Association Between Antibiotic Resistance and Integron Class2 Among Commonsal *Escherichia coli* Genotypic Groups

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الخلاصة:

العلاج بالمضادات الحياتية لا يؤثر فقط على البكتيريا المسببة للأمراض، ولكن يؤثر أيضا على الكائنات المجهرية المتعايشة في أمعاء الأنسان، والتي قد تكون بمثابة خزانات للجينات المقاومة للمضادات الحياتية. غالبا ما تعتبر الاشيريشيا القولونية البرازية (إي كولاي) كمؤشر جيد لتحديد الضغط الذي يفرضه استخدام المضادات الحياتية. الهدف من هذه الدراسة هو تحديد مجموعة التنميط الجيني للأي كولاي المتعايشة وتحديد وتيرة الانتكرون الصنف 2والمقاومة للمضادات الحياتية فيما بينها. كذلك الكشف عن علاقة الانتكرون الصنف 2 مع المقاومة للمضادات الحياتية (مقاومة لمضاد حياتي واحد أو اكثر) والتي قد تنقل أفقيا في المجتمعات البكتيرية. في هذه الدراسة تم عزل 301عزلة من الأي كولاي المتعايشة من براز أفراد أصحاء أعمارهم تتراوح من 1 الى 80عاما. و قد تم اختبار حساسية جميع العزلات ضد 16 مضاد حيوي في حين تم استخدام تفاعلات انزيم البلمرة المتسلسلة التقليدية للكشف عن الانتكرون الصنف 2 و تفاعلات انزيم البلمرة المتسلسلة المتعددة لتحليل التنميط الجيني. وأظهرت النتائج الحالية أن مجموعة B2 تمثل الغالبية العظمى من العزلات التي جمعت (63٪) تليها المجموعة A (23٪) وD (14٪) لكن لم يتم العثورعلى سلالات تنتمي إلى مجموعة B1. كما كشفت النتائج أن 10٪ من العزلات تمتلك الأنتكرون الصنف 2 والتي تعود بصورة رئيسية الى المجموعة B2. لوحظ مقاومة عالية بالنسبة لمعظم المضادات الحياتية خصوصا امبسلين والاموكسلين و لينكومايسين و سيفالكسين في حين كانت المقاومة لأميكاسين والجنتاميسين أقل شيوعا. مقاومة المضادات الحيوية ظهرت أساسا في العزلات الحاوية على الأنتكرون صنف 2. في الختام، ارتفاع معدل انتشار المقاومة للمضادات الحياتية خصوصا بين العزلات الحاوية على الانتكرون صنف 2 وهذا يشير إلى دور المتعايشة المعوية كمخزن ومصدر لمقاومة المضادات الحياتية.

Abstract

Antibiotic therapies can not affect only on the pathogenic bacteria, but also commensal microorganisms in the humans gut, which might serve as a reservoir of antimicrobial resistance genes. fecal *Escherichia coli* (*E. coli*) is often considered as a good indicator for selection pressure imposed by antimicrobial use. The goal of this study was to determine genotyping groups of commonsal *E. coli* and investigate the frequency of integrons class 2 and antibiotic resistance among them. So detect the association of integron class2 with antibiotic resistance (single and multiple drug resistance) that may be transport horizontally in bacterial populations. In this study 301isolates of commonsal *E. coli* were isolated from stools obtained from healthy individuals with age from 1to 80 years. All isolates were tested for their susceptibility against 16 antimicrobial agents and subjected to conventional polymerase chain reactions (PCR) for detection integrons and multiplex PCR for genotyping analysis**.** Present results showed that group B2 represent the majority of the collected isolates

(63%) followed by group A (23%) and D (14%) but no strains were found to belong to group B1. Also results revealed that 10% of isolates have integron class 2 that mainly related to genotypic group B2. High resistance observed for most antibiotics especially ampicilln, amoxcillin, lincomycin, cephalexin while resistance to amikacin and gentamicin was less common. Antibiotic resistance mainly appeared in integron positive isolates. In conclusion, high prevalence of antibiotic resistance especially among integron class 2 positive isolates that indicated role of gut flora as reservoir and source of antibiotic resistance.

Key words: Commensal *E. coli*, integron class 2, antibiotic resistance, multiple drug resistance

Introduction

Multidrug resistance to antibiotics among *Enterobacteriaceae* is of large concern in clinical settings and is a major public health issue. The consume of antimicrobial agents is the main selective force in the propagation and maintenance of antimicrobial resistance (1,2,3). Furthermore, the acquisition of resistance genes by horizontal transfer through plasmids and transposons plays an important role in the rife of resistance genes and in the development of multidrug resistance in bacterial populations (3,4). A substantial portion of commensal fecal flora in humans is resistant to antibiotics commonly used to treat bacterial infections. Commensal fecal floras are considered to be a potential reservoir for antimicrobial resistance genes and play an essential role in the ecology of antibiotic resistance of bacterial populations (8,16). Commensal *E. coli* have been exploited as sensitive indicators in the surveillance of antimicrobial resistance (3,5). Genotyping analysis have shown that *E. coli* strains divided into four main genotypic groups (A, B1, B2, and D) and that virulent extra-intestinal strains fundamentally related to group B2 and lesser extent to group D, whereas most commensal strains belong to group A and B1. Genotypic grouping was done based on the Clermont triplex PCR method using primers targeted three genetic markers, *chuA*, *yjaA* and TspE4.C2 (6). Commensal *E. coli* strains efficiently exchanging genetic materials with other bacteria such as *Shigella*, *Salmonella*, *Yersinia* and *Vibrio*, as well as pathogenic *E. coli* (7,8,9). Recently this exchange of many different and diverse genes responsible for antibiotic resistance have been correlated to genetic structures named integrons, that integrate and mobilize individual gene cassettes encoding antimicrobial resistance determinants (10,11). There are many classes of integron have been indicated and clarified by their respective integrase (*IntI*) genes. Class 2 integron (IntI2)were present among *Acinetobacter*, *Shigella*, *Escherichia coli* and *Salmonella* isolates (12) also may be detected in other bacteria in

subsequent studies. The class 2 integron is related to the class 1 integron (46% amino acid identity) and both these integrons are also present in resistant intestinal *E. coli* isolated from different hosts in the community (13,14). Unfortunately most studies on antimicrobial agents and resistance genes focused on pathogenic bacteria. Although the commensal bacteria are act as important reservoir of antibiotic resistant genes and they are capable of facilitating the pervasion of resistant genes to pathogenic strains. Also most genotypic studies on *E. coli* in Iraq and other countries carried out on pathogenic *E. coli* isolates which are considered as a causative agent for recurrent urinary tract infection and other diseases, and little attention was paid on the importance of the multiple antibiotic resistance among commensal *E. coli*. So we believed that under taking such studies is not only beneficial for providing new information, but also important for initiating the first step towards future studies.

Materials and Methods

Fecal sample collected from 301 healthy individuals with age from 1 to 80 years that including : health students, hygiene workers and officers and their family in College of Medicine of Al-Qadisiya University also samples collected from lab staff, physicians and their family of Al-Diwaniya teaching hospital. About 1g of stool is collected from each individuals at sterile containers and directly transported to lab. Cary-Blair transported media are used to preserve samples that not immediately bring to microbiology lad. The primarily identification of *E. coli* was done depending on cultures (culture on MacConkey agar plats than subculture on Eosin Methylene Blue agar) that confirmed by biochemical tests and microscopically examination with Gram's stain. After primary identification of *E. coli*, isolates were subjected to susceptibility testing by disc-diffusion method**.** Antibiotic resistance of 16 antibiotic discs (ampicillin, amoxicillin, amikacin, norfloxacin, streptomycin, trimethoprim, lincomycin, gentamicin, cephalexin, ciprofloxacin, nalidixic acid, chloramphenicol, cefotaxime, ceftriaxone, tetracyclin and oxytetracyclin) was determined. Molecular study, conducted by conventional PCR for amplification primer of conserved region of *IntI2* gene (forward: 5-GGTCAAGG ATCTGGATTTCG-3 and reverse: 5-ACATGCGTGTAAATCATCGTC-3) with 788bp fragment **(16).** The primer pairs that used for genotyping were *ChuA* (1:5 – GACGAACCAACGGTCAGGAT-3 and 2:5-TGCCGCCAGTACCAA AGACA-3), *YjaA* (1:5-TGAAGTGTCAGGAGACGC TG-3 and 2:5-ATGGAGAATGCGTTCCTCAAC-3), and *TspE4C2* (1:5-GAGTAATG TCGGGGCATTCA-3 and 2*:* 5-CGCGCCAACAAAGTATTACG-3) which generate 279bp, 211bp, and 152bp fragments respectively(15). **Statistical analysis:** Statistical analyses were performed by the Statistical Package for Social Sciences

version 17 for Windows software and Microsoft Excel 2010.Statistical analyses curried out by using $\chi 2$ test or Fisher's exact test. A *P* -value of <0.05 was considered to be statistically significant.

Results

Genotyping analysis and prevalence of integron class 2

Result of multiplex PCR (figure 1) showed that group B2 represent the majority of the collected isolates (190 isolates, 63%) followed by group A (69 isolates, 23%) and D (42 isolates, 14%) but no strains were found to belong to group B1(figure 2). *IntI2* gene seen in 30 (10%) of tested isolates (figures 3&4). Integron class 2 mainly demonstrated in group B2 (28 isolates, 15%) flowed by group A (2isolates, 3%) but not detected in any isolates related to group D as in figure (5).

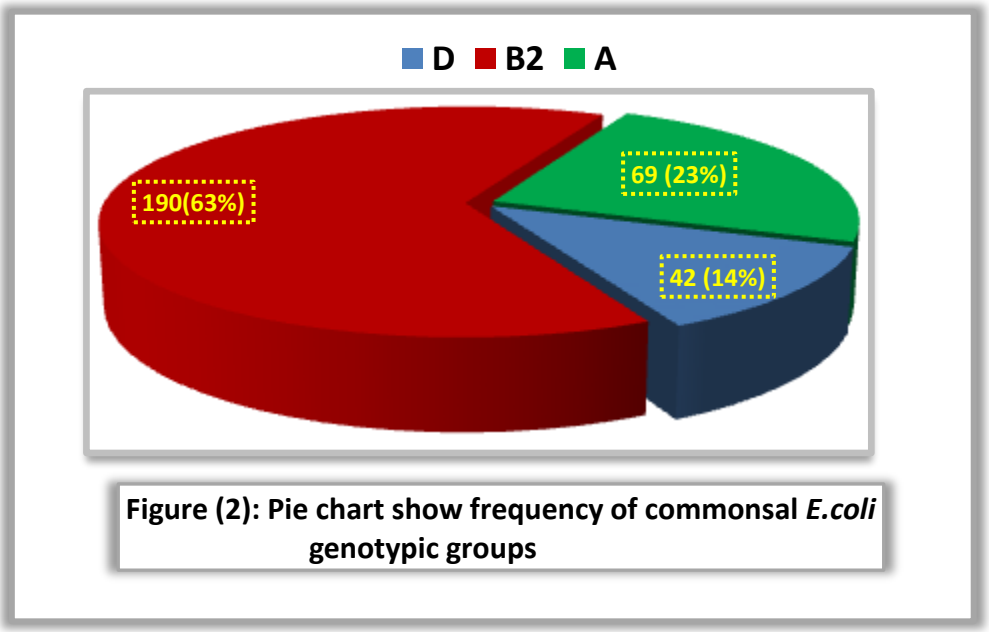

Figure (2): Pie chart show frequency of commonsal *E.coli* genotypic groups

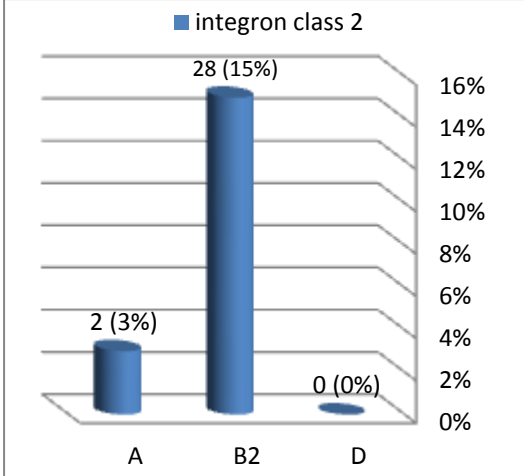

Figure (5) : distribution of integron class 2 among genotyping groups

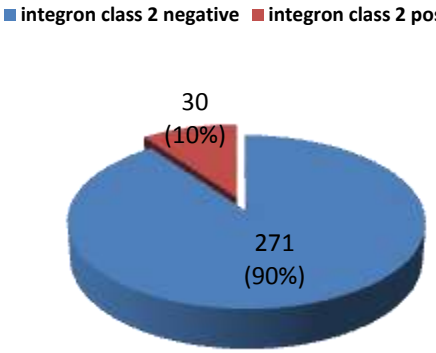

Figure (4): frequency of integron class 2 among commonsal *E. coli*

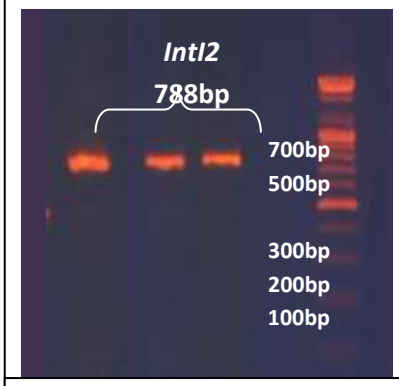

Figure (3): PCR amplification of *IntI 2* gene of commonsal *E. coli* isolates.

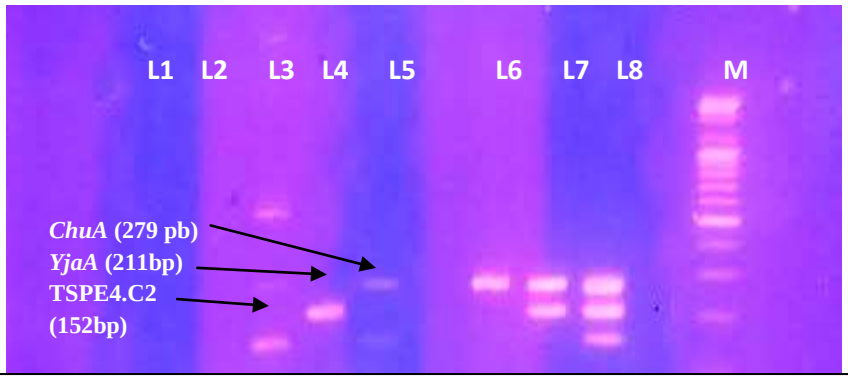

Figure (1): Triplex PCR profiles specific for *E. coli* phylogenetic groups. Each combination of *chuA* and *yjaA* gene and DNA fragment TSPE4.C2 amplification to detection phylogenetic groups and their subgroups of *E. coli* strain. Lines 1 and 2 represent group A (subgroup A0); lines 3 and 5 represent group D(subgroup D2), lines 4 include group A (subgroup A1) and line 6 represent group D (subgroup D1); lines 7 and 8 represent group B2 ($B2_2$ and $B2_3$ respectively). Line M contained ladder

High resistance of commonsal *E. coli* are observed against most tested antibiotic agents especially ampicillins (100%), amoxicillin (100%), cephalexin (100%), lincomycin (100%) and cefotaxime (93%). Compared antibiotic resistance of *IntI2* positive with *IntI2* negative isolates reflected that *IntI2* positive group A significant association with resistance to norfloxacin ($p = 0.0001$), ciprofloxacin ($p = 0.0001$), ceftriaxone ($p = 0.022$), amikacin ($p < 0.0001$), gentamicin ($p < 0.0001$), chloramphenicol ($p = 0.019$) and trimethoprim(0.004) while *IntI2* positive group B2 are significantly association with resistance to cefotaxime ($p = 0.001$), streptomycin ($p = 0.002$), tetracycline ($p < 0.0001$), chloramphenicol ($p = 0.0007$) and ceftriaxone ($p = 0.033$) (table 1).

Table (1): Relationship between *IntI2* gene and antibiotic resistance among genotypic groups.

Antimicrobial agent	Total (N = 301)	*IntI2* gene positive genotyping groups					
		A (N=2)			B2 (N=28)		
	R N(%)	R N(%)	X^2	P value	R N(%)	X^2	P value
Pencillines							
Ampicillin	301 (100)	2 (100)	2.992	0.091	28 (100)	0.052	0.770
Amoxcillin	301 (100)	2 (100)	2.992	0.091	28 (100)	0.052	0.770
Cephalosporin							
Cefotaxime	280 (93)	2 (100)	2.992	0.091	28 (100)	10.21	0.001
Ceftriaxone	188 (63)	2 (100)	2.46	0.022	14 (50)	6.601	0.033
Cephalexin	301 (100)	2 (100)	2.992	0.091	28 (100)	0.052	0.770
Quinolones							
Nalidixic acid	168 (56)	1 (50)	2.74	0.940	14 (50)	3.011	0.122
Norfloxacin	92 (31)	1 (50)	15.99	0.0001	9 (33)	0.0019	0.90
Ciprofloxacin	77 (26)	1(50)	18.49	0.0001	7 (25)	0.0488	0.995
Aminoglycsides							
Amikacin	42 (14)	1 (50)	55.08	< 0.0001	0 (0)	10.06	1.033
Gentamicin	64 (21)	2 (100)	55.08	<0.0001	5 (18)	3.225	0.111
Streptomycin	161 (53)	0(0)	0.972	0.559	14 (50)	2.10	0.002
Tetracyclines							
Oxytetracycline	201 (67)	2 (100)	4.255	0.088	20 (71)	0.112	0.908
Tetracycline	223 (74)	2(100)	4.255	0.097	28(100)	0.112	< 0.0001
Phenicol							
Chloramphenicol	105 (35)	1 (50)	6.14	0.019	14 (50)	6.133	0.0007
Anti-Folate							
Trimthoprim	202(67)	2 (100)	11.01	0.004	14 (50)	0.017	0.189
Lincosamid							
Lincomycin	301 (100)	2 (100)	2.942	0.085	28 (100)	0.052	0.770

N = Number; R = Resistance; Significant *P* - value < 0.05

All integrons positive isolates are resist to more than five antibiotics (table 2). On other hand 50% of *IntI2* gene positive group A resist to 15 and 13 antibiotics (P < 0.05) while *IntI2* gene positive group B2 significantly associated (P < 0.05) with resist to 6 (14%), 9(21%), 13 (25%) and 15 (14%) antibiotics. Many resistance patterns reported among integrons positive isolates and most common resistance patterns are AM-AX-L-CL and AM-AX-L-CL-CTX and by compared resistance patterns between integron positive and integron negative isolates, *P* -value are determent. Correlation between resistance patterns and *IntI2* gene positive genotypic groups are appeared mainly in group A and B2 (table 3). Integron class 2 positive group A significantly associated (p <0.05) with most resistance patterns except AM-AX-L-CL (P = 0.091), CTX-CRO-CL (P= 0.078), AX-CTX-T-TE-S-CL-TMP-AM-L (P= 0.630), AX-CTX-T-C-CRO-S-TE-CL-TMP-NA-L (P = 0.864), NA-CTX-CIP-T-C-CRO-S-

TE-NOR-TMP (P = 0.864), NA-CTX-T-CRO-S-TE-TMP (P = 0.630), AX-CTX-T-C-CRO-S-TE-CL-TMP-AM-L (P = 0.914) and AX-CTX-T-CRO-S-TE-CL-TMP-AM-L (P= 0.681). Integron class 2 positive group B2 significantly associated ($p < 0.05$) with resistance patterns; AM-AX-L-CL-CTX (P= 0.001), AM-AX-L-CL-CTX –TE (P< 0.0001), C-TMP-L (P = 0.0044), CTX-CRO-CL (P = 0.001), CRO- TE-CL-TMP (0.002), AX-CTX-T-C-CRO-S-TE-CL-TMP-NA-L (P = 0.0001), NA-CTX-CIP-T-C-CRO-S-TE-NOR-TMP (0.004), NA-CTX-T-CRO-S-TE-TMP (P = 0.0311) and T-TE-TMP (P = 0.0355) and AX-CTX-T-C-CRO-S-TE-CL-TMP-AM-L (P = 0.0001)

Table (2): Compared MDR between integron class 2 positive and negative genotypic groups

Number of antibiotics	Group A			Group B2		
	IntI2 positive (N=2)	*IntI2* negative (N=67)	*P* value	*IntI2* positive (N=28)	*IntI2* negative (N=162)	*P* value
	R N (%)	R N (%)		R N (%)	R N (%)	
4	0 (0)	0 (0)	NS	0 (0)	0 (0)	NS
5	0 (0)	9(13)	NS	0 (0)	11(7)	NS
6	0 (0)	14(21)	NS	4(14)	10 (6)	< 0.05
8	0 (0)	7(10)	NS	2 (8)	11(7)	NS
9	0 (0)	8(12)	NS	6(21)	10(6)	< 0.05
10	0 (0)	9(13)	NS	0 (0)	13(8)	NS
11	0 (0)	6(9)	NS	2 (7)	53(33)	NS
12	0(0)	6(9)	NS	0 (0)	21 (13)	NS
13	1(50)	8(12)	<0.05	7 (25)	11(7)	< 0.05
14	0 (0)	0 (0)	NS	3 (11)	22 (14)	NS
15	1 (50)	0 (0)	< 0.05	4 (14)	0 (0)	< 0.05

R= Resist; N = Number; NS= Non-Significant (P > 0.05)

Table (3): Distribution of MDR among *IntI 2* positive genotypic groups

Resistance patterns	A (N=2)		B2 (N=28)	
	R N(%)	P value	R N(%)	P value
AM-AX-L-CL	2 (100)	0.091	28(100)	0.770
AM-AX-L-CL-CTX	2 (100)	0.005	28(100)	0.001
AM-AX-L-CL-CTX-TE	2 (100)	0.463	28(100)	< 0.0001
AM-AX-L-CL-CTX-TE-NA	2 (100)	0.0907	14 (50)	0.111
AM-AX-TE-T	2 (100)	0.0491	19(68)	0.377
NOR-CIP-NA	2 (100)	0.0006	5 (18)	0.507
C-TMP-L	2 (100)	< 0.0001	14 (50)	0.0044
CTX-CRO-CL	2 (100)	0.078	21(75)	0.001
NOR-CIP-NA-CTX-CRO-CL	2 (100)	0.0002	5 (18)	0.422
AX-NA-CTX-T-CL-TMP-AM-L	2 (100)	0.0121	14 (50)	0.611
AX-CTX-T-TE-S-CL-TMP-AM-L	0 (0)	0.630	12 (43)	0.409
CRO-TE-CL-TMP	2 (100)	0.0121	19 (68)	0.002
AX-AM-CTX-CL-TE-T-CIP-NOR-AN-TMP-C-L-AK-CN-CRO	2 (100)	< 0.0001	0 (0)	0.822
AX-CTX-T-C-CRO-S-TE-CL-TMP-NA-L	0 (0)	0.864	7 (25)	0.0001
AX-CTX-T-CRO-TE-CL-TMP-NA-L	2 (100)	0.0002	9(32)	0.333
AX-CTX-T-CRO-TE-CL-TMP-L	2(100)	0.0121	14 (50)	0.645
NA-CTX-CIP-T-C-CRO-S-TE-NOR-TMP	0 (0)	0.864	7(8)	0.004
NA-CTX-T-CRO-S-TE-TMP	0 (0)	0.630	12 (43)	0.0311
CTX-T-CRO-TE-TMP	2 (100)	0.0121	14 (50)	0.663
T-TE-TMP	2 (100)	0.0213	16 (58)	0.0355
AX-CTX-T-CRO-TE-CL-AM-L	2 (100)	0.0031	14 (50)	0.617
AX-CTX-T-C-CRO-S-TE-CL-TMP-AM-L	0 (0)	0.914	7 (25)	0.0001
AX-CTX-T-CRO-S-TE-CL-TMP-AM-L	0 (0)	0.681	12 (43)	0.2113

***Abbreviations: Number (N), Ampicillin (AM), Amoxicillin (AX), Amikacin (AK), Gentamicin (CN), Streptomycin (S), Trimethoprim (TMP), Lincomycin (L), Norfloxacin (NOR), Ciprofloxacin (CIP), Nalidixic Acid (NA), Chloramphenicol (C), Cefotaxime (CTX), Ceftriaxone (CRO), cephalexin (CL), Tetracyclin (TE) and oxytetracyclin (T).**

Discussion

Genotyping analysis of this study reported that most commonsal *E. coli* strains related to group B2 flowed by A and lesser extent to group D and not revealed any isolate belong to group B1whereas numerous previous studies recorded that virulent extra-intestinal strains of *E. coli* mostly belong to group B2 and, to a lesser extent, to group D, while most commensal strains belong to group A and B1, this result can illustrate by studies of other researchers that found strains that

involved in commonsal *E. coli* genotypic groups not or less virulent in compared with strains that belong to pathogenic *E. coli* genotypic groups for example many of virulence factor detected in group B2 or A that cause UTI or diarrhea not determent in strains of group B2 or A that included in commonsal *E. coli* this mean that commonsal *E. coli* constituted from commonsal strains that associated with special properties regardless on genotypic analysis (17,18). Commonsal *E. coli* isolates in the current study mainly related to group B2 followed by group A and this similar to results of many studies in various populations (19,20,21,33). Massot and his coworkers remember the frequency of isolation of group B2 strains has increased with a parallel decrease of that of group A strains that may be a consequence of modifications in the lifestyle of the Parisian population that included changing food processing and hygiene procedures (33). Absent of strains of group BI in gut flora may be because gut conditions that not optical for their growth or may be those strains very sensitive for antimicrobial agents that used in extensive manner in this population and these results agreement with study in Tokyo in 2002 by Obata-Yasuoka *et al.*, who found B2 conformed 44% of commonsal *E. coli* isolates while B1 not detected in any isolate (0%) (22). Also study of Derakhshandeh *et al.*,(2013) in Iran include pathogenic and commonsal *E. coli* collected from different samples not found BI group but this results unlike other researches that found A and B1 groups conformed high frequencies among commonsal *E. coli* isolates (6,20,23). The differences in distribution of the genotypic groups among the strains of geographically distinct populations in different studies may be due to the health status of the host, geographic climatic conditions, dietary factors, use of antibiotics, host genetic factors, also some *E. coli* strains may also be primarily adapted to the gut conditions of certain populations (24). All these causes may be lead to dramatic shifts in the proportions of some groups (such as B2 &A) in many populations as France, Japan, Sweden and others (25). Therefore this study suggested that genotypic analysis used for molecular identification *E. coli* but not distinguishing between commonsal and pathogenic *E. coli*. Present results show increase dissemination of integron among commonsal *E. coli* isolates this may be because of the widespread of horizontal transfer of mobile elements such as plasmids and transposones that associated with integrons between commensal *E. coli* isolates and *Enterobacteriaceae* (3,28). In general present results showed high integron prevalence appeared in group B2 compared with other genotypic groups and this results unlike results of Skurnik *et al.*, (2005) who reported that *E. coli* B2 genotypic group strains tend to carry less integrons (*IntI1*gene (4%) and *IntI2* gene (0%)) than other genotypic groups in commensal environments (27). More ever misuse and spread resistance genes of pencillines, cephalosporines, lincomycin, tetracyclines and trimthoprim in human and animals associated with seen of high resistance

of these antibiotics among commonsal *E. coli* (26). All isolates in present study are multiple drug resistance isolates and are resist to at least four antibiotics but integron positive isolates are resist to at least six antibiotics this may be related to present many resistance genes associated with integrons or may be due to present more than one integron in the same isolate and each one carry out different resistance genes for different antibiotics. Current results in line with study of Phongpaichit *et al*., (2008) who found multiple drug resistance was more frequent in integron-positive isolates (89%) than those in integron-negative *E. coli* (57%)(7). Also integrons positive genotypic groups significantly associated with most resistance patterns particularly groups B2 and A that indicated role of integrons that found in plasmid or chromosome as major means for transporting and incorporating antibiotic resistance genes (29) and this results like previous study of Cocchi *et al*., (2007) that observed nonpathogenic commensal *E. coli* strains (mainly genotypic group A) represent an important reservoir of integrons, and consequently of multiple antibiotic resistance gene cassettes and this is in agreement with the hypothesis that virulent strains may acquire these factors from commensal strains and survive in an environment where a high antibiotic pressure is present (12,30). The present study detected isolates in genotypic groups possessing integrase gene and failed to express antibiotic resistance patterns and this findings agreement with some investigators that found antibiotic impressible *E. coli* and *salmonella sp* have particular resistance genetic cassettes within integron but the expression of that gene cassettes is weakling (7,31,32) and probability that these sensitive strains may not contain resistant cassette or the expression of their gene cassette is weak. Also, that may be a product of the interval between the promoter and the cassette that transcribe the gene cassettes. additional cause might be a weak promoters versions or mutation in the area between two special sequences of the promoter(31,32).

Conclusion

Current research showed the majority of commonsl *Escherichia coli* isolates related to B2 genotype that have highest container of integron class 2 among genotypic groups also present study reflected high resistant to most tested antibiotic agents and significant correlation between integron and many antibiotic resistances (single or multiple).

References

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