J Infect Chemother 25 (2019) 285–288

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Case Report

Emergence of a carbapenem-resistant and colistin-heteroresistant *Enterobacter cloacae* clinical isolate in Japan☆

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ARTICLE INFO

Article history:
Received 9 March 2018
Received in revised form
8 August 2018
Accepted 4 September 2018
Available online 29 September 2018

Keywords:
Carbapenem resistance
Class C β-lactamase ACT-2
Colistin heteroresistance
Enterobacter cloacae

ABSTRACT

A carbapenem-resistant and colistin-heteroresistant clinical isolate of *Enterobacter cloacae* was obtained from an inpatient in Okinawa, Japan. The minimum inhibitory concentrations of both imipenem and meropenem were 32 μg/mL. The isolate showed heteroresistance to colistin using the Etest method and resistance to colistin using the broth microdilution method. It had a disrupted *ompC* and a mutation in the promoter region of bla_{ACT-2}, but did not harbor any genes encoding carbapenemase. The disruption of *ompC* and the mutation in bla_{ACT-2} was associated with the carbapenem resistance of this isolate. This isolate also had mutations in *pmrAB* and *phoPQ* encoding two-component regulatory systems, which may be associated with colistin heteroresistance.

1. Introduction

The emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) is a serious problem worldwide, as CRE induce infections with high mortality rates and limited treatment options [1]. Mechanisms of carbapenem resistance in *Enterobacteriaceae* include the overproduction of AmpC, the loss of porin due to the disruption of *omp* genes and the production of acquired carbapenemases [2].

Colistin heteroresistance was firstly described by Li et al. [3] in 2006 both in clinical isolates of *Acinetobacter baumannii* from Australia and in *A. baumannii* ATCC19606 (a type strain) obtained from the American Type Culture Collection. Subsequently, colistin heteroresistance was reported in isolates of *Enterobacter cloacae* and *A. baumannii* in 2007 in the Netherlands [4], isolates of *A. baumannii* from a patient with post neurosurgical meningitis in 2009 in Argentina [5], carbapenemase-producing *A. baumannii* isolates in 2011 in Greece [6], *A. baumannii* isolates from a patient with post neurosurgical meningitis in 2013 in Iran [7], an *E. cloacae* isolate in 2014 in the USA [8], a *Klebsiella pneumoniae* isolate in 2015 in South Africa [9], and *K. pneumoniae* isolates in 2016 in Portugal [10]. This report describes a clinical isolate of carbapenem-resistant and colistin heteroresistant *E. cloacae* in Japan.

2. Case report

A 78-year-old Japanese woman with aortic regurgitation and diabetes mellitus was hospitalized from July to September 2015 at a university hospital (UHR). Aortic valve replacement surgery was done in August 2012. After the surgery, she was suspected to have pneumonia. Methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* were isolated from a sputum sample. She was treated with daptomycin for suspected MRSA infection for a month, with doripenem for a week, with ciprofloxacin for three weeks, and with meropenem for nine days, because colistin was not used for the treatment. The patient did not have a history of overseas travel history. She had diarrhea and an isolate of *E. cloacae* RYU7 was obtained from her stool sample. Bacteria were identified

☆ ICMJE Statement: All authors meet the ICMJE authorship criteria.

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https://doi.org/10.1016/j.jiac.2018.09.004
1341-321X/

using VitekMS (bioMeriux, France), with identification confirmed by 16SrRNA sequencing. The minimum inhibitory concentrations (MICs) of antibiotics were determined using the broth microdilution method [11]. Colistin susceptibility was assessed using the Etest (bioMeriux, France). Genomic DNA of the isolate was extracted using DNeasy Blood & Tissue kits (QIAGEN, Tokyo, Japan) and sequenced by MiSeq (Illumina, San Diego, CA). Sequence data were analyzed using CLC genomics workbench version 8.0 (CLC bio, Tokyo, Japan). Sequences of drug-resistance genes, including genes associated with resistance to aminoglycosides, β-lactams and fluoroquinolones, were determined using ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/). Sequences of genes encoding outer membrane proteins (*ompA*, *ompC* and *ompF*) and those encoding two-component systems (*phoPQ* and *pmrAB*), were determined using CLC genomics workbench version 8.0. Multilocus sequence typing (MLST) of *E. cloacae* [12] based on contig data was deduced using the PubMLST (http://pubmlst.org/ecloacae/) database.

E. cloacae RYU7 was found to be a multidrug-resistant strain, as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [13], i.e. it was resistant to all β-lactams tested including carbapenems, gentamycin and ciprofloxacin, but susceptible to amikacin (Table 1). RYU7 displayed heteroresistance to colistin using the Etest method, in that it appeared sensitive to colistin, with an MIC of 0.094 μg/mL, but colonies appeared in the inhibition zone (Fig. 1). In contrast, the isolate showed resistance to colistin when tested using the broth microdilution method. RYU7 possessed the bla_{TEM-1}, bla_{SHV-12}, bla_{ACT-2}, *aadA2*, and *aac(6')-IIc* genes, but did not harbor any carbapenemase-encoding genes. RYU7 was found to have a disrupted gene encoding an outer membrane protein, *ompC* [14], as well as a point mutation in a quinolone-resistance-determining region of *gyrA*, with an amino acid substitution of Asp87Met in GyrA [15]. RYU7 possessed mutations in genes encoding two-component regulatory systems, *phoPQ* and *pmrAB*, which have been associated with colistin resistance [16] (Table 2). *E. cloacae* RYU7 belonged to ST53. Only one *E. cloacae* isolate classified as ST53 has been reported to date in Japan (http://pubmlst.org/ecloacae/), but not in any other regions.

A comparison of the sequences of genes encoding two-component systems of *mgrB*, *phoPQ* and *pmrAB* in RYU7 and ATCC13047 showed RYU7 (a parental strain) had several mutations with amino acid substitutions, including a mutation in *mgrB*, six mutations in *phoP*, 26 in *phoQ*, 15 in *pmrA* and five in *pmrB* (Table 2).

Table 1
Antimicrobial susceptibility profile of *E. cloacae* RYU7.

	MICs (μg/mL)
	Broth microdilution
Ampicillin	>512
Amikacin	1
Cefepime	64
Cefmetazole	>256
Ceftazidime	>1024
Ciprofloxacin	16
Colistin	512
Doripenem	64
Gentamycin	256
Imipenem	32
Meropenem	32
Panipenem	128
Piperacillin	>512
Piperacillin-tazobactam	512
Tigecycline	16
Tobramycin	4

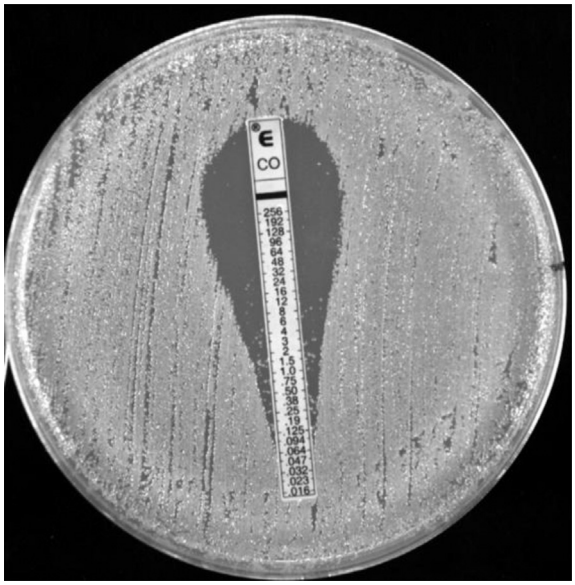

Fig. 1. Etest for colistin susceptibility of *E. cloacae* RYU7. Although RYU7 had an MIC of 0.094 μg/mL, colonies appeared in the inhibition zone.

3. Discussion

The carbapenem resistance of *E. cloacae* RYU7 is likely due to the synergistic effects of the hyperproduction of AmpC β-lactamase (ACT-2) and the loss of porin. ACT-2 hyperproduction in *E. cloacae* clinical isolates has been associated with reduced susceptibility to carbapenems [17]. Carbapenem resistance of *K. pneumoniae* has been reported due to the combination of bla_{ACT-1} β-lactamase production and porin inactivation [18].

Colistin heteroresistance in *E. cloacae* may be associated with mutations in genes encoding two-component regulatory systems (*mgrB*, *phoPQ* and *pmrAB*). For example, insertional inactivation of *mgrB*, the regulator of PhoQ/PhoP, has been found to result in colistin resistance in *K. pneumoniae* [19]. A single amino acid change (Asp191Thr) in PhoP is involved in colistin resistance, resulting in colistin heteroresistance in *K. pneumoniae* [9]. In addition, a single amino acid substitution (Thr157Pro) in PmrB has been found responsible for colistin resistance in *K. pneumoniae* [20]. To our knowledge, there have been no reports on mutations associated with both colistin resistance and colistin heteroresistance in *E. cloacae* due to different methods to test drug susceptibility, although colistin heteroresistance has been detected in an isolate of *E. cloacae* [8]. A study using a mouse infection model using colistin-heteroresistant *E. cloacae* clinical isolates revealed that, in the absence of colistin, innate immune defenses led an increased frequency of the resistant subpopulation, leading to inefficacy of subsequent colistin therapy, which may elucidate an enigmatic cause of antibiotic treatment failure [21].

To our knowledge, this is the first report of a colistin-heteroresistant *E. cloacae* in Japan. Although the isolate had several mutations in genes encoding two-component systems responsible to colistin resistance, we were unable, to identify the mutations responsible for colistin heteroresistance. Further investigations are necessary to determine the molecular mechanisms of colistin heteroresistance in *E. cloacae* and also to improve diagnostic kits to detect colistin heteroresistance in clinical laboratories. It is also necessary to establish adequate chemotherapy against infections with colistin-heretoresist pathogens.

Table 2
Amino acid mutations in RYU7 two-component regulatory systems.

Reference strains[a]	Genes Amino acid mutations
ATCC13047	
	mgrB
	I10V
	phoP
	A80V, L129I, A141L, S142A, N145D, H207Q
	phoQ
	K2R, R3RG, L4I, M5L, M9L, Q69R, S71T, V102I, H133I, R134L, K141Q, D150N, P168L, D169N, V178I, R190M, G193S, V196I, I212V, S244T, R297K, M298L, D431V, S448A, G464S, S484L
	pmrA
	A19G, S21A, A31G, A38S, V71I, D72A, N89T, S93A, Q120L, C143R, Q144R, S145D, L146Q, P147A, R218S
	pmrB
	E70D, T73S, V74T, R76S, T77A

[a] Amino acid sequences in RYU7 were compared with those in ATCC13047.

Contributors

TK and JF conceptualized and designed the study. KU, TT and KS analyzed the data and interpreted the results. KU and TS collected samples and screened drug-resistant isolates. All authors have read and agreed on the manuscript.

Ethical statements

This study was approved by the Ethical Committee, University of the Ryukyus (approval number: 890).

Funding

This study was supported by grants from Okinawa Communicable Disease Research Hub Formation Promotion Project, Okinawa Prefectural Government Commissioned Projects For Fiscal Year 2016, Japan; Research Program on Emerging and Re-emerging Infectious Diseases from Japan Agency for Medical Research and Development (AMED) Grant Number 18fk0108061, Japan and JSPS KAKENHI Grant Number 16K19133, Japan.

Conflict of interest

None to declare.

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