European Review for Medical and Pharmacological Sciences 2023; 27: 1147-1154

Genetic analysis of multidrug-resistant and AmpC-producing *Citrobacter freundii*

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Abstract. – OBJECTIVE: During the last decade, antimicrobial resistance within pet animals has received worldwide concern owing to their close contact with humans and the possibility of animal-human co-transmission of multidrug-resistant bacteria. This study examined phenotypic as well as molecular mechanisms associated with antimicrobial resistance in a multidrug-resistant, and AmpC-producing *Citrobacter freundii* recovered from a dog suffering from kennel cough in.

MATERIALS AND METHODS: The isolate was recovered from a two-year-old dog suffering from severe respiratory manifestations. Phenotypically, the isolate was resistant to a wide range of antimicrobial agents including, aztreonam, ciprofloxacin, levofloxacin, gentamicin, minocycline, piperacillin, sulfamethoxazole-trimethoprim, and tobramycin. PCR and sequencing confirmed that the isolate harbors multiple antibiotic resistance genes, such as bla_{CMY-48} and bla_{TEM-1B} which mediate resistance to B-lactams, and *qnrB6* which mediate resistance to quinolone antibiotics.

RESULTS: Multilocus sequence typing confirmed that the isolate belongs to ST163. Due to the unique characteristics of this pathogen, the whole genome sequencing was performed. In addition to the previously confirmed antibiotic resistance genes by PCR, the isolate was also confirmed to harbor other resistance genes which mediate resistance to aminoglycoside (*aac(3)-IId, aac(6')-Ib-cr, aadA16, aph(3")-Ib,* and *aph(6)-Id)*, macrolides [*mph(A)),* phenicols *(floR)*, rifampicin (ARR-3), sulphonamides (*sul1* and *sul2*), trimethoprim *(dfrA27)*, and tetracycline *(tet(A)* and *tet(B)*].

CONCLUSIONS: The results presented in this study confirm that pets are possible sources of highly pathogenic multidrug-resistant microbes with unique genetic characteristics taking into consideration the high potential for their dissemination to humans, which can undoubtedly develop of severe infections in these hosts.

Key Words:

Multidrug-resistance, AmpC beta-lactamase, Citrobacter freundii, Quinolone, Animal human transmission, Pets.

Introduction

Antimicrobial resistance (AMR) is a global issue that is quickly developing with huge financial implications[1]. According to recent estimates, the economic costs of AMR ranged from £3-11 billion to US $100 trillion[2]. There has recently been an increase in reports that explain and lend support to the possibility of a connection between AMR emergence in human populations and that in animal species[3-5]. Global attention has increased due to the rise of AMR in pet animals[3,4,6-10]. The significant potential of animal-human transmission of highly pathogenic bacteria that exhibit a variety of distinct resistance mechanisms, such as carbapenemases, ESBLs, and mcr, is the reason for the growing interest in antimicrobial resistance among pets[3]. Recently, human pathogens, for example, have been isolated in dogs[3,4,10]. Additionally, pathogenic strains of the same extended-spectrum beta-lactamases and ampC beta-lactamases (ESBL/AmpC) have been identified in both dogs and humans[11]. Gram-negative bacteria, particularly Enterobacteriaceae, are a major source of concern because they spread, particularly ESBL and AmpC-producers, from pets[3-11]. *Citrobacter freundii* is one of the major Enterobacteriaceae group that was associated with the emergence and development of AMR mechanisms including resistance to β-Lactam and quinolones antibiotics which represent the major categories of antimicrobials used for clinical human use[12-16].

The WHO has classified β-lactam and quinolone antibiotic classes as "critically important" for human clinical use[17]. Unfortunately, their widespread use in clinical human and veterinary fields leads to the emergence and spread of resistance[18-20]. Among the various mechanisms of β-lactam resistance, ESBLs/AmpCs have received special attention because they are able

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to inactivate monobactams, cephalosporins and penicillin inducing resistance to a wide range of antimicrobial agents[18]. Quinolone resistance on the other hand is caused by functional mutations in DNA topoisomerase IV and DNA gyrase, the drug target enzymes, as well as in the regulatory genes of bacterial membrane efflux pumps[4,5,18,21]. Furthermore, Low resistance levels are also brought on by bacteria acquiring resistance genes, like the plasmid-mediated resistance gene *qnr*[21]. The rapid dissemination of *qnr* genes among bacterial species ultimately induced a special concern for this resistance mechanism among healthcare workers[18,21].

The poor treatment outcomes and high morbidity/mortality rates linked to producers of ESBL/AmpC, as well as quinolone resistance isolates, have fueled research interest in elucidating the molecular mechanism of β-lactam resistance, particularly in pet animals, that serve as a possible source of transmission of human infection[3,4]. Therefore, it is of special interest to fully evaluate the molecular mechanism of highly resistant pathogens associated with pet infection that may be transmitted to humans. Herein, we fully elucidate the molecular mechanism of multidrug-resistant and AmpC-producing *C. freundii* from pets.

Materials and Methods

Bacterial Isolation

The bacterial isolate was recovered from the ocular charge of two years old male dog that suffered from severe respiratory signs, including sneezing, serous nasal discharge, nasal ulcer, ocular inflammation, and ocular discharge. The animal was subjected to careful clinical examination with special attention to the respiratory system. For bacterial isolation, the ocular swab was enriched in 5 ml of tryptic soya broth for 16-18 hours at 37°C then striking on MacConkey agar. After 24-hour period of incubation, suspected colonies were isolated and kept in 20% glycerol stocks for further investigations[4,5].

Bacterial Identification and DNA Extraction

The bacterial isolates were identified by MALDI-TOF-MS as previously described[22]. The DNA was extracted using boiling lysate method as described previously[1,18].

Phenotypic Carbapenemase ESBL, and AmpC Detection

Phenotypic identification of bacteria that produce carbapenemase enzyme was applied using a modified method for carbapenem inactivation (mCIM) on a Mueller-Hinton agar plate with both meropenem discs as described previously[22]. Detection of ESBL and AmpC production in bacterial isolate was performed by D68C AmpC & ESBL set of detection (Mast Diagnostics, Mast Group Ltd., Merseyside, UK) according to the manufacturer's instructions as previously reported. The indicator organism, *E. coli* ATCC 25922, was used as negative control. As positive controls, we used ESBL, carbapenemase, and AmpC-producers from our previous studies[4,5,22-25].

Genotypic Detection of Carbapenemase-Encoding Genes and Other Resistance Genes

The isolate was also tested for different classes of resistance mechanisms (Table I), including other β-lactamases (bla_{CTX-M}, bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{SHV}, bla_{AAC}, bla_{EBC}, and bla_{FOX}), 16S rRNA methylases (*armA*, *npmA*, *rmtA*, *rmtB*, *rmtC*, and *rmtD*), plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB*, *qnrS*, and *qnrC*), and the quinolone efflux pump determinant *qepA* gene as previously described[26-31]. The sequencing of all the PCR products was performed as previously described[32,33].

Antimicrobial Susceptibility Testing

The isolate was tested for susceptibility to imipenem (IPM), meropenem (MEPM), doripenem (DRPM), aztreonam (AZT), piperacillin (PIPC), piperacillin/tazobactam (PIP/TAZ), ceftazidime (CAZ), cefepime (CFPM), cefozopran (CZOP), sulbactam/cefoperazone (SBT/CPZ), gentamicin (GM), tobramycin (TOM), amikacin (AMK), levofloxacin (LVFX), ciprofloxacin (CPFX), fosfomycin (FOM), minocycline (MINO), sulfamethoxazole-trimethoprim (ST), and colistin (CL) were determined using a MicroScan Walk Away and Microscan Class II Negative bacteria series dry antibiotic plates (Beckman Coulter, Inc., Brea, CA, USA). Susceptibility test was performed according to the manufacturers' directions and the susceptibility to each antimicrobial agent was determined according to the CLSI breakpoint[34].

Multilocus Sequence Typing (MLST)

MLST was performed according to the *Citrobacter* sequence typing website (https://pubmlst.org/organisms/citrobacter-spp).

Table I. Primers used in this study.

Primer name	Sequence (5′-3′)	Target	Ref.
β-lactamases			
CTXM7	GCG TGA TAC CAC TTC ACC TC	$bla_{\text{CTX-M}}$-1 group	26
CTXM8	TGA AGT AAG TGA CCA GAA TC		
CTXM17	TGA TAC CAC CAC GCC GCT C	$bla_{\text{CTX-M}}$-2 group	26
CTXM18	TAT TGC ATC AGA AAC CGT GGG		
CTXM19	CAA TCT GAC GTT GGG CAA TG	$bla_{\text{CTX-M}}$-8/25/26 group	26
CTXM20	ATA ACC GTC GGT GAC AAT T		
CTXM11	ATC AAG CCT GCC GAT CTG GTT A	$bla_{\text{CTX-M}}$-9 group	26
CTXM12	GTA AGC TGA CGC AAC GTC TGC		
SHV_F	AGCCGCTTGAGCAAATTAAAC	*SHV-1/variant*	27
SHV_R	ATCCCGCAGATAAATCACCAC		
TEM-F	CATTTCCGTGTCGCCCTTATTC	$bla_{\text{TEM-1/-2/variant}}$	27
TEM-R	CGTTCATCCATAGTTGCCTGAC		
MOXMF	GCT GCT CAA GGA GCA CAG GAT	$bla_{\text{MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11}}$	28
MOXMR	CAC ATT GAC ATA GGT GTG GTG C	$bla_{\text{LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1}}$	28
CITMF	TGG CCA GAA CTG ACA GGC AAA		
CITMR	TTT CTC CTG AAC GTG GCT GGC		
DHAMF	AAC TTT CAC AGG TGT GCT GGG T	bla_{DHA}	28
DHAMR	CCG TAC GCA TAC TGG CTT TGC		
ACCMF	AAC AGC CTC AGC AGC CGG TTA	bla_{ACC}	28
ACCMR	TTC GCC GCA ATC ATC CCT AGC		
EBCMF	TCG GTA AAG CCG ATG TTG CGG	$bla_{\text{MIR-1, ACT-1}}$	28
EBCMR	CTT CCA CTG CGG CTG CCA GTT		
FOXMF	AAC ATG GGG TAT CAG GGA GAT G	$bla_{\text{FOX-1 to FOX-5b}}$	28
FOXMR	CAA AGC GCG TAA CCG GAT TGG		
Plasmid-mediated quinolone resistance			
qnrA-F	ATTTCTCACGCCAGGATTTG	*qnrA*	29
qnrA-R	TGCCAGGCACAGATCTTGAC		
qnrB-F	CGACCTKAGCGGCACTGAAT	*qnrB*	29
qnrB-R	GAGCAACGAYGCCTGGTAGYTG		
qnrS-F	ACTGCAAGTTCATTGAACAG	*qnrS*	29
qnrS-R	GATCTAAACCGTCGAGTTCG		
Quinolone efflux pump determinant			
qepA-F	AACTGCTTGAGCCCGTAGAT	*qepA*	30
qepA-R	GTCTACGCCATGGACCTCAC		
16S rRNA methylases			
armA-F	GGTGCGAAAACAGTCGTAGT	*armA,*	31
armA-R	TCCTCAAATATCCTCTATGT		
npmA-F	CGGGATCCAAGCACTTTCATACTGACG	*npmA*	31
npmA-R	CGGAATTCCAATTTTGTTCTTATTAGC		
rmtA-F	CTAGCGTCCATCCTTTCCTC	rmtA	31
rmtA-R	TTTGCTTCCATGCCCTTGCC		
rmtB-F	GGAATTCCATATGAACATCAACGATGCC	*rmtB*	31
rmtB-R	CCGCTCGAGTCCATTCTTTTTTATCAAGT		
rmtC-F	CGAAGAAGTAACAGCCAAAG	*rmtC*	31
rmtC-R	GCTAGAGTCAAGCCAGAAAA		
rmtD-F	TCATTTTCGTTTCAGCAC	*rmtD*	31
rmtD-R	AAACATGAGCGAACTGAAGG		

Whole-Genome Sequencing and in Silico Data Analysis

The isolate was subjected to whole-genome sequencing for the complete characterization of antimicrobial resistance. Briefly, using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), extraction of total genomic DNAs of the isolates was performed according to the manufacture's protocol. The whole-genome sequencing was performed employing an Illumina NovaSeq

using 150 bp paired-end sequencing. De novo assembly was performed using Platanus Genome Assembler[35]. Assembled contig sequence data were uploaded to https://pubmlst.org/rmlst/ and species identification was performed by ribosomal multilocus sequence typing (rMLST)[36]. Sequence data were also submitted to multilocus sequence typing (MLST)[37], PlasmidFinder and plasmid multilocus sequence typing (pMLST)[38], to identify sequence type of identified species, known plasmid incompatibility groups and plasmid sequence types, respectively. Acquired resistance genes were detected by ResFinder[39].

Results

Antimicrobial Susceptibility Testing

Phenotypically the isolate was resistant to a wide range of antimicrobials, including β-lactams (aztreonam and piperacillin), quinolones (ciprofloxacin, levofloxacin), aminoglycoside (gentamicin and tobramycin), tetracycline (minocycline), and sulphonamides (sulfamethoxazole-trimethoprim) (Table II).

Table II. Phenotypic characteristics of *C. freundii* from dog.

Antibiotic	MIC	MIC Interpretation
AMK	≤ 8	Susceptible
AZT	8	Intermediate
CAZ	≤ 4	Susceptible
CFPM	≤ 2	Susceptible
CL	N/R	Susceptible
CPFX	> 2 (R)	Resistant
CPZ/SBT	≤ 16/8	Susceptible
CZOP	≤ 4	Susceptible
DRPM	≤ 1	Susceptible
FOM	≤ 4	Susceptible
GM	> 8	Resistant
IPM	2	Intermediate
LVFX	> 4	Resistant
MEPM	≤ 1	Susceptible
MINO	> 8	Resistant
PIPC	> 64	Resistant
PIPC/TAZ	≤ 8	Susceptible
ST	> 2/38	Resistant
TOB	>8	Resistant

Tested antibiotic are: imipenem (IPM), meropenem (MEPM), doripenem (DRPM), aztreonam (AZT), piperacillin (PIPC), piperacillin/tazobactam (PIP/TAZ), ceftazidime (CAZ), cefepime (CFPM), cefozopran (CZOP), sulbactam/cefoperazone (SBT/CPZ), gentamicin (GM), tobramycin (TOM), amikacin (AMK), levofloxacin (LVFX), ciprofloxacin (CPFX), fosfomycin (FOM), minocycline (MINO), sulfamethoxazole–trimethoprim (ST), and colistin (CL).

Phenotypic Carbapenemase, Esbl, and Ampc Detection

Phenotypic carbapenemase detection confirmed that the isolate is intermediate by mCIM test. While the isolates were negative for ESBL production and positive for AmpC production by D68C AmpC & ESBL detection set.

Genotypic Detection of Carbapenemase-Encoding Genes and Other Resistance Genes and Whole-Genome Sequencing

PCR and sequencing using the previously identified primers (Table I), showed that the isolate harbored different antibiotic resistance genes such as bla_{CMY}, bla_{TEM}, and *qnr*. WGS results (Table III) confirmed that β-lactams resistance is due to harboring bla_{CMY-48}, bla_{TEM-1b} genes. Quinolone resistance is basically attributed to the co-harboring of the plasmid-mediated quinolone resistance genes (PMQR) *aac(6')-Ib-cr* and *qnrB6* (Table III). Furthermore, WGS confirmed that the isolates harbor different resistant determinants that mediate resistance to wide range of antimicrobials such as *mph(A)* which mediates resistance to macrolide, *floR* which mediates resistance to phenicol, *ARR-3* which mediates resistance to rifampicin, *sul1* and *sul2* which mediate resistance to sulphonamides, *tet(A)* and *tet(B)* which mediate resistance to tetracycline, *and dfrA27* which mediate resistance to trimethoprim.

Multilocus Sequence Typing

MLST analysis of the strain by PCR as well as its confirmation by WGS (Table III) confirmed that the *C. freundii* strain belongs to ST163.

Discussion

Citrobacter spp. are one of the most common commensal inhabitants of the intestinal tract of humans and other animals. Additionally, they have also been isolated from other different sources such as water, sewage, and soil[12]. Among *Citrobacter* spp., *C. freundii* is the most common *Citrobacter* species causing infections in humans and animals[13]. Infection with *C. freundii* has been recently complicated by the confirmation that this pathogen is often resistant to multiple antibiotics classes, elucidating that both clinical, animal, and environmental strains may harbor different antimicrobial resistance determinants[12,13]. Multiple recent studies[12,14,15] have confirmed the emergence

Table III. Full genotypic of *C. freundii* from dog.

Characterization	Criteria for characterization	Result
Isolate confirmation,	Identified Species (rMLST)	*Citrobacter freundii*
ST identification and Plasmid Inc	ST (MLST)	ST163
	Plasmid Inc groups by PlasmidFinder	IncFIB(K)
Antibiotic resistant genes	β-lactams	bla_{CMY-48}, bla_{TEM-1B}
identification by ResFinder	Carbapenem	-
	Aminoglycoside	*aac(3)-IId, aac(6')-Ib-cr,aadA16, aph(3'')-Ib, aph(6)-Id*
	Colistin	-
	Fosfomycin	-
	Fusidicacid	-
	Glycopeptide	-
	Macrolide	mph(A)
	Nitroimidazole	-
	Oxazolidinone	-
	Phenicol	floR
	Quinolone	aac(6')-Ib-cr,qnrB6
	Rifampicin	*ARR-3*
	Sulphonamide	*sul1,sul2*
	Tetracycline	*tet(A),tet(B)*
	Trimethoprim	*dfrA27*
PointFInder	Mutations	-

of highly resistant *Citrobacter* spp. in human clinical settings, on the other hand, few reports[16] characterized the emergence of multidrug-resistant *Citrobacter* spp. in animals. Therefore, this study fully characterized a multidrug-resistant *C. freundii* recovered from a dog suffering from severe respiratory signs in Egypt.

The extensive use of these antibiotics in hospitals[1,18] as well as in animals[3-5] may act as a selective pressure for the development of such determinants. Among these antibiotics, special concern is paid to β-lactams and quinolones, as these classes of antibiotics have been identified by WHO as critically important for human use[17]. The absence of carbapenems resistance (imipenem, meropenem, and doripenem) in this strain matches with the previous results from pets[4] and small ruminants[5]. This may be due to the fact that carbapenems are not allowed for animal use in Egypt[4,5] as well as other countries and are preserved only for human use as a last resort for treatment of severe infections caused by multidrug-resistant bacteria[1,22,23].

PCR and sequencing using the previously identified primers (Table I), showed that the isolate harbored different antibiotic resistance genes, such as bla_{CMY}, bla_{TEM}, and *qnr*. Due to the unique resistant character of the isolate, it was subjected to whole genome sequencing (WGS) to fully characterize its genetic nature (Table III). WGS has different advantages over PCR that fully characterize the genetic nature of the isolate, and therefore, it is nowadays rapidly growing as an essential tool for its capacity to greatly enhance understanding and knowledge of clinical microbiology and infectious diseases[40]. WGS results emphasized that β-lactams resistance is due to harboring bla_{CMY-48}, bla_{TEM-1b} genes. Most importantly, bla_{CMY-48} is one of the most common AmpC-encoding genes which has received worldwide attention, since the majority of the detected blaAmpC genes from pet animals were blaCMY, that was identified in the Netherlands[6], France/Spain[7], Denmark[8], Italy[9], and Tunisia[10].

In this study, quinolone resistance is basically attributed to the co-harboring of PMQR *aac(6')-Ib-cr* and *qnrB6* (Table III), explaining the phenotypic resistance to ciprofloxacin, levofloxacin (Table II). *aac(6')-Ib-cr* gene is also responsible for the resistance to aminoglycosides beside other identified resistant determinants, including *aac(3)-IId*, *aadA16*, *aph(3'')-Ib*, *aph(6)-Id* which elucidates the phenotypic resistance to gentamicin and tobramycin. The detected PMQR associated with *aac(6')-Ib-cr* and *qnrB6* in this study is of special interest as these resistance determinants are easily spread among bacterial species, as well as among animals and human isolates by plasmid mobility[4,5,18,21]. Functionally, quinolone resistance is attributed to these genes due to they are responsible for encoding

a protein (pentapeptide repeat family), that has blocked action on ciprofloxacin on the purified DNA topoisomerase IV and gyrase[4,5,18,21]. Our results support the previous finding that *qnr* is the most prevalent PMQR gene among pets in Egypt[4], as well as in other European countries such as France/Spain[7] and the UK[41].

MLST of the strain by confirmed that the *C. freundii* strain belongs to ST163. Interestingly, this is the first time to identify *C. freundii* ST163 from animals as well as the first time for its identification in Egypt and the entire of Africa. According to *C. freundii* MLST website, only three isolates with ST163 were previously identified (https://pubmlst.org/organisms/citrobacter-spp). Surprisingly, two *C. freundii* ST163 isolates were recovered from humans in China and Israel and one *C. freundii* ST163 was recovered from the environment in Canada. This finding is of special importance as it confirms the cross-transmission of *C. freundii* ST163 between humans, animals, and the environment. It also highlights the importance of developing a "one health approach" to overcome its dissemination in a high epidemic manner.

Conclusions

This is the first report identifying *C. freundii* ST163 from animals in Egypt after its previous identification in humans and the environment. The close contact between pets and humans may act as a potential source for its dissemination to the human environment and the development of severe infection. Therefore, special regulations such as enhancing wise antibiotic use in the veterinary field and establishing infection control measures are necessary to overcome the dissemination of antimicrobial resistance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

The author received no financial support for this article's research and/or publication.

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Authors' Contribution

The study concept and design, data acquisition, analysis and interpretation, and manuscript writing were prepared by Shahira Hassoubah.

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