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Characterization of antibiotic resistance integrons harbored by Romanian *Escherichia coli* uropathogenic strains

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Abstract

Because little is known about the integrons which constitute an important means of spreading resistance in bacteria circulating in Romania, this study aimed to detect antibiotic resistance gene cassettes embedded in integrons in a convenient collection of 60 ciprofloxacin-resistant Escherichia coli isolates of various phylogroups, associated with community-acquired urinary tract infections. Characterization of the integrons was accomplished by PCR, restriction fragment length polymorphism typing, and DNA sequencing of each identified type. More than half of the tested E. coli strains were positive for integrons of class 1 (31 strains) or 2 (1 strain). These strains derived more frequently from phylogenetic groups A (15 of 21 strains), B1 (10 of 14 strains), and F (3 of 4 strains), respectively. While 20 strains carried class 1 integrons which could be assigned to nine types, eleven strains carried integrons that lacked the 3'-end conserved segment. The attempts made to characterize the gene cassettes located within the variable region of the various integrons identified in this study revealed the presence of genes encoding resistance to trimethoprim, aminoglycosides, beta-lactams or chloramphenicol. The evidence of transferable resistance determinants already established in the autochthonous E. coli strains highlights the need for improved control of resistance-carrying bacteria.

Keywords: *Escherichia coli, urinary tract infections, integrons, antibiotic resistance*

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Introduction

Reports from all over the world indicate an increasing number of infections caused by bacteria with resistance to the antimicrobial agents currently used in medical practice (1-3). *Escherichia coli* is one of the species able to adapt to diverse environments and pressures appearing at their level, including those resulted from the use of antibiotics. Over the years, *E. coli* members have evolved a complex arsenal of strategies to withstand the action of various antibiotics (4-5).

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A significant proportion of the genes encoding the mechanisms underlying these strategies are part of small mobile genetic elements called gene cassettes. Integrons are genetic platforms capable of incorporating such cassettes by site-specific recombination and providing a promotor for the expression of the antibiotic resistance determinants contained in them. They gain mobility by association with transposons or plasmids and, thereby, play a major role in the spread of the antibiotic resistance genes by horizontal gene transfer (6-7).

Multi-country evidence of the widespread integron-associated resistance and of the prominent role of class 1 integrons in creating novel combinations of resistance genes in *E. coli* population has been gathered (8-10). However, almost no information regarding these aspects is available from Romania even though it is one of the European countries with excessive antibiotic consumption and high levels of antimicrobial resistance (https://ecdc.europa.eu/en/publications-data/ecdc-country-visit-romania-discuss-antimicrobial-resistance-issues). This study aimed to provide a summary indication of the presence and the variety of integron-associated antibiotic resistance genes harbored by the Romanian *E. coli* strains in order to shed light on their profile otherwise invisible in the international pool of data on integrons. The research was carried out on *E. coli* isolates with resistance to fluoroquinolones, the most frequently expressed resistance phenotype identified among the invasive *E. coli* reported by Romania to the European Antimicrobial Resistance Surveillance Network (EARS-Net) (European Centre for Disease Prevention and Control. Surveillance of antimicrobial resistance in Europe – Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017. Stockholm: ECDC; 2018).

Material and methods

Strain collection

The study took advantage of a convenient collection of 60 *E. coli* strains resistant to ciprofloxacin and susceptible to extended-spectrum cephalosporins, collected between 2014-2015, that were provided anonymously by a private clinical laboratory from the community. The strains originated from the urine specimens of non-hospitalized adults and had already been explored for the molecular basis of the fluoroquinolone resistance (mutations in chromosomal *gyrA*, *gyrB*, *parC* genes and presence of plasmid-borne *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr* genes), phylogenetic background and ST131 status (11). The collection comprised strains derived from the phylogenetic group A (21strains), B1 (14 strains), B2 (10 strains), C (8 strains), D (3 strains), and F (4 strains). Of note, two *E. coli* strains that were assigned to phylogroups A and B1, respectively had been identified as *qnr*-positive. Additionally, two strains belonging to phylogenetic groups B2 and C harbored the *cr* variant of *aac(6')-Ib* gene coding for aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin. Regarding the B2 phylogenetic group strains, 9 strains were members of the O25b/ST131 clone.

PCR detection and characterization of class 1, class 2, and class 3 integrons

To determine whether they carried integrons, the *E. coli* strains were PCR screened for the integrase genes *intI1*, *intI2,* and *intI3* with published primers designed to amplify conserved regions of the respective genes (12). The template DNA used in the PCR assays was prepared from cell suspensions resulted from overnight culture suspended in 200 µl of sterile distilled water, boiled for 15 minutes, and centrifuged.

For the characterization of the variable regions of the class 1 and 2 integrons, all the integrase-pos-

itive *E. coli* strains detected were further subjected to PCR and sequencing assays.

In the PCRs performed for the *intI1*-positive *E. coli* strains primers corresponding to the 5'- and 3'- conserved segments (5' and 3'CS) of the integrons were used, as described by Levesque et al. (13). For class 2 integrons, the PCR was performed with the primers attI2-F and orfX-R, as described by Machado et al. (14).

Additional primers (15-16) of which some designed for this study (17) were used for the amplification of the variable regions if the integrase-positive strains were found negative in the PCR assays with the 5'CS and 3'CS primers. All the primers used in this study are listed in Table 1. The JL-D2 primer paired with the 5'CS standard primer was used for the case of the insertion of IS*26* which presumably caused the deletion of the typical 3'CS region (18). Three nested sequence-specific primers corresponding to the *intI1* gene, 5'CS region, and *attI1* integron-integration site were paired with short arbitrary degenerate primers (i.e. AD1, AD2, and AD3) previously used by Liu et al. in a thermal asymmetric interlaced (TAIL)-PCR protocol (19). Briefly, in the initial PCR amplification of this protocol the specific primer used was intI1 primer paired with each of the AD primers. The secondary reaction used the primary PCR product with the nested 5'CS-specific primer while in the third reaction, the specific product was favored to amplify using the *attI*-specific primer.

For the preliminary typing of integrons, the gene cassette regions amplified using the standard

Table 1. Primers used in this study to identify and to characterize the integrons of ciprofloxacin-resistant *E. coli* strains with urinary origin

Primer	5'-3'sequence	Reference
Int1 - F	GTTCGGTCAAGGTTCTGG	(12)
Int1 - R	CGTAGAGACGTCGGAATG	
Int2 - F	CAAGCATCTCTAGGCGTA	
Int2 - R	AGAAGCATCAGTCCATCC	
Int3 - F	CATCAAGCTGCTCGATCA	
Int3 - R	ACAACTCTTGCACCGTTC	
5'CS	GGCATCCAAGCAGCAAG	(13)
3'CS	AAGCAGACTTGACCTGA	
attI2 - F	GACGGCATGCACGATTTGTA	(14)
orfX - R	GATGCCATCGCAAGTACGAG	
aad1 - 68F	ATCTCGAACCGACGTTGC	This study - Gene Runner v. 6.5.51 (17)
ISUnCu1 - 519F	CCGTCTGCTGCATATCGTC	This study - Gene Runner v. 6.5.51 (17)
aadA1-162F	GACCGTAAGGCTTGATGAAAC	This study - Gene Runner v. 6.5.51 (17)
cml - 599R	AGCTGCGACCATTGCAAGC	This study - Gene Runner v. 6.5.51 (17)
oxa30 - 305F	TGGAGATCTGGAACAGCAATC	This study - Gene Runner v. 6.5.51 (17)
aadA1 - 229R	AGAATCTCGCTCTCTCCAGG	This study - Gene Runner v. 6.5.51 (17)
IS26 - F	CGCATCACCTCAATACCTT	(17)
att1 - F	TTATGGAGCAGCAACGATGT	(16)
qacH - 183 F	AAATCCAAGCAATAGCTGCC	(15)
cmlA1 - 863F	TGCAACAGTCGTGCTCGGTC	This study - Gene Runner v. 6.5.51 (17)
AD1	NTCGASTWTSGWGTT	(19)
AD2	WGTGNAGWANCANAGA	
AD3	AGWGNAGWANCAWAGG	

5'CS and 3'CS primers were subjected to restriction fragment length polymorphism (RFLP) with *Pst*I endonuclease. Identical RFLP patterns obtained for amplicons with the same size were considered to be indicative of similar gene cassette content.

Characterization by sequencing of a representative PCR product of each distinct class 1 or class 2 RFLP-type integrons was further performed assuming that the remaining strains have the same gene cassette arrangement as those selected for sequencing. The amplicons were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and subjected to sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (ThermoFisher Scientific) and the same primers used for PCRs (5'CS/3'CS and attI2/orfX primers). To complete the double strand sequencing of amplicons >2000 bp, additional specific primers were used (Table 1). Sequencing of the DNA fragments corresponding to the variable regions of the atypical integrons was also performed with the specific primers used for the PCR amplification. All sequencing reaction products were purified with the DyeEx 2.0 Spin kit (Qiagen) to remove unincorporated dye terminators prior to capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems).

DNA sequence similarity searches were carried out against sequences deposited in the GenBank database of the National Centre for Biotechnology Information via the Blast network service (http://www.ncbi.nlm.nih.gov) and in the Repository of Antibiotic resistance Cassettes (20).

Results

Integron carriage in uropathogenic E. coli strains and their association with phylogenetic groups

The PCR screening of the presence of integrase genes showed that 31 of 60 strains were positive for class 1 integron-associated integrase *intI1* and only one strain carried *intI2* gene indicative of class 2 integrons. None of the urinary strains carried both class 1 and class 2 integrases and also there was no *intI3*-positive strain identified. Of note, the *E. coli* strains with *qnr* or *aac(6')-Ib-cr* genes were not among the integrase-positive strains. The *intI*-positive strains belonged to various phylogenetic groups. Specifically, the *intI1*-bearing strains belonged to phylogenetic groups A (14 of 21 strains), B1 (10 of 14 strains), B2 (1 of 10 strains), C (2 of 8 strains), D (1 of 3 strains), and F (3 of 4 strains) while the *intI2*-positive strain was derived from the phylogenetic group A.

Characterization of E. coli class 1 and 2 integrons

For 20 (64.5%) *intI1*-positive *E. coli* strains, the PCR products used for the analysis of gene cassettes were obtained using the standard 5'CS/3'CS primers.

The 5'CS/3'CS PCR products ranged in size from approximately 600 to 3250 bp (Table 2a). Their subsequent *PstI* RFLP analysis distinguished 9 distinctive types, designated I to IX in descending order of size. Integrons assigned to RFLP types I, III, IV, VII, VIII and IX were identified in single strains. RFLP types II, V, and VI integrons were shared by several strains: type V by 9 strains, type VI by 3 strains, and type II by 2 strains, respectively.

For 11 (35.5%) *intI1*-positive strains, no amplicon could be obtained with the standard primer pair, the variable regions being characterized by pairing the 5'CS primer with other specific primers, selected on the basis of the known integron sequences included in GenBank or by using the TAIL-PCR approach with arbitrary primers. Following the DNA sequencing of these strains, insertion sequences (i.e. IS1, IS26), *mobC* or *qacH* genes were found downstream of the variable re-

gions. Nonetheless, in one strain the variable region failed to be amplified, despite several PCR variants performed (Table 2b).

Sequencing analysis performed to characterize typical and atypical integrons showed that with the exception of the typical integron assigned to RFLP type IX which carried only an *estX* gene cassette, the rest were linked to antibiotic resistance and carried at least one gene cassette conferring resistance to trimethoprim (i.e. *dfrA1*, *dfrA5*, *dfrA7*, *drfA12*, *dfrA14*, *dfrA16*, and *dfrA17*), aminoglycosides (i.e. *aadA1*, *aadA1a*, *aadA2*, *aadA5*, *aadA22*, and *aadB*), beta-lactams (i.e. *blaOXA-30*, *blaPSE-1*) or chloramphenicol (i.e. *cmlA1*, *cmlA6*). Insertion sequences (i.e. IS1, IS26, ISUnCu1) were found in several integrons.

Overall, on the basis of the sequenced integrons the aminoglycoside adenylyltransferase genes *aadA* were inferred to be the most common gene cassettes embedded in integrons while the *dfrA* genes coding for dihydrofolate reductases type A were second in prevalence. The combination of *dfrA17* and *aadA5* cassettes was inferred to be common among the Romanian *intI1*-positive strains. More complex cassette arrays were also detected. Specifically, the configuration *dfrA16-bla*PSE-1-*aadA2-cmlA1-aadA1* adjacent to *qacH* gene was present in one of the integrons whose variable region could not be amplified

Table 2. Characteristics of the class 1 integrons detected in Romanian ciprofloxacin-resistant *E. coli* strains with urinary origin

a) typical integrons

No. of isolates/ Phylogenetic background	Approximate size of 5'CS-3'CS PCR products (bp)	Variable region content
1/A	3250	*aadB – aadA1a-2R*[1)] *– cmlA6*
2/B1	3000	*aadA1/aadB*[2)] *– aadA1a –ISUnCu1*
1/C	2000	*blaOXA-30 – aadA1a*
1/A	1850	*dfrA12 – orfF – aadA2*
6/A, 2/B1, 1/F	1700	*dfrA17 - aadA5*
1/A, 1/B1, 1/F	1600	*dfrA1 – aadA1a*
1/A	1000	*aadA22*
1/B1	750	*dfrA7*
1/C	650	*estX*

1) aadA1a cassette with aadA2R spacer; 2) nucleotides 1-17 of aadA1 cassette followed by nucleotides 2-end of aadB cassette

b) atypical integrons

No. of isolates/ Phylogenetical background	Downstream of the variable region	Variable region content
1/A	IS26	*dfrA5*
1/B1	IS26	*dfrA14*
2/A	IS26	*dfrA14*
2/A	*mobC*	*dfrA14*
1/B2, 1/F	IS1	*dfrA17*
1/B1	IS26	*dfrA17-aadA5*
1/B1	*qacH*	*dfrA16-blaPSE-1-aadA2-cmlA1-aadA1*
1/B1	-	-

using the standard 5'CS/3'CS primers. Of note, two hybrid gene cassettes formed between *aadA1* and *aadB*, and *aadA1* and *aadA2* cassettes, respectively were also identified. Also, insertion sequences IS26 elements identified downstream of the variable regions of atypical integrons were found to have different orientations.

Table 2 gives an overview of the representative gene cassette arrays identified in the class 1 integrons characterized in this study. Complete gene sequences of gene cassette arrays from this study were deposited in GenBank (accession no. MH208290 to MH208305).

Regarding the class 2 integron detected, the amplicon of about 2200 bp generated for its variable region harbored *dfrA1-sat2-aadA1* configuration.

Discussion

In 2017, the ECDC team that conducted a visit to Romania to specifically discuss and assess the situation regarding prevention and control of antimicrobial resistance concluded that the levels of antimicrobial resistance were a serious concern of this country, recommending to the national authorities to designate antimicrobial resistance as a national public health threat encompassing all regions (https://ecdc.europa.eu/en/publications-data/ecdc-country-visit-romania-discuss-antimicrobial-resistance-issues). The recommendation to increase the capacity of the microbiology laboratory was also made considering both the better orientation of treatment strategies at national level and the national contribution to the coherent and meaningful surveillance of resistance at European level.

Aiming to deliver much better laboratory data in order to fill some of the information gaps concerning the pathogens reported in the Romanian population, we extended the investigation of *E. coli* strains collected from UTIs to demonstrate the presence of integron-like structures with resistance gene cassettes embedded in them. All the urinary strains sampled shared the susceptibility to third generation cephalosporines but not to ciprofloxacin, the first-line antibiotic preferred by the Romanian practitioners for non-complicated UTI episodes. Also, 30% of them were resistant to gentamycin and twice as much to the combination trimethoprim-sulfamethoxazole.

For this study, the algorithm for integron detection and characterization relied on the use of molecular tools found to be appropriate and successful in studying integrons in conjunction with the search of publicly available comprehensive DNA sequence information. The presence of integrons was initially screened through their most distinctive components, the integron-integrases, the strains which contained integrase-encoding genes being recorded as integron-bearing strains. To validate if they carried integrons encoding antibiotic resistance, these strains were subjected to a structural analysis of the integrons performed in the following way: PCR amplification to confirm the presence of gene cassettes in the integron hot-spot insertion regions, amplicon endonuclease restriction to ascertain whether the amplified variable regions of the same size had the same genetic content and thus delineate integron types, nucleotide sequencing to identify the integron-associated resistance genes.

Integrons containing genes involved in diverse resistance mechanisms were found in 53% of these strains and class 1 integrons made up the majority of them, which is in concordance with previous studies, emphasizing the high prevalence of integrons in *E. coli* isolates with clinical origins, including UTIs, and indicating class 1 integrons as predominant (21-22). The substantial proportion of class 1 integron-positive strains found in the Romanian strains also confirmed the high frequency of integron-carriers previously found across other fluoroquinolone resistant *E. coli* strains collected from different regions of the world (23-26). Although trans-

ferable plasmid-mediated quinolone resistance genes *qnr* and *aac(6')-Ib-cr* found embedded in the gene cassettes of integrons have been described, the relationship between the presence of integrons and resistance to fluoroquinolones in *E. coli* isolates has not been fully elucidated to date (27-29). In this study, the few strains positive for *qnr* or *aac(6')-Ib-cr* genes did not harbor integrons.

Regarding the content of the class 1 integrons identified in the Romanian strains, known and largely spread resistance gene cassettes were found and in general their presence correlated well with the phenotypic resistance. Similar to other reports on urinary *E. coli* strains, among the gene cassettes detected, those carrying genes for combined streptomycin/spectinomycin resistance (*aadA* genes) and trimethoprim resistance (*dfrA* genes) were most prevalent (22, 30-31). Moreover, the inferences drawn from the representative integron variants sequenced indicated *dfrA17 - aadA5* cassette array, which was significantly linked to human strains as the most common (32).

It is worth mentioning that more than one third of the class 1 integrons detected in this study had gene cassette arrays "non-amplifiable" with a very popular primer pair that has been used to identify the integron-associated resistance genes in many studies. Strains probably carrying truncated integrons lacking 3'-end-conserved segments were also described by others but their significance has not been clarified yet (33-36).

Regarding the association of integrons and *E. coli* phylogenetic groups, several studies reported different results. While some authors reported no significant differences in the distribution of integrons among the various phylogenetic groups described (22, 37), others indicated that integrons were more commonly associated with certain phylogroups, such as B2 (38-39) or D (40). Of the eight recognized *E. coli* phylogroups, six were represented in this study, but integrons were mostly found in the strains derived from the phylogroups F, A, and B1 although the former was rare overall.

Because of the limitations of this study consisting of the small number of strains investigated and the fact that inferences were made when assessing the diversity of integron variants, conclusions must be cautiously drawn. However, we consider that the study was a good choice for baseline characterization of community-acquired integrons. The evidence that a pool of transferable resistance determinants has already been established and disseminated across the autochthonous *E. coli* strains highlighted the need for improved control of the spread of resistance-carrying bacteria. In this context, the usefulness of more studies on molecular mechanisms of both resistance and resistance dissemination to complement the existent resistance surveillance data at national level is undeniable. Moreover, without the benefit of sharing high quality microbiological data it is difficult to generate a consistent map of the risks posed by the antibiotic resistant pathogens all over the world.

Authors' contribution

M.O. (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing –review & editing);

M.C.M (Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Writing – original draft;);

A.S.C (Data curation; Formal analysis; Investigation; Methodology; Resources; Writing – original draft;);

D.C (Data curation; Formal analysis; Investigation; Methodology; Resources; Writing – original draft;);

V.C. Data curation; Formal analysis; Investigation; Resources; Writing – original draft;);

C.R.U. (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Project administration; Writing – original draft; Writing –review & editing).

Conflict of interests

None to declare.

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