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First report on class 1 integrons and trimethoprim-resistance genes from *dfrA* group in uropathogenic *E. coli* (UPEC) from the aleppo area in syria

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Horizontal gene transfer (HGT) introduces advantageous genetic elements into pathogenic bacteria using tools such as class1 integrons. This study aimed at investigating the distribution of these integrons among uropathogenic *E. coli* (UPEC) isolated from patients in Aleppo, Syria. It also set to uncover the frequencies of the clinically relevant DfrA1 and DfrA17,7, as well as various associations leading to reduced susceptibility. This study involved 75 Trimethoprim-resistant *E. coli* isolates from in- and outpatients with urinary tract infections (UTIs) from 3 major hospitals in Aleppo. Bacterial identification, resistance and extended-spectrum-β-lactamase (ESBL) production testing were performed according to Clinical Laboratory Standards Institute guidelines. Detection of integrons and DfrA genes was done using PCR and statistical significance was inferred through χ^2 (Fisher's) test. Class1 integrons were detected in 54.6% of isolates while DfrA1 and DfrA17,7 were found in 16% and 70.6% of tested samples respectively. Furthermore, only DfrA17,7 were strongly associated with class1 integrons, as were reduced susceptibility to the majority of individual antibiotics, multidrug resistance and ESBL production. This study demonstrated the high prevalence of class1 integrons among UPEC strains in Aleppo, Syria, as well as their significant associations with MDR. This data give information for local healthcare provision using antibiotic chemotherapy.

Introduction

Urinary tract infections (UTIs) are among the most prevalent bacterial infections in humans. This constitutes a substantial financial and social burden on healthcare providers in developed countries such USA,[1] and even more so for developing countries. *Escherichia coli*—the most prominent member of the family of *Enterobacteriaceae*—is the number one cause of UTIs.[2] It is not uncommon for UTIs to be treated empirically with broad-spectrum antibiotics spurring more antibiotic resistance. The dissemination of resistance elements has been aided to a great extent by horizontal gene transfer. The latter process uses a number of biological tools, most notably of these tools are integrons.[3] Not only can integrons harbor a number of resistance gene cassettes in tandem, but also provide a local promoter for their transcription. Furthermore, integrons are capable of expanding their collection of promoterless gene cassettes through the actions of specialized site-specific recombination enzymes; intI. Therefore they operate as fully equipped site-specific recombination systems which can reside on other mobile genetic elements such as transposons and plasmids to horizontally transfer resistance encoding genes between bacterial species, particularly within the *Enterobacteriaceae* family.[4]

Trimethoprim was a widely-used and cheap antibiotic for treating UTIs, it inhibits the enzyme dihydrofolate reductase, which is involved in the cellular biosynthesis and growth. To neutralize this inhibition bacterial cells make use of modifications in the gene encoding dihydrofolate reductase (*dfr*) resulting in Trimethoprim resistance.[5] The association between integrons and bacterial resistance necessitates frequent identification and monitoring of integrons on the local level. Since improper use of antibiotics imposes higher levels of selective pressure, this type of epidemiological studies is most needed in developing countries such as Syria where antibiotics misuse is commonplace. With the total lack of data from our region, the objective of this study was to investigate the molecular epidemiology of integrons and certain resistance genes among isolates of uropathogenic *E. coli* in Aleppo, Syria. Additionally we set to uncover the level of association between MDR and ESBL production with the presence of integrons, thus providing the basis for better healthcare decisions in this context.

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Table 1. Antibiotic susceptibility phenotypes of tested isolates

Escherichia coli No. tested =75	Percent susceptible
Amoxicillin-Clavulanic acid (code CT0223B)	42.66
Cefepime (code CT0771B)	58.66
Ceftazidime (code CT0412B)	56
Cefotaxime (code CT0166B)	49.33
Nitrofurantoin (code CT0036B)	96
Piperacillin-Tazobactam (code CT0725B)	62.66
Aztreonam (code CT0264B)	61.33
Amikacin (code CT0107B)	90.66
Imipenem (code CT0455B)	100
Cefoxitin (code CT0119B)	81.33
Tigecycline (code CT1841B)	100
Ceftriaxone (code CT0417B)	48
Trimethoprim-Sulfamethoxazole (code CT0052B)	20
Ampicillin-Sulbactam (code CT0520B)	12
Tobramycin (code CT0056B)	50.66
Ciprofloxacin (code CT0425B)	66.66
Nalidixic acid (code CT0031B)	44
Tetracycline (code CT0054B)	44
Chloramphenicol (code CT0013B)	77.33

Results

Urine samples were collected from UTI patients during the study period in order to provide 104 unique isolates, which were studied to uncover antibiotic resistance phenotypes, as previously published by the current authors.[6] Out of the total number of tested isolates 75 (72.1%) were resistant to Trimethoprim, and only these were taken for further phenotypic and molecular investigations in this study. **Table 1** is an antibiogram that summarizes susceptibility patterns of Trimethoprim-resistant isolates. With Tigecycline and Imipenem eliciting zero resistance, and < 10% resistance was associated with each of Nitrofurantoin and Amikacin. The lowest rate of susceptibly was recorded with Ampicillin-Sulbactam (12%).

Class 1 integrons were detected in 41 out of 75 Trimethoprim resistant isolates, which amounts to 54.66%. Evidence of ESBL production was found in 46 isolates (61.33%), while 53 isolates (70.66%) could be classified as multidrug resistant. The highly significant association between carrying class 1 integrons and testing positive for ESBL production is shown in **Table 2**, which also displays a similarly significant association between multidrug resistance and class 1 integrons ($p < 0.0001$). 40 MDR isolates (out of 53) were found to be ESBL producers, and only six ESBL-producing *E. coli* isolates did not belong to the MDR category. Thus the association between the two characteristics is highly significant ($p = 0.0002$).

Upon investigating the presence of Trimethoprim-resistance genes *dfrA7,17* and *dfrA1* in the tested group we found that 53 isolates (70.66%) harbored *dfrA7,17* while *dfrA1* was detected in 12 isolate (16%) only. The presence of *dfrA7,17* genes was tightly linked to class 1 integrons ($p < 0.0001$), this was not the case with *dfrA1* as shown in **Table 2**. Only seven isolates (< 10%) harbored both genes simultaneously. A number of highly significant associations were found between decreased susceptibility to certain antibiotics and carrying the genes *dfrA7,17*. This was evident with Cephalosporins (Cefepime, Ceftazidime, Cefotaxime and Ceftriaxone), Tobramycin, Ciprofloxacin, Nalidixic acid and Trimethoprim/Sulfamethoxazole with p values = 0.01. No such associations were found with other tested antibiotics. The presence of *dfrA1* genes did not correlate significantly with decreased susceptibly to the antibiotics used in this study.

Very strong associations ($p < 0.0001$) were noted between resistance to Cephalosporins, Nalidixic acid, Ciprofloxacin and Trimethoprim/Sulfamethoxazole and the presence of class 1 integrons in tested isolates. In fact the same general trend was observed with the majority of commonly used antibiotics except for Chloramphenicol, Tetracycline and Ampicillin-Sulbactam (**Table 3**).

Discussion

Integrons can serve as a vital tool for bacterial survival against antibiotics because they offer a unique platform for assembling and expressing multiple genetic elements in the bacterial cell. Integrons are associated with in-house recombination/integration systems and equipped with a promoter for effective transcription. The structure of these genetic elements is very dynamic because it is affected by a number of factors that differ from one region to another, most importantly, antibiotics choice and misuse. Moreover, there is a severe paucity of data on integrons and related genetic elements from the Middle East in general; despite the high relevance of such information for a region where antibiotic surveillance is rarely practiced.

Four classes of integrons (1–4) have been identified so far, but the clinical significance of classes 2, 3 and 4 in the context of antibiotic resistance is dwarfed by that of class 1 integrons.[7] This study reports for the first time the prevalence of class 1 integrons in Aleppo, Syria to be 54.66% among UPEC isolates from in- and outpatients.

International information regarding integron frequencies vary between different geographical locations and clinical settings, with a majority of studies focusing on outpatients. One geographically comprehensive study has been conducted in 16 western European countries and Canada,[8] it detected class 1 integrons in 57.6% of tested Trimethoprim-resistant UPEC isolates from non-hospitalized patients. Despite the apparent similarity in the levels of class 1 integron frequencies, the European/Canadian figure is notably higher because it involves community-acquired infections only. Studies involving samples from hospitalized patients report higher levels of class 1 integrons, for example a Korean study[9] showed in 2004 that 69% of Trimethoprim-resistant isolates harbored class 1 integrons. Both studies seem to report higher frequencies than the current study, probably due to geographic and temporal differences.

Table 2. Association between susceptibility to individual antibiotics, ESBL and MDR status and the presence of class 1 integrons in uropathogenic *E. coli* isolates from Aleppo, Syria

Antibiotic resistance-related phentype		Number of isolates		Association with integrons (p value)[a]
		With class1 integrons	Lacking class 1 integrons	
Amoxicillin-Clavulanic acid	Susceptible	11	21	**0.0045**
	Non susceptible	30	13	
Cefepime	Susceptible	14	30	**<0.0001**
	Non susceptible	27	4	
Ceftazidime	Susceptible	12	30	**<0.0001**
	Non susceptible	29	4	
Cefotaxime	Susceptible	9	28	**<0.0001**
	Non susceptible	32	6	
Nitrofurantoin	Susceptible	41	31	**0.0091**
	Non susceptible	0	3	
Piperacillin-Tazobactam	Susceptible	21	26	**0.318**
	Non susceptible	20	8	
Aztreonam	Susceptible	17	29	**0.0012**
	Non susceptible	24	5	
Amikacin	Susceptible	34	34	**0.014**
	Non susceptible	7	0	
Cefoxitin	Susceptible	28	33	**0.002**
	Non susceptible	13	1	
Ceftriaxone	Susceptible	9	27	**<0.0001**
	Non susceptible	32	7	
Trimethoprim-Sulfamethoxazole	Susceptible	1	14	**<0.0001**
	Non susceptible	40	20	
Ampicillin-Sulbactam	Susceptible	2	7	0.0701
	Non susceptible	39	27	
Tobramycin	Susceptible	13	25	**0.0005**
	Non susceptible	28	9	
Ciprofloxacin	Susceptible	17	33	**<0.0001**
	Non susceptible	24	1	
Nalidixic acid	Susceptible	7	26	**<0.0001**
	Non susceptible	34	8	
Tetracycline	Susceptible	14	19	0.0669
	Non susceptible	27	15	
Chloramphenicol	Susceptible	28	30	0.0535
	Non susceptible	13	4	
dfrA1	Present	5	7	0.3594
	Absent	36	27	
dfrA7,17	Present	39	14	**<0.0001**
	Absent	2	20	
ESBL status	Producers	34	12	**<0.0001**
	Non-producers	7	22	
Multidrug resistance status	MDR	41	12	**<0.0001**
	Non-MDR	0	22	

[a]p values indicating significance are in bold.

Table 3. Association between susceptibility to individual antibiotics and the presence of *dfrA17,17* in 75 uropathogenic *E coli* isolates from Aleppo, Syria

Antibiotic		Number of isolates		Association with genes (p value)[a]
		Dfra7,17 +ve	Dfra7,17 –ve	
Amoxicillin-Clavulanic acid	Susceptible	19	13	0.077
	Non susceptible	34	9	
Cefepime	Susceptible	23	21	**p < 0.0001**
	Non susceptible	30	1	
Ceftazidime	Susceptible	23	19	**0.0007**
	Non susceptible	30	3	
Cefotaxime	Susceptible	18	19	**p < 0.0001**
	Non susceptible	35	3	
Nitrofurantoin	Susceptible	52	20	0.2041
	Non susceptible	1	2	
Piperacillin-Tazobactam	Susceptible	30	17	0.1189
	Non susceptible	23	5	
Aztreonam	Susceptible	28	18	0.0213
	Non susceptible	25	4	
Amikacin	Susceptible	46	22	0.0980
	Non susceptible	7	0	
Cefoxitin	Susceptible	41	20	0.2101
	Non susceptible	12	2	
Ceftriaxone	Susceptible	18	18	**0.0003**
	Non susceptible	35	4	
Trimethoprim-Sulfamethoxazole	Susceptible	3	12	**p < 0.0001**
	Non susceptible	50	10	
Ampicillin-Sulbactam	Susceptible	5	4	0.4350
	Non susceptible	48	18	
Tobramycin	Susceptible	34	4	**0.0003**
	Non susceptible	19	18	
Ciprofloxacin	Susceptible	29	21	**0.0004**
	Non susceptible	24	1	
Nalidixic acid	Susceptible	14	19	**p < 0.0001**
	Non susceptible	39	2	
Tetracycline	Susceptible	23	10	1.0000
	Non susceptible	30	12	
Chloramphenicol	Susceptible	37	21	0.0161
	Non susceptible	16	1	

[a]p values indicating significance are in bold.

With total lack of comparable research in Syria the closest point of reference geographically and temporally would be a relatively recent study from Lebanon.[10] According to the latter study 30% of UPEC isolates from two hospitals in Lebanon showed evidence of class 1 integrons, and nearly all these isolates (96.7%) were resistant to Trimethoprim/Sulfamethoxazole. The prevalence of integrons in Gram negative bacilli in northwestern Turkey was investigated by Sandalli et al.[11] with 27 integron-positive isolates out of 72 community-acquired *E. coli* infections (urinary and otherwise). A lower level of integron prevalence was reported by an Iranian study[12] which detected integrons in 16.6% of isolates from children with UTIs, and only less than half of these isolates carried *intI1*. This low prevalence may be due to limiting the study to non-hospitalized children from a sparsely urbanized locality (Jahrom, Iran). Interestingly an up-to-date study from the same country reported a much higher rate of integrons reaching 50.3% in isolates from UTI patients,[13] which is quite comparable to the data presented in this study. However, there are many caveats in formulating solid conclusions about such country to country differences due to diverse sampling strategies which can result in highly variable frequencies.

This study focused in particular on the most commonly encountered Trimethoprim-resistant genes in the context of medical practice; *dfrA*1 and *dfrA7,17*. The frequencies of *dfrA*1 and *dfrA7,17* were found to be 16% and 70.66% respectively. As with integron prevalence values; different studies gave diverse accounts from different parts of the world. Our results regarding the higher prevalence of *dfrA7,17* over *dfrA*1 echoed many findings in a number of similar studies from Lebanon,[10] Denmark, The Netherlands,[8] Korea[9] and Australia.[14] Conversely, *dfrA*1 appeared to be more prevalent than *dfrA7,17* in Spain, Portugal, France, Belgium[8] and Turkey.[11] These differences can be attributed to a number of factors ranging from diverse sampling schemes to the genetic drift affecting horizontally transferred resistance genes.

The extremely high correlation between reduced susceptibility to individual antibiotics, MDR and ESBL production on one side and harboring integrons on the other in Aleppo corroborated well with the results from several international studies, albeit to a lesser extent. Examples include Fallah et al.[13] and Farshad et al.[12] from Iran, and Mathai et al.[15] from southern India. In fact all integron studies reported strong association between class 1 integrons and antibiotic resistance genes such as *dfrA* and *aadA* genes.[10,16,17] Thus decreased susceptibility to antimicrobials is likely to be the result of antibiotic resistance genes being carried along the same vectors (transposable elements and conjugative plasmids) as integrons. The high significance of the correlation between low susceptibility to Cephalosporins and presence of class 1 integrons in this study probably reflects the widespread misuse of this class of antibiotics in Syria. Thus detecting class 1 integrons can have a predictive value of co-resistance to antibiotics in this context. Ampicillin-Sulbactam was the only β-lactam that did not show decreased susceptibility with integrons; however this is likely to be caused by the extremely high resistance to this agent so that any changes in susceptibility would be practically undetectable. Susceptibility to Tetracycline and Chloramphenicol did not change significantly with presence of class 1 integrons, because the corresponding resistance genes may have been lost from integron-carrying plasmids due to their limited use in Syria.

In conclusion, this study presents unprecedented data about the frequency of class 1 integrons in the Aleppo governorate in Syria along with *dfrA*1 and *dfrA7,17* that mediate Trimethoprim resistance. There is urgent need for expanding this type of investigations into the molecular epidemiology of genetic elements underlying antibiotic resistance in this part of the world. Additionally more effort is required for disseminating this information locally and internationally and formulating relevant guidelines accordingly in order to attain better levels of healthcare provision.

Materials and Methods

Clinical isolates. This study was conducted at three university hospitals in Aleppo, Syria from September to November 2011. It involved 75 UTI patients; 26 men and 49 women. Non-repetitive isolates were collected from these patients and they have been selected from a larger group on the basis of being Trimethoprim resistant. The tested cohort consisted of 43 outpatients and 32 inpatients, of the latter 8 were catheterized, and all obtained isolates were non-repetitve. Patients' medical history was obtained to infer hospitalization status (inpatients being hospitalized for ≥ 48 h). Written informed consent was obtained from patients who provided the samples. The study protocol including the consent procedure was approved by the scientific council and the Ethical Committee at the University of Aleppo. The diagnosis of UTIs was based on microscopic findings of > 5 White Blood Cells/high power field and a colony count of 10^5 CFU/ml of a single pathogen using standard procedures.[18]

Table 4. Sequences of primers used for detecting antibiotic resistance determinants in this study

Target	Primer name	Primer sequence
dfr1	dfr1-forward	5'-TGG TAG CTA TAT CGA AGA ATG GAG-3'
	dfr1-reverse	5'-TAT GTT AGA GGC GAA GTC TTG GGT A-3'
dfr7,17	dfr7,17-forward	5'-ACA TTT GAC TCT ATG GGT GTT CTT C-3'
	dfr7,17-reverse	5'-AAA ACT GTT CAA AAA CCA AAT TGA A-3'
class 1 cassette regions	Hep58 (forward)	5'-TCA TGG CTT GTT ATG ACT GT-3'
	Hep59 (reverse)	5'-GTA GGG CTT ATG CAC GC-3'

Phenotypic study. Urine samples were inoculated onto Nutrient and MacConkey agar with 0.001 ml calibrated loops by a semi-quantitative technique. *Escherichia coli* was identified by conventional biochemical tests[18] using mini API® ID32E system (BioMerieux, 32400). The organisms were maintained at (–80°C) in glycerol stocks. Antimicrobial susceptibility testing was performed by standard disc diffusion method on Mueller-Hinton agar as recommended by the guidelines of Clinical and Laboratory Standards Institute (CLSI).[19] Nineteen antibiotics were used (Oxoid, codes are listed in **Table 1**) as well as Trimethprim (Oxoid, CT0076B). Multi-drug resistance (MDR) was defined as resistance to 3 or more unrelated antibacterial agents. ESBL production was determined using disc diffusion method and double disc synergy diffusion test (DDST) according to CLSI guidelines.[19] We used (*E. coli* ATCC 25922) as a negative control and (*E. coli* ATCC 35218) as a positive control.

PCR amplification. Bacterial DNA was extracted from a single *E. coli* colony using QIAprep Spin Miniprep Kit (QIAGEN GmbH, 27104) according to manufacturer's instructions, then stored at –20°C as a template DNA stock. Class 1 integrons were amplified using the primers hep58 and hep59 as described.[20] While the primer pairs [dfr1-f, dfr1-r] and [dfr7&17-f, dfr7&17-f] (VBC-Biotech, custom primers) were used for the detection of *dfrA1* and *dfrA7,17* respectively, according to Grape et al.[21] **Table 4** represents primer sequences in full.

Statistical analyses. The statistical Package for the Social Sciences (SPSS) version 19.0 was used. And the significance of associations were established using the Fisher's exact test ($p < 0.05$ was considered significant).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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