resistance genes in the enteric *E. coli* highlights the role of normal bacterial flora as a significant source of genetic determinants that encodes resistance to various antimicrobials. The existence of different types of *sul* genes seems to be due to the heavy load of sulfonamides and other antibiotics which are usually prescribed.

High prevalence of *int1* gene was found in resistant strains indicating widespread distribution of resistant determinants in the community. Restrictive utilization of all antimicrobials is recommended in order to minimize the expansion of antibiotic resistance problem among different bacterial strains, particularly in children.

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