Antibiotic resistance profiling of pathogenic Enterobacteriaceae from Cluj-Napoca, Romania

Anca Farkas^{1,*}, Emma Tarco², Anca Butiuc-Keul³

Abstract

Introduction Members of the family Enterobacteriaceae are commonly identified in the clinical laboratory, being responsible for a substantial range of infections. This study aimed to investigate phenotypic and genotypic resistance traits in pathogenic Enterobacteriaceae isolated from outpatients in Cluj-Napoca, Romania.

Methods Pathogenic Enterobacteriaceae were isolated from urinary tract infections, wound infections and persistent diarrhea in a private laboratory from Cluj-Napoca, Romania. Bacterial strains were biochemically identified and subjected to antimicrobial susceptibility testing by disk diffusion. The carriage of antibiotic resistance genes and of class 1 integron were assessed by PCR.

Results *E. coli* and *Enterobacter* spp. were the most prevalent pathogens. High levels of resistance were observed against folate pathway inhibitors (74%), fluoroquinolones (49%) and penicillins (44%). The incidence of carbapenem resistance was 3%. The strains displaying phenotypic resistance were able to produce β -lactamase enzymes encoded by *bla*_{TEM}, *bla*_{TEM-1}, *bla*_{SHV-1} and *bla*_{CTX-M}, aminoglycoside modifying enzymes due to the carriage of *aac(3)-IIIa*, *aac(6)-II* and *aac(6)-Ie-aph(2'')*, to possess fluoroquinolones resistance due to *qnrS* DNA gyrase protection proteins and resistance to folate pathway inhibitors due to dihydropteroate synthases encoded by *sul1*, *sul2* and *sul3* genes. The high frequency of *int11* integrase was associated to sulphonamide resistance ($r=0.48$; $p<0.001$) and also to fluoroquinolone resistance ($r=0.27$; $p=0.011$), but no significant associations in the co-occurrence of specific antibiotic resistance genes and *int11* were found in pathogenic Enterobacteriaceae.

Conclusions An important proportion of pathogenic Enterobacteriaceae were multidrug resistant, due to a wide diversity of mechanisms encoding genetic resistance.

Keywords Antibiotic susceptibility, resistance genes, *int11*, urinary tract infections, wound infections, persistent diarrhea.

Introduction

Members of the family Enterobacteriaceae are commonly isolated in the clinical laboratory, being responsible for a variety of infections: enteric disease, urinary tract infections, wound and burn infections, bloodstream infections, pneumonia, ocular, ear and sinus infections. Various *Escherichia coli* serotypes, or strains

belonging to *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Proteus*, *Providencia*, *Salmonella*, *Yersinia* and other genera have long been responsible for a substantial percentage of nosocomial and community-acquired infections.¹

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Antimicrobial resistant (AMR) bacteria exert a great burden on healthcare systems, management of the infectious diseases becoming progressively complicated. In Romania, the incidence of multidrug resistant (MDR) Enterobacteriaceae reaches one of the highest levels, as indicated by the latest annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Recently, significant increases were observed for third-generation cephalosporin and aminoglycoside resistance, as well as for combined resistance to fluoroquinolones, aminoglycosides and third-generation cephalosporins.²

Antibiotic resistance genes (ARGs) can arise and spread rapidly in different bacteria. This way, AMR is more available than ever before to pathogenic organisms, narrowing the options of clinical medicine for drug regimens used to treat bacterial infection. The spread of particular ARG types or AMR strains can widely occur from patient to patient in hospitals, from person to person in the community or between the clinical settings and the environment.³

Gram-negative bacteria possess naturally occurring resistance mechanisms, such as the chromosomally mediated β -lactamases. TEM-type are the first plasmid-mediated ARGs. The genes *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} are frequently found in Enterobacteriaceae. A variety of extended-spectrum β -lactamases (ESBLs) arise by mutations of the parent genes and of other common genes. VIM- and NDM-type metallo- β -lactamases, OXA-48 and KPC appear as the most successful enzymes and may threaten the efficacy of carbapenems in the near future.⁴

The combination of mutagenesis with the ability of ARGs to transfer as part of integrons, gene cassettes, transposons, or integrative conjugative elements results in the capacity of resistance genes to virtually reach all bacterial types.⁵ In Gram-negative bacteria, particularly the Enterobacteriaceae, ARGs and associated mobile elements contained by plasmids often cluster together in large multiresistance regions.⁶ More recently, genetic determinants conferring resistance to fluoroquinolones, aminoglycosides and sulphonamides, such as the *qnr*, *aac(6')*-*Ib-cr*

and *sul* genes were found to be associated with β -lactamase ARGs in complex integron structures, and most frequently with class 1 integron.^{7,9} Thus, class 1 integrons pose major clinical concern, being among the biggest contributors to the problem of MDR infections. In pathogenic Enterobacteriaceae the cassette arrays can vary, but overall they have certain common, albeit not universal, characteristic features.¹⁰

Previous Romanian studies of resistance in clinical Enterobacteriaceae were focused on detection of β -lactamases, ESBLs and carbapenemases.¹¹⁻¹⁴ Even with a global surveillance, dynamics of antimicrobial resistance occurring both under antibiotic treatment and in the environment are poorly understood. Therefore, a continuous monitoring of a diversity of mechanisms is imperative. Most of the existing information on the antibiotic resistance patterns is based on samples from hospitalized patients and limited information is available regarding outpatient infections.

The aim of the present work was to analyze the antimicrobial susceptibility profiles in pathogenic Enterobacteriaceae from outpatients and to characterize a variety of genetic mechanisms responsible for resistance. Enzymes involved in drug alteration (β -lactamases, aminoglycoside modifying enzymes), catalytic enzymes (in the folic acid biosynthesis) and protecting proteins (for the DNA-enzyme complexes from the action of quinolones) have been investigated. Also, the prevalence of class 1 integron and the association with phenotypic and genotypic resistance was assessed in all Enterobacteriaceae isolates.

Methods

Bacterial isolates

A cross-sectional study was conducted to assess the mechanisms of antibiotic resistance among Enterobacteriaceae isolated from outpatient infections in Cluj-Napoca, Romania. A collection of 68 non-susceptible bacterial strains were investigated for AMR and ARG patterns. From January to March 2016, clinical specimens were collected from individual patients addressing a private laboratory located in the center of the city. After the inoculation of

samples into specific culture media including blood agar, MacConkey agar, deoxycholate citrate Leifson agar, Hektoen enteric agar and eosin methylene blue agar and following overnight incubation, typical presumptive Enterobacteriaceae colonies were purified and recultured. The isolates were identified according to standard methods by colony appearance, pigment production, catalase test, oxidase test, Gram staining, triple sugar iron fermentation, motility, indole, urease production, citrate utilization, lysine decarboxylase, phenylalanine deaminase detection and oxidation-fermentation of sugars. For confirmation, isolates were speciated with the API-20E biochemical kits (bioMérieux, Marcy-l'Étoile, France). Enteropathogenic *E. coli* (EPEC) strains isolated from fecal samples were serogrouped by slide agglutination using O-specific antisera (Bio-Rad, Hercules, USA).

Antimicrobial susceptibility testing

All bacterial strains were subjected to antimicrobial susceptibility testing against six antibiotic classes: penicillins, cepheems, carbapenems, fluoroquinolones, aminoglycosides and folate pathway inhibitors. Enterobacteriaceae isolated from urinary tract infections (UTIs) and from wound infections (WIs) were tested for resistance against up to 20 individual drugs. The panel for EPEC strains isolated from patients with persistent diarrhea (PD) included up to 15 antibiotics (Supplementary Table 1). Antibiotic susceptibility testing was performed by disk diffusion in Mueller-Hinton agar plates. Following overnight incubation, the inhibition zone diameters were measured and the results were interpreted according to CLSI guidelines.¹⁵ MDR was defined as non-susceptibility to at least one agent in three or more antimicrobial categories.

Antibiotic resistance genes screening

Bacterial strains displaying phenotypic resistance were included in the molecular testing for detection of ARGs conferring resistance to the corresponding antibiotic classes. The carriage of class I integron integrase was

screened in all isolates. PCR amplifications were performed in 25 µL reaction mix containing: nuclease-free water, DreamTaq Green PCR master mix, forward and reverse primers to a final concentration of 0.5 µM, and DNA template as fresh bacterial suspensions. The PCR reaction conditions were set up as follows, using a thermocycler TProfessional TRIO (Analytik Jena, Jena, Germany): initial denaturation at 94°C for 5 min; 35-fold repeated cycles for denaturation, annealing (Supplementary Table 2) and synthesis; a final elongation at 72°C for 8 min. Agarose gel electrophoresis was used for separation of DNA amplicons. Data acquisition and analysis were performed using the BDA Digital Compact System and BioDocAnalyze Software (Analytik Jena). Molecular reagents and oligonucleotide primers were purchased from Cleaver Scientific (Rugby, UK), Eurogentec (Seraing, Belgium), Lonza (Basel, Switzerland) and Thermo Fisher Scientific (Massachusetts, USA).

Statistical analysis

Inferential statistics methods were used to calculate the proportions of AMR and MDR Enterobacteriaceae as well as the abundances of ARGs and *intI1* in bacterial strains displaying clinical resistance. Correlations between the AMR and ARG numbers were explored. Resistant and susceptible phenotypes (with the intermediate resistance phenotype considered as susceptible) and positive/negative genotypes were converted to binary matrices (1-0). It was verified whether the phenotypic or genotypic resistance was associated with the presence of *intI1* gene, while linear and logistic regression models were used to assess the degree of relationship. Two-sided significance level was set at 0.05, inferred using the Real Statistics Resource Pack software for Microsoft Excel.¹⁶

Results

A total of 68 non-susceptible bacterial strains were analyzed during this study: 61 isolates from UTIs (90%), three isolates from WIs (6%) and four isolates from patients with PD (4%). Biochemical identification confirmed 47 *E. coli*

strains (of which 4 were EPEC), 13 *Enterobacter* spp., 5 *Proteus* spp., 2 *Citrobacter* spp. and 1 *Providencia* spp. Multiple resistance up to 14 individual drugs belonging to maximum six antimicrobial categories was observed (Figure 1). High levels of resistance were displayed for folate pathway inhibitors (74%), fluoroquinolones (49%) and penicillins (44%). Carbapenem resistance was observed in 3% of the isolates. A proportion of 41% of the resistant strains were MDR (Table 1).

Nine β -lactamase genes were screened during this study, and only four were detected: *bla*_{TEM}, *bla*_{TEM-1}, *bla*_{SHV-1} and *bla*_{CTX-M}. The most frequent ARGs were *bla*_{TEM}, found in 24 out of 30 strains resistant to penicillins. *bla*_{TEM-1} encoding the parent enzyme of TEM-type ESBLs was found in 16 isolates. *bla*_{CTX-M}, the most influential mechanism providing cephalosporin resistance in Enterobacteriaceae, was present in 8 out of 21 bacterial strains. Three genes responsible for resistance to fluoroquinolones (*qnrA*, *qnrB* and *qnrS*) were investigated, *qnrS* genes being detected in three out of 33 isolates resistant to ciprofloxacin or norfloxacin. Six ARGs encoding aminoglycoside modifying enzymes were screened by PCR. *aac(3)-IIIa*, *aac(6')-II* and *aac(6')-Ie-aph(2'')* were detected in 5 out of 16 isolates phenotypically resistant to gentamicin. Resistance to folate inhibitors pathway was encoded by *sul1*, *sul2*, and *sul3* genes in 25 of the 45 isolates resistant to trimethoprim-sulfamethoxazole (Figure 2). Three isolates contained only the *sul1* gene, 15 other isolates contained only the *sul2* gene and one isolate contained only the *sul3* gene. *sul1*, *sul2* and *sul3* genes occurred simultaneously in one strain, both *sul1* and *sul2* were found in 5 isolates, while both *sul2* and *sul3* were found in five strains. *sul1* and *sul3* genes were not observed concomitantly. A significant proportion of pathogenic Enterobacteriaceae harbored class 1 integrons, the *int11* gene being detected in 40 strains (59%) (Figure 2).

With regard of the sample origin, the *bla*_{TEM}, *bla*_{TEM-1} and *bla*_{CTX-M} genes were detected in samples from various infections. The *bla*_{SHV-1} β -lactamase as well as the genes conferring resistance to fluoroquinolones and

aminoglycosides were not observed in samples from WIs, being found frequently in EPEC strains from PD samples. Sulphonamide resistance genes were mostly associated with UTIs, and were not detected in WIs. Regarding the taxonomic incidence, ARGs particularly occurred in *Citrobacter* and *E. coli* (particularly EPEC), were less frequent in *Enterobacter* and *Proteus* species, and were not found in *Providencia* isolates (Table 2).

Phenotypic and genotypic profiles of some bacterial strains require special attention. Despite the wide AMR pattern to 14 drugs, *Enterobacter* spp. E1 was found to carry only two resistance genes. *Citrobacter* spp. E25, extensively resistant to eleven antibiotics, expressed seven ARGs. Two *E. coli* strains, EPEC E39 and E53, resistant to eleven and eight drugs, were found to possess a plethora of ARGs, of 9 and 7 genes, respectively. All these 4 strains were positive for *int11*. In addition, the simultaneous presence of *sul1* and *sul3* genes in strain E25 suggests the coexistence of multiple integrons (Table 3).

Statistically significant correlations were observed between the levels of phenotypic and genotypic resistance ($r=0.60$; $p<0.001$). Despite the variable resistance patterns overall, two significant correlations between the integron and the phenotypic resistance were found. The presence of *int11* was correlated to trimethoprim-sulfamethoxazole resistance ($r=0.48$; $p<0.001$) and also to fluoroquinolone resistance ($r=0.27$; $p=0.011$). ARG abundances were linked to *int11* carriage ($r=0.33$; $p=0.006$) but no significant associations in the co-occurrence of specific ARGs and *int11* were found in pathogenic Enterobacteriaceae.

Discussion

Most of the resistant Enterobacteriaceae were isolated from UTIs, *E. coli* and *Enterobacter* spp. prevailing in clinical infections. *Klebsiella* species were not detected in this study, although other Romanian papers reported them among the most frequent pathogens.^{12-14,17} The frequency of MDR Enterobacteriaceae causing infections in patients from North-Western Romania was similar to that reported in the South-Eastern part of the

Figure 1. Antimicrobial resistance in clinical Enterobacteriaceae

Table 1. Frequencies of resistant and multidrug resistant Enterobacteriaceae

Phenotypic resistance to	Total isolates Resistant/N (%)	UTIs Resistant/N	WIs Resistant/N	PD Resistant/N
Penicillins	30/68 (44)	24/61	2/3	4/4
Ampicillin	9/9 (100)	6/6	-	3/3
Piperacillin	3/3 (100)	2/2	-	1/1
Ticarcillin	3/3 (100)	1/1	1/1	1/1
Amoxicillin-clavulanate	12/13 (92)	7/8	1/1	4/4
Ampicillin-sulbactam	17/62 (27)	15/57	1/1	1/4
Piperacillin-tazobactam	1/6 (17)	1/3	0/2	0/1
Cephems	21/68 (31)	16/61	2/3	3/4
Cefuroxime	7/16 (44)	4/10	1/2	2/4
Ceftazidime	5/6 (83)	3/4	1/1	1/1
Ceftriaxone	15/60 (25)	11/54	1/2	3/3
Cefotaxime	15/56 (27)	13/53	1/2	1/1
Cefepime	5/12 (42)	4/9	0/2	1/1
Carbapenems	2 (3)	2/61	0/3	0/4
Imipenem	0/55 (0)	0/49	0/2	0/4
Ertapenem	2/9 (22)	2/8	0/1	-
Meropenem	2/2 (100)	2/2	-	-
Fluoroquinolones	33/67 (49)	29/61	1/2	3/4
Ciprofloxacin	9/15 (60)	5/9	1/2	3/4
Norfloxacin	26/56 (46)	26/56	-	-
Aminoglycosides	16/68 (24)	14/61	0/3	2/4
Gentamicin	15/60 (25)	13/53	0/3	2/4
Amikacin	1/60 (2)	1/57	0/3	-
Tobramycin	1/8 (13)	1/8	-	-
Folate pathway inhibitors	45/61 (74)	39/55	2/2	4/4
Trimethoprim-sulfamethoxazole	45/61 (74)	39/55	2/2	4/4
MDR strains	28/68 (41)	23/61	2/3	3/4

MDR – multidrug resistant; PD – persistent diarrhea; UTIs – urinary tract infections; WIs – wound infections.

country.¹⁸ Carbapenems are still valid options for the treatment of MDR strains of Enterobacteriaceae, and imipenem was found to be the most effective drug.

The TEM-1 β -lactamase is able to hydrolyze ampicillin at a higher rate than that for

carbenicillin, oxacillin, or cephalothin, and displays negligible activity against extended-spectrum cephalosporins. The SHV-1 enzyme has the same hydrolytic profile as TEM-1, conferring resistance to broad-spectrum penicillins such as

Table 2. Antibiotic resistance genes and class 1 integron integrase detected in Enterobacteriaceae isolated from clinical samples

Category	Gene	Mechanism	n/N isolates from			Found in
			UTIs	WIs	PD	
β-lactamases	<i>bla</i> _{TEM}	β-lactamase enzymes, ESBLs	18/24	2/2	4/4	<i>E. coli</i> , <i>Enterobacter</i> , <i>Citrobacter</i> , <i>Proteus</i>
	<i>bla</i> _{TEM-1}	β-lactam hydrolytic enzymes	11/24	1/2	4/4	<i>E. coli</i> , <i>Enterobacter</i> , <i>Citrobacter</i>
	<i>bla</i> _{TEM-2}	β-lactamase	0/24	0/2	0/4	-
	<i>bla</i> _{SHV-1}	β-lactam hydrolytic enzymes	2/24	0/2	1/4	<i>E. coli</i> , <i>Enterobacter</i>
	<i>ampC</i>	β-lactamase	0/16	0/2	0/3	-
	<i>bla</i> _{CTX-M}	β-lactamase	4/16	1/2	3/3	<i>E. coli</i> , <i>Enterobacter</i> , <i>Proteus</i>
	<i>bla</i> _{NDM-1}	Metallo β-lactamase	0/16	0/2	0/3	-
	<i>bla</i> _{VIM-1}	Metallo β-lactamase	0/16	0/2	0/3	-
	<i>bla</i> _{OXA-48}	β-lactamase	0/16	0/2	0/3	-
Fluoroquinolone resistance genes	<i>qnrA</i>	Quinolone resistance protein	0/29	0/1	0/3	-
	<i>qnrB</i>	Quinolone resistance protein	0/29	0/1	0/3	-
	<i>qnrS</i>	Quinolone resistance protein	1/29	0/1	2/3	-
	<i>qnrS</i>	Pentapeptide repeat family protecting the DNA gyrase				<i>E. coli</i> , <i>Enterobacter</i>
	<i>aac(3)-I</i>	Aminoglycoside modifying enzymes	0/14	0/0	0/2	-
	<i>aac(3)-IIIa</i>	Aminoglycoside modifying enzymes	1/14	0/0	0/2	<i>E. coli</i>
	<i>aac(6')-Im</i>	Aminoglycoside N-acetyltransferase	0/14	0/0	0/2	-
	<i>aac(6')-II</i>	Aminoglycoside modifying enzymes	2/14	0/0	2/2	<i>E. coli</i> , <i>Citrobacter</i>
	<i>aac(6')-Ie-aph(2'')</i>	Aminoglycoside N-acetyltransferase	0/14	0/0	1/2	<i>E. coli</i>
Aminoglycoside resistance genes	<i>aac(6')-Ie-aph(2'')</i>	Aminoglycoside O-phosphotransferase				<i>E. coli</i>
	<i>aac(6')-Ie-aph(2'')-Ia</i>	Aminoglycoside modifying enzymes	0/14	0/0	0/2	-
	<i>aac(6')-Ie-aph(2'')-Ia</i>	Aminoglycoside N-acetyltransferase				-
	<i>aac(6')-Ie-aph(2'')-Ia</i>	Aminoglycoside O-phosphotransferase				-
	<i>sul1</i>	Dihydropteroate synthase type 1	9/39	0/2	0/4	<i>E. coli</i> , <i>Enterobacter</i> , <i>Citrobacter</i> , <i>Proteus</i>
	<i>sul2</i>	Dihydropteroate synthase type 2	18/39	0/2	3/4	<i>E. coli</i> , <i>Enterobacter</i> , <i>Citrobacter</i>
	<i>sul3</i>	Dihydropteroate synthase type 3	6/39	0/2	0/4	<i>E. coli</i> , <i>Citrobacter</i>
	<i>intI1</i>	DNA integration, DNA recombination, DNA binding	22/61	0/3	3/4	<i>E. coli</i> , <i>Enterobacter</i> , <i>Citrobacter</i> , <i>Providencia</i>
	<i>intI1</i>	DNA integration, DNA recombination, DNA binding				<i>E. coli</i> , <i>Enterobacter</i> , <i>Citrobacter</i> , <i>Providencia</i>

ESBLs –extended-spectrum β-lactamases; n – positive strains; N – total tested strains; PD – persistent diarrhea; UTIs – urinary tract infections; WIs – wound infections.

ampicillin, ticarcillin and piperacillin but not to oxyimino-substituted cephalosporins. In this screening, the presence of the *bla*_{TEM-1} gene was highly related to phenotypic resistance to ampicillin, while the carriage of *bla*_{SHV-1} was

additionally associated to third and fourth generation cephalosporins resistance. In the past, TEM-1 β-lactamases were described as susceptible to inhibitors such as clavulanic acid and sulbactam. The present study reveals that all the

Figure 2. Genetic resistance in clinical Enterobacteriaceae

Table 3. Characteristics of multidrug resistant strains with exceptional resistance profiles

Isolate	Strain identification	Origin	AMR phenotype	ARG genotype	<i>int11</i>
E1	<i>Enterobacter</i> spp.	UTI	PRL, TIC, AMC, SAM, TZP, CAZ, CRO, CTX, FEP, ETP, MEM, NOR, TOB, SXT	<i>bla</i> _{TEM} , <i>sul2</i>	+
E25	<i>Citrobacter</i> spp.	UTI	PRL, SAM, CAZ, CRO, CTX, FEP, ETP, MEM, CIP, CN, SXT	<i>bla</i> _{TEM} , <i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-1} , <i>aac</i> (6')-II, <i>sul1</i> , <i>sul2</i> , <i>sul3</i>	+
E39	EPEC	PD	AM, PRL, TIC, AMC, CXM, CAZ, CRO, FEP, CIP, CN, SXT	<i>bla</i> _{TEM} , <i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-1} , <i>bla</i> _{CTX-M} , <i>qnrS</i> , <i>aac</i> (6')-II, <i>aac</i> (6')-Ie-aph(2''), <i>sul2</i>	+
E53	EPEC	PD	AMC, SAM, CXM, CRO, CTX, CIP, CN, SXT	<i>bla</i> _{TEM} , <i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-1} , <i>bla</i> _{CTX-M} , <i>qnrS</i> , <i>aac</i> (6')-II, <i>sul2</i>	+

AM – ampicillin; AMC – amoxicillin-clavulanate; CAZ – ceftazidime; CIP – ciprofloxacin; CN – gentamicin; CRO – ceftriaxone; CTX – cefotaxime; CXM – cefuroxime; ETP – ertapenem; FEP – cefepime; MEM – meropenem; NOR – norfloxacin; PD – persistent diarrhea; PRL – piperacillin; SAM – ampicillin-sulbactam; SXT – trimethoprim-sulfamethoxazole; TIC – ticarcillin; TOB – tobramycin; TZP – piperacillin-tazobactam; UTI – urinary tract infection; “+” – *int11* positive.

*bla*_{TEM-1}-carrying bacteria were resistant to antibiotic combinations such as amoxicillin-clavulanate or ampicillin-sulbactam. This mechanism was attributed to the TEM-1 β -lactamase hyperproduction.¹⁹ TEM-1 and TEM-2 are not ESBLs. Therefore, detection of strains carrying the *bla*_{TEM} but not the *bla*_{TEM-1} or *bla*_{TEM-2} genes suggests that approximately 16% of the penicillin-resistant strains carry *bla*_{TEM}-type ESBL derivatives.

The *ampC* gene was not detected in study strains, while the *bla*_{CTX-M} group was present. *ampC* plasmid genes are derived from the chromosomal *ampC* genes of various Enterobacteriaceae and ancestors of the *bla*_{CTX-M}

group of β -lactamases.⁶ Most of CTX-M enzymes exhibit powerful activity against cefotaxime and ceftriaxone but not ceftazidime, and the presence of the *bla*_{CTX-M} gene was associated to ceftriaxone resistance. *bla*_{NDM}, *bla*_{VIM} and *bla*_{OXA}-type genes, a significant cause of carbapenem resistance in Enterobacteriaceae, recently found in bacterial strains from hospitalized patients in Romania,¹¹⁻¹³ were not detected during this study. Qnr proteins, encoded by ARGs such as the *qnrS* gene, belong to the pentapeptide repeat family capable of protecting DNA gyrase from drug inhibition, being responsible for resistance in the presence of quinolones at therapeutic levels.

The resistance mechanism of *aac*(3)-IIIa and *aac*(6')-II genes relies on aminoglycoside N-

acetyltransferase, which modifies aminoglycosides by acetylation. In addition, the bifunctional *aac(6')-Ie-aph(2'')* gene modifies aminoglycosides by phosphorylation, being responsible for high-level aminoglycoside resistance. These ARGs are not common for Enterobacteriaceae. They were previously observed in *Pseudomonas*, *Enterococcus* or *Staphylococcus* species.^{20,21} Other aminoglycoside ARGs, such as *aac(3)-I*, *aac(6')-Im* or *aac(6')-Ie-aph(2'')-Ia*, known as frequently present in Enterobacteriaceae, were not detected during this study.

The *sul* genes encode for dihydropteroate synthases that are not inhibited by sulphonamides, catalyzing the formation of the immediate precursor of folic acid. The more frequent dissemination of *sul2* gene, as observed in this study, in 47% of the isolates, could be attributed to the efficacy and stability of type II enzyme. The gene frequency distribution *sul2* > *sul1* > *sul3* is characteristic for pathogenic *E. coli* in Europe and Canada,²² while the pattern of increased *sul1* frequency over *sul2* was observed in Australia and Korea.^{23,24} Distribution of *sul* genes in the 3'-conserved segment provides evidence on class 1 integrons diversity: classical integrons contain the *sul1* gene while non-classical integrons lack the *sul1* cassette, and the *sul3* gene is present.

Horizontal gene transfer adds an important dimension to infectious pathology, particularly when an ARG can transfer resistance to multiple otherwise unrelated pathogens.²⁵ A variety of penicillin, aminoglycoside and sulphonamide resistance determinants are often found as part of transferrable gene cassettes in integrons,^{6,10,20} and therefore the high frequency of class 1 integrons in pathogenic Enterobacteriaceae displaying AMR.

This study has several limitations. First, a small number of bacterial isolates were sampled in a single facility, over a period of only 3 months. Therefore, the results of the survey may not be generalizable for the entire population. Nevertheless, it brings relevant data on the magnitude of resistance in non-hospitalized patients. Second, the PCR technique is unable to detect the whole range of ARGs and subsequent

variants. From hundreds of genes known by far, molecular detection was limited to a selection of ARGs. This set of genes was chosen to cover a diversity of resistance mechanisms with wide occurrence and/or clinical significance for pathogenic Enterobacteriaceae.

Conclusions

Pathogenic Enterobacteriaceae isolated in Cluj-Napoca displayed high levels of phenotypic resistance against folate pathway inhibitors, fluoroquinolones and penicillins. The incidence of carbapenem-resistance was very low. Forty-one percent of the strains were MDR.

A wide diversity of mechanisms encoding genetic resistance were found to be involved in AMR. The strains displaying phenotypic resistance were able to produce β -lactamase enzymes, aminoglycoside modifying enzymes, fluoroquinolone protection proteins as well as enzymes able to confer resistance to sulphonamides. Class 1 integron integrase was detected in 59% of the resistant isolates and significant associations were found between the carriage of *int11* gene and the levels of phenotypic and genotypic resistance. Correlations between the presence of *int11* and resistance to sulphonamides and also regarding the concomitant carriage of *int11* and *sul2* genes were found.

Authors' contributions statement: AF designed and supervised the study, analyzed the data and drafted the manuscript. ET and ABK carried out the laboratory work and reviewed the manuscript. All authors reviewed and approved the final version of the manuscript.

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Supplementary table 1. Antibiotics used in antimicrobial susceptibility testing for pathogenic Enterobacteriaceae

Antimicrobial agent	Disc content (µg)	Origin of isolates		
		UTIs	WIs	PD
Penicillins				
Ampicillin (AM)	10	+	+	+
Piperacillin (PRL)	100	+	+	+
Ticarcillin (TIC)	75	+	+	+
Amoxicillin-clavulanate (AMC)	20/10	+	+	+
Ampicillin-sulbactam (SAM)	10/10	+	+	+
Piperacillin-tazobactam (TZP)	100/10	+	+	+
Cephems				
Cefuroxime (CXM)	30	+	+	+
Ceftazidime (CAZ)	30	+	+	+
Ceftriaxone (CRO)	30	+	+	+
Cefotaxime (CTX)	30	+	+	+
Cefepime (FEP)	30	+	+	+
Carbapenems				
Imipenem (IPM)	10	+	+	+
Ertapenem (ETP)	10	+	+	-
Meropenem (MEM)	10	+	+	-
Fluoroquinolones				
Ciprofloxacin (CIP)	5	+	+	+
Norfloxacin (NOR)	10	+	+	-
Aminoglycosides				
Gentamicin (CN)	10	+	+	+
Amikacin (AK)	30	+	+	-
Tobramycin (TOB)	10	+	+	-
Folate pathway inhibitors				
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	+	+	+

PD – persistent diarrhea; UTIs – urinary tract infections; WIs – wound infections.

Supplementary table 2. Primers and PCR conditions used in this study

Antibiotic class	Target gene	Primers sequence F/R (5' – 3')	Amplicon size (bp)	Annealing temperature
Penicillins	<i>bla</i> _{TEM}	ATAAAATTCTTGAAGACGAAA/ GACAGTTACCAATGCTTAATC	1080	50°C
	<i>bla</i> _{TEM-1}	GGTCGCCGCATACACTATTC/ ATACGGGAGGGCTTACCATC	500	57°C
	<i>bla</i> _{TEM-2}	AAGTAAAGATGCTGAAGATAAGTTGG / GATCTGTCTATTTTCGTTTCATCCATAG	737	61°C
	<i>bla</i> _{SHV-1}	GCGTTATATTCGCCTGTGTATTAT/ GCCTGTTATCGCTCATGGTAATG	385	61°C
Cephems	<i>amp</i> C	AGAAGGACCAGGCACAGATC/ CTCGGCATTGGGATAGTTGC	671	57°C
	<i>bla</i> _{CTX-M}	GTGAAACGCAAAAGCAGCTG/ CCGGTCGTATTGCCTTTGAG	400	55°C
	<i>bla</i> _{NDM-1}	GGTTTGGCGATCTGGTTTTTC/ CGGAATGGCTCATCACGATC	621	55°C
Carbapenems	<i>bla</i> _{VIM-1}	GATGGTGTGGTTCGCATATC/ ACGGCACAACCACCGTATAG	449	57°C
	<i>bla</i> _{OXA-48}	ATGGCAAGAAAACAAAAGTTGG/ TTGAGCACTTCTTTTGTGATGG	705	53°C
	<i>qnrA</i>	AGTTTGATGGTTGCCGCTTT/ TCTTCATTGATCTGCACGCC	541	53°C
Fluoroquinolones	<i>qnrB</i>	TCGTGCGATGCTGAAAGATG/ CCGAATTGGTCAGATCGCAA	368	55°C
	<i>qnrS</i>	TGATCTCACCTTCACCGCTT/ GAGTTCGGCGTGGCATAAAT	496	55°C
	<i>aac</i> (3)-I	ACCTACTCCCAACATCAGCC/ TCTTCCCGTATGCCCAACTT	329	55°C
Aminoglycosides	<i>aac</i> (3)-IIIa	GCATGCCTCACTTAAAGCGA/ ACCGTTTCTTCCAAGCATCG	514	55°C
	<i>aac</i> (6')-Im	GGCTGACAGATGACCGTGTCTTG/ GTAGATATTGGCATACTACTCTGC	303	61°C
	<i>aac</i> (6')-II	AGCGACCGACTCTTGATGAA/ GGCTTGTCGTGTTTGAACC	414	53°C
	<i>aac</i> (6')-Ie- <i>aph</i> (2'')	CCAAGAGCAATAAGGGCATA/ CACTATCATAACCACTACCG	220	53°C
	<i>aac</i> (6')-Ie- <i>aph</i> (2'')-Ia	CCAAGAGCAATAAGGGCATA/ CACTATCATAACCACTACCG	280	53°C
	<i>sul1</i>	AGGCATGATCTAACCCTCGG/ GGCCGATGAGATCAGACGTA	665	57°C
	<i>sul2</i>	GACAGTTATCAACCCGCGAC/ GAAACAGACAGAAGCACCGG	380	57°C
Folate pathway inhibitors	<i>sul3</i>	GTGGGCGTTGTGGAAGAAAT/ AAAAGAAGCCCATACCCGGA	370	55°C
Class 1 integron integrase	<i>intI1</i>	CGTGCCGTGATCGAAATCCAG/ TTCGTGCCTTCATCCGTTTCC	371	60°C