Journal of Medical Microbiology and Infectious Diseases

JoMMID
ISSN: 2345-5349
eISSN: 2345-5330

Identification of Carbapenemase within Class 1 Integron Structure in Intrinsically Colistin-resistant Enterobacteriaceae

Negin Bolourchi[1], Omid Azizi[2], Mahdi Rohani[1], Soha Seyyedi Abhari[1], Armaghan Soltani Shirazi[1], Farzad Badmasti[1, 3]*

[1]Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran; [2]Health Sciences Research Center, Torbat Heydariyeh University of Medical Sciences; [3]Microbiology Research Center (MRC), Pasteur Institute of Iran

ARTICLE INFO

Original Article

Keywords: Antimicrobial resistance, Carbapenemase, Integron, Plasmid

Received: 22 Sep. 2021
Received in revised form: 11 Oct. 2021
Accepted: 02 Oct. 2021
DOI: 10.52547/JoMMID.9.4.203

***Correspondence**
Email: fbadmasti2008@gmail.com
Tel: +982166405535
Fax: +982166405535

ABSTRACT

Introduction: Identification of carbapenem resistance genes in intrinsically colistin-resistant Enterobacteriaceae (ICRE), i.e., *Proteus* spp., *Providencia* spp., *Serratia* spp., and *Morganella* spp., is necessary for preventing such life-threatening bacteria. Here, we characterized carbapenemase resistance genes and their carriage within class 1 integron structures in clinical intrinsically colistin-resistant Enterobacteriaceae. **Methods:** Clinical samples were collected from six general hospitals in Tehran and Torbat-e-Heydarieh, Iran, from 2018 to 2020. The isolates were identified by standard microbiological techniques and 16s rRNA sequencing. The resistance to imipenem and colistin was determined by disk diffusion and minimum inhibitory concentration (MIC) assays. PCR-sequencing was performed to detect primary carbapenemase-encoding genes, including $bla_{\text{OXA-48}}$, bla_{IMP}, bla_{VIM}, bla_{KPC}, and bla_{NDM}. **Results:** Three *Proteus mirabilis*, three *Providencia stuartii*, and two *Serratia marcescens* isolates were identified. The MIC_{IMP} values of all isolates were >16 µg/ml. The $bla_{\text{VIM-1}}$ gene and $bla_{\text{KPC-2}}$ genes were detected in five and four isolates, respectively. Cassette arrays consisting of $bla_{\text{VIM-1}}$-*aacA7-dhfrA1-aadA1*were detected in five isolates and *aadB* in one isolate. The $bla_{\text{VIM-1}}$ gene in all $bla_{\text{VIM-1}}$ positive isolates was located on class 1 integrons, while the $bla_{\text{KPC-2}}$ carriage was not related to these genetic elements. The *in silico* analysis of the large plasmids carrying $bla_{\text{VIM-1}}$-*aacA7-dhfrA1-aadA1* array belonging to the IncA/C incompatibility group showed transmissibility among Enterobacteriaceae members. **Conclusions:** Transferable plasmid harboring $bla_{\text{VIM-1}}$ in ICREs can pose a severe threat to public health. More investigations are required to depict the epidemiology of these underestimated carbapenem-resistant Enterobacteriaceae members.

INTRODUCTION

The risk of antibiotic resistance is rapidly increasing worldwide [1]. Carbapenems are considered the most effective antibiotics for the treatment of healthcare-associated infections [2]. Carbapenem-resistant Enterobacteriaceae (CRE) are considered a major emerging threat to global public health due to the rapid emergence in clinical settings worldwide, limited treatment options, and high morbidity and mortality rates associated with infections [3]. However, the overuse of carbapenems has led to the emergence of carbapenem resistance driven mainly by the production of carbapenemases such as OXA-48, GES, KPC, IMP, VIM, and NDM [1, 2]. The CRE was listed among the first "critical priority pathogens" by the World Health Organization (WHO) in 2017 [4, 5].

The increasing number of multidrug-resistant (MDR) Gram-negative bacteria, particularly carbapenem-resistant Enterobacterales, has led to using colistin as the antimicrobial of last resort [6]. However, resistance to carbapenems seems to be underestimated in less-studied Enterobacteriaceae members such as *Proteus* spp., *Serratia* spp., and *Providencia* spp. [2, 7]. Among the Enterobacteriaceae members, *Proteus* spp., *Providencia* spp., *Edwardsiella* spp., *Serratia marcescens*, and *Morganella morganii* are considered intrinsically colistin-resistant Enterobacteriaceae (ICRE). Consequently, only a few antibiotics remain available for the clinical treatment of CRE-infected patients [8, 9].

Despite the lower isolation rate among these bacteria compared to highly prevalent Enterobacteriaceae

[DOI: 10.52547/JoMMID.9.4.203]

e.g., *Escherichia coli* and *Klebsiella pneumoniae*, the detection and reporting of carbapenem resistance in ICREs are highly significant [10]. Because the drug of choice against these bacteria is carbapenems, they are considered almost the only therapeutic options [11]. Furthermore, the emergence of carbapenem resistance in ICREs is a critical medical situation. Therefore, early detection of ICRE isolates can significantly control their spread in healthcare systems. In the present study, we reported carbapenem-resistant intrinsically colistin-resistant *P. mirabilis*, *S. marcescens*, and *P. stuartii* in clinical specimens. Also, we characterized the structure of class 1 integron harboring carbapenemase genes.

MATERIAL AND METHODS

Bacterial isolates. Clinical samples were collected from six general hospitals in Tehran and Torbat-e-Heydarieh, Iran, from 2018 to 2020, and isolates were identified by standard microbiological techniques, including colony morphology, Gram stain, oxidase, and a conventional biochemical test panel. Species identification was performed by PCR-sequencing of the 16s rRNA [12].

Antimicrobial susceptibility testing. Antimicrobial susceptibility was determined using the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2020 [13]. The antimicrobials tested included amoxicillin (10µg), cefotaxime (30µg), ceftazidime (30µg), ciprofloxacin (5µg), gentamycin (10µg) and imipenem (10µg). Susceptibility to colistin was determined by the agar dilution method based on clinical breakpoints defined by CLSI [13]. The minimum inhibitory concentrations (MICs) of the CRE isolates to imipenem were determined by the microdilution method according to the recommendations and interpretation criteria of the CLSI [13]. The strain *E. coli* ATCC 25922 was used as a control in antimicrobial susceptibility testing.

Molecular detection of carbapenemases and class 1 integrons. Whole DNA was extracted from bacterial isolates by the boiling method as previously described [14]. The CRE isolates were screened for the carbapenemases including $bla_{\text{OXA-48}}$, bla_{IMP}, bla_{VIM}, bla_{KPC}, and bla_{NDM} using PCR, as previously described [15], with the primers designed by others (Table 1), and PCR conditions reflected elsewhere [16]. All integron-positive strains were screened for variable regions of the class 1 integron using primers 5'-CS /3'-CS. Sequencing of the purified DNA was performed by the Bioneer Company with Sanger dideoxy chain termination method using Applied Biosystems 3730/3730Xl DNA Analyzers (Applied Biosystems). The nucleotide sequence analysis was performed using the BLAST tool at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were manually analyzed using CLC workbench software version 20 (CLC Bio, Aarhus, Denmark).

Plasmid analysis. A collection of plasmids carrying class 1 integron was retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/), and the plasmids incompatibility (Inc) was determined by the online web tool PLSDB (https://ccb-microbe.cs.uni-saarland.de/plsdb/) [17]. The presence of relaxasome complex in plasmids was determined using the online web tool oriTfinder to determine their conjugative capability (https://bioinfo-mml.sjtu.edu.cn/oriTfinder/) [18]. This web tool was developed to facilitate the rapid detection of *oriT*, the origin of transfer sequence in bacterial plasmids. It can also detect the T4CP and T4SSs as components involved in plasmids horizontal transfer. The online web tool CARD identified all antimicrobial resistance genes on plasmids (https://card.mcmaster.ca/analyze/rgi) [19]. Plasmid sequence alignment was performed by Mauve version 2.4.0, an open-free software for multiple alignments of genomic sequences that exhibit the arrangement of gene cassettes within different sequences

Table 1. Primers used for amplification of 16s rRNA, carbapenemase-encoding genes, and class 1 integrons

Primer	Primer sequence (5'→3')	Product size (bp)	Reference
IMP	F: GGAATAGAGTGGCTTAAYTCTC R:CCAAACYACTACGTTATCTKGAG	189	[37]
VIM	F: AGTGGTGAGTATCCGACAG R:ATGAAAGTGCGTGGAGAC	261	[37]
NDM	F: GGCAGCACACTTCCTATCTC R:GTTGATCTCCTGCTTGATCC	155	[37]
KPC	F: ATCGCCGTCTAGTTCTGCTG R:CCCTCGAGCGCGAGTCTA	850	[37]
OXA48	F: TTGGTGGCATCGATTATCGG R:CATCAAGTTCAACCCAACCG	658	[37]
16S rRNA	F: AGAGTTTGATYMTGGCTC R:CAKAAAGGAGGTGATCC	~1500	[37]
intI1	F: TCTCGGGTAACATCAAGG R: AGGAGATCCGAAGACCTC	250	[16]
Conserved segment of class 1 integrons	5′-CS: GGCATCCAAGCAGCAAG 3′-CS: AAAGCAGACTTGACCTGA or 3′-CS:GAAGCGGCGTCGGCTTGA	Variable	[16]

[DOI: 10.52547/JoMMID.9.4.203]

RESULTS

Demographic data. Nine non-duplicated carbapenem-resistant, intrinsically colistin-resistant Enterobacteriaceae isolates were identified (Table 2) in the clinical isolates originated from urine (n=5), blood (n=2), sputum (n=1), and surgery site (n=1). The patients' age ranged from 41 to 66 years, except for one 27-year-old male. Three species, including *P. mirabilis* (n=4), *P. stuartii* (n=3), and *S. marcescens* (n=2), were identified by biochemical and molecular tests. Four out of five urine samples contained *P. mirabilis*.

Table 2. The data for carbapenem-resistant, intrinsically colistin-resistant Enterobacteriaceae isolates

No.	Strain	Isolation source	Gender/Age	Hospital source	Patient's outcome	MIC (µg/ml) of imipenem	Antimicrobial resistance Profile*	Carbapenemase genes	*intI1*	Variable region of class 1 integrons
1	*P. stuartii*	Urine	F/53	A	Discharge	>32	IPM, CTX, AMX, GEN	bla_{VIM-1}	+	bla_{VIM-1}-*aacA7-dhfrA1-aadA1*
2	*P. stuartii*	Tracheal	M/46	B	Death	>64	IPM, GEN, CAZ, AMX	bla_{KPC-2}	-	-
3	*P. stuartii*	Blood	M/61	B	Death	>16	IPM, CTX, GEN	bla_{VIM-1}	+	bla_{VIM-1}-*aacA7-dhfrA1-aadA1*
4	*P. mirabilis*	Urine	F/41	C	Discharge	>32	IPM, CTX, GEN, CIP, AMX	bla_{VIM-1}	+	bla_{VIM-1}-*aacA7-dhfrA1-aadA1*
5	*P. mirabilis*	Urine	F/55	B	Death	>64	IPM, CTX, CIP, CAZ, AMX	bla_{VIM-1}	+	bla_{VIM-1}-*aacA7-dhfrA1-aadA1*
6	*P. mirabilis*	Urine	M/47	C	Death	>32	IPM, GEN, CIP, AMX	bla_{VIM-1}	+	bla_{VIM-1}-*aacA7-dhfrA1-aadA1*
7	*P. mirabilis*	Urine	M/66	D	Discharge	>32	IPM, CTX, GEN, CIP, AMX	bla_{KPC-2}	+	*aadB*
8	*S. marcescens*	Surgery	F/27	E	Discharge	>16	IPM, CTX, AMX	bla_{KPC-2}	-	-
9	*S. marcescens*	Blood	M/58	F	Death	>32	IPM, CTX, GEN, AMX	bla_{KPC-2}	-	-

*AMX: amoxicillin, CAZ: ceftazidime, CTX: cefotaxime, CIP: ciprofloxacin, GEN: gentamycin, IMP: imipenem, M: Male, F: Female.

Antimicrobial resistance test. All isolates were resistant to imipenem by disk diffusion test. The MIC of imipenem among the bacterial isolates ranged from 32 to 64 µg/ml for *P. mirabilis* and *S. marcescens* and 16 to 64 µg/ml for *P. stuartii*. As tested by the agar dilution method, the MIC for colistin was >8 µg/ml for all isolates.

Molecular detection of carbapenemases. PCR screening for the carbapenem-resistant genes among the CRE isolates revealed only bla_{VIM-1} and bla_{KPC-2}. One *P. stuartii*, one *P. mirabilis*, and two *S. marcescens* isolates were positive for bla_{KPC-2}, and three *P. mirabilis* and two *P. stuartii* isolates were positive for bla_{VIM-1}. The bla_{IMP}, bla_{NDM}, and bla_{OXA-48} genes were not detected among the isolates. Table 2 shows the data on carbapenem-resistant, intrinsically colistin-resistant Enterobacteriaceae isolates.

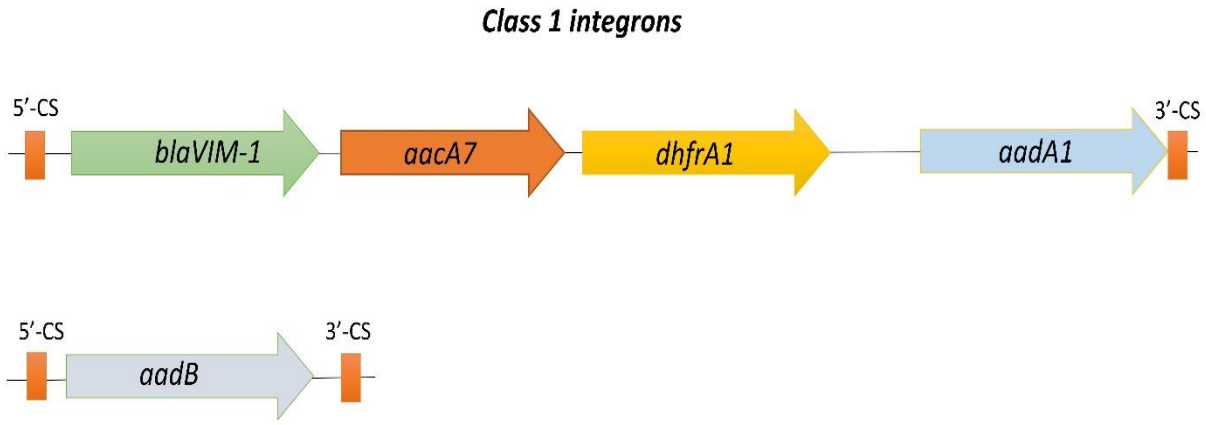

Fig. 1. The gene cassette arrays consisting of bla_{VIM-1}-*aacA7-dhfrA1-aadA1* and *aadB*. The bla_{VIM-1} gene carried on class 1 integrons in all positive VIM isolates, while the bla_{KPC-2} gene was not associated with class 1 integrons.

[DOI: 10.52547/JoMMID.9.4.203]

Molecular detection of class 1 integron. PCR detected the presence of integrase gene intI1 in 6 out of 9 isolates. Class 1 integrons were widespread among these clinical isolates. However, there was no *intI1* in *S. marcescens*. The class 1 integrons cassette arrangements were characterized by amplification-sequencing of the internal variable regions (IVRs). Sequencing confirmed the presence of cassette arrays consisting of *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* (5/9) and *aadB* (1/9). The *bla*$_{VIM-1}$ gene in all isolates was on class 1 integrons, while the *bla*$_{KPC-2}$ gene was not related to class 1 integrons (Fig. 1).

Plasmid analysis harboring *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* array. We collected eight plasmids from the GenBank database. Two categories of plasmid harbored *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* array, including plasmids (IncA/C) with >100 Kbp and ~52% CG content, isolated from different bacteria. The second group was plasmids (IncR-1) with <100 Kbp and ~53% CG content, isolated from *K. pneumoniae*. Characterization of resistance genes on plasmid showed that the first category had heterogenic resistance genes compared to the second group. All plasmids (except pG06) had intact conjugal apparatus. See Table 3. Genetic similarities were observed among all eight plasmids. The alignment of plasmid sequences showed that plasmids CP031573, JQ824049, CP029718, CP032168, CP031584, and CP031610 (the first group) possessed consensus genetic structures with different arrangements. The CP032168 and CP031584 (both belonging to *K. pneumoniae*) had identical nucleotide arrangements. KU665641 and CP023926 in the second group had consensus structures lacking large compartments of the first group. See Fig. 2.

Table 3. Plasmid analysis harboring *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* array among Enterobacteriaceae

Plasmid name	Accession number	DNA size (bp)	CG%	Bacterium source	Inc typing	oriT, relaxase, T4SS and T4CP	Other resistance genes
pIncAC2	CP031573	164095	52.45%	*E. hormaechei*	IncA/C	*oriT* (+), relaxase (+), and T4CP (+)	*ant* (2")-Ia, *qnrA1*, *bla*$_{TEM-1}$, *mphA*, *qacEdelta1* and *sul1*
pTC2	JQ824049	180184	52.51%	*P. stuartii*	IncA/C	*oriT* (+), relaxase (+), and T4CP (+)	*mphA*, *bla*$_{SHV-5}$, *dfrA12*, *aph*(3')-Ia, *qacEdelta1* and *sul1*
Unnamed	CP029718	158987	52.56%	*E. cloacae*	IncA/C	*oriT* (+), relaxase (+), and T4CP (+)	*mphA*, *QnrA1*, *dfrA12*, *qacEdelta1* and *sul1*
Unnamed	CP032168	158987	52.56%	*K. pneumoniae*	IncA/C	*oriT* (+), relaxase (+), and T4CP (+)	*mphA*, *qnrA1*, *dfrA12*, *qacEdelta1*and *sul1*
pIncAC2	CP031584	164082	52.45%	*K. pneumoniae*	IncA/C	*oriT* (+), relaxase (+), and T4CP (+)	*ant* (2")-Ia, *qnrA1*, *bla*$_{TEM-1}$, *mphA*, *qacEdelta1* and *sul1*
pIncAC2	CP031610	160292	52.51%	*E. coli*	IncA/C	*oriT* (+), relaxase (+), and T4CP (+)	*mphA*, *qnrA1*, *dfrA12*, *qacEdelta1* and *sul1*
pG06	KU665641	53618	53.02%	*K. pneumoniae*	IncR	*oriT* (+), relaxase (-), and T4CP (-)	*ant* (3")-IIa, *aph*(6)-Id, *aph*(3")-Ib, *dfrA12*, *qacEdelta1* and *sul1*
Unnamed	CP023926	78541	53.50%	*K. pneumoniae*	IncR	oriT(+), relaxase (+),and T4CP (-)	*ant*(3")-IIa, *aph*(6)-Id, *aph*(3")-Ib, *dfrA12*, *qacEdelta1* and *sul1*

Nucleotide sequence accession numbers. The *bla*$_{VIM-1}$ and *bla*$_{KPC-2}$ gene sequences and the *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* and *aadB* arrays were deposited at DDBJ/ENA/GenBank under the accession MZ359738, MZ359739, MZ541989, and MZ541990, respectively.

DISCUSSION

Nosocomial infections caused by carbapenem-resistant ICRE pose a severe clinical threat and contribute to pan-drug resistance. Reports suggest that the prevalence of carbapenem-resistant ICRE is increasing worldwide [20, 21].

The mortality rate of our ICRE isolates was high; five out of nine patients (55%) were expired, similar to a report from Greece [22]. Unfortunately, the patient's outcomes are rarely available in many other reports on naturally colistin-resistant Enterobacteriaceae. Therefore, determining the average mortality rate caused by carbapenem-resistant ICRE is complicated and requires further investigations.

Of the four *P. mirabilis* isolates, only one harbored *bla*$_{KPC-2}$, while the rest were positive for *bla*$_{VIM-1}$, indicating a higher prevalence of *bla*$_{VIM-1}$ in our *P. mirabilis* isolates. The *bla*$_{KPC-2}$-harboring *P. mirabilis* was first reported in the United States (US) in 2008 [23], followed by other reports from china in 2010 and Brazil in 2015 [24, 25]. Reports of Metallo beta-lactamase *bla*$_{VIM}$-like genes in *P. mirabilis* are available from Greece and Italy in 2006 and 2008, respectively, and

[DOI: 10.52547/JoMMID.9.4.203]

more recently from Bulgaria [25-27]. The *bla*$_{VIM}$-like carriage can be utilized to determine the levels of carbapenem resistance in *P. mirabilis*. Bontron *et al.* (2019) determined the *blaVIM-1* copy numbers in four susceptible P. mirabilis variants using quantitative PCR; comparing the results with the imipenem MIC values suggested that the phenotypic resistance degree to carbapenems in *bla*$_{VIM-1}$ positive *P. mirabilis* depends on the copy number. [28].

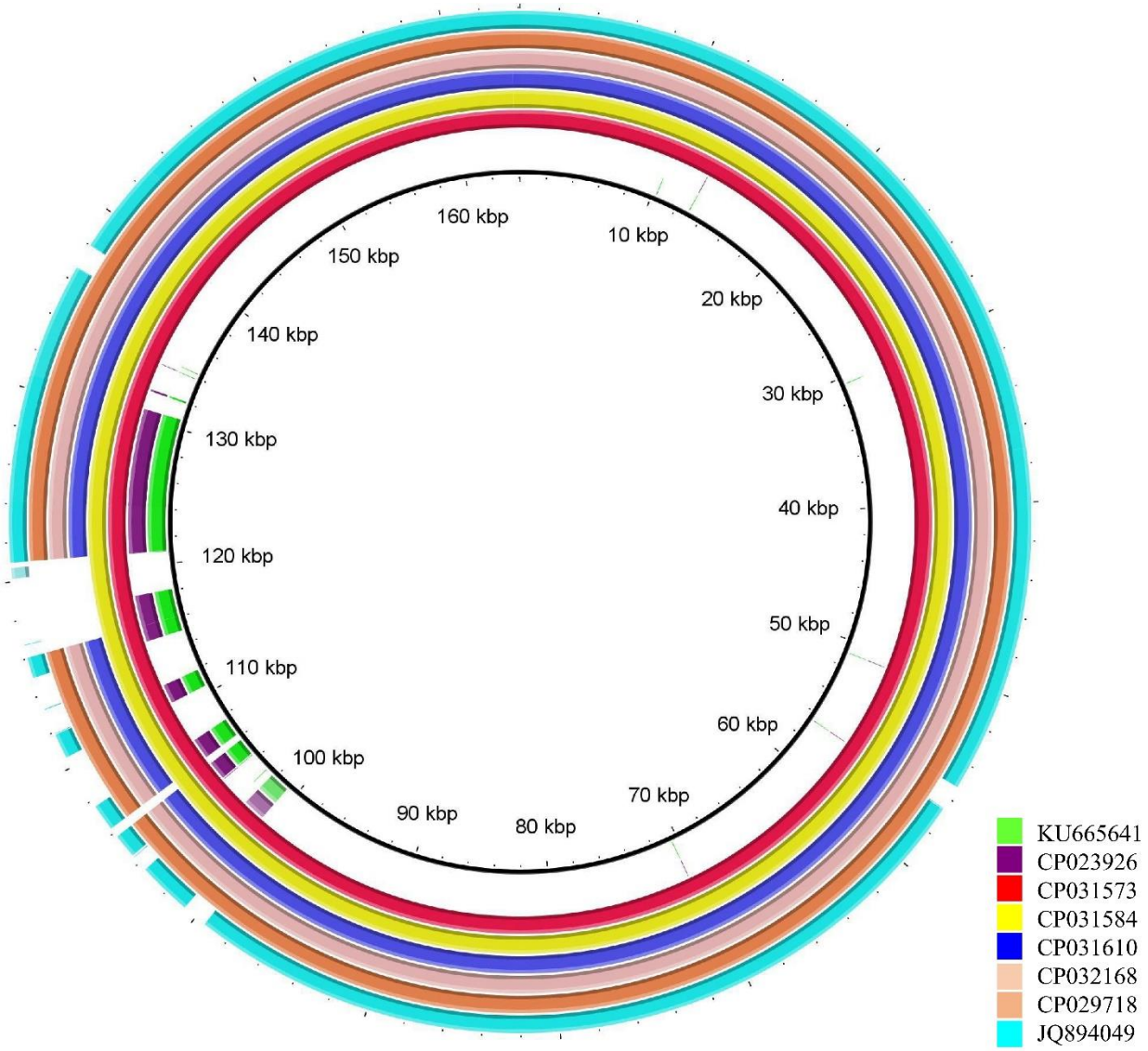

Fig. 2. Multiple sequence alignments of plasmids harboring *bla*$_{VIM-1}$*-aacA7-dhfrA1-aadA1* array among Enterobacterales. Sequences comparing showed that larger plasmids CP031573, JQ824049, CP029718, CP032168, CP031584, and CP031610 possessed consensus genetic structures with different arrangements (the first group), while smaller plasmids KU665641 and CP023926 were categorized in the second group.

Today, 12 carbapenem-resistant *P. stuartii* incidents comprising 39 cases and three outbreak reports are available worldwide [29]. There are reports for *bla*$_{KPC-2}$ and *bla*$_{VIM}$-like for this bacterium from Saudi Arabia, Japan, Taiwan, and other countries [29-31]. We detected three non-duplicated clinical isolates with three different sources, two of which produced *bla*$_{VIM-1}$ and one produced *bla*$_{KPC-2}$.

In our study, both *S. marcescens* isolates encoded *bla*$_{KPC-2}$; this bacterium typically exhibits carbapenem resistance by encoding different *bla*$_{KPC}$ genes, first reported in China [32]. Previously, *S. marcescens* was a less commonly reported bacterium in the clinical setting [33]. However, reports from Brazil and China of clinical carbapenem-resistant *S. marcescens* strains showed its clinical importance. In these studies, most isolates were

[DOI: 10.52547/JoMMID.9.4.203]

blaKPC-2 positive, indicating a high prevalence of this type of plasmid-mediated carbapenemase..

The sequencing results indicated that all *bla*$_{VIM-1}$ positive isolates carried this gene within the same class 1 integron structure. The *bla*$_{VIM-1}$ carriage in our intrinsically colistin-resistant CRE isolates was associated with *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* cassettes. The blaVIM$_{-1}$ carriage by this structure was previously reported in *P. stuartii* isolates in 2016 [34]. Similarly, *bla*$_{VIM-1}$-*aacA7-dhfrA1-aacA1* was identified in *bla*$_{VIM-1}$ positive *P. mirabilis* isolates in Switzerland in 2019. The integron mapping indicated that integron class 1 truncated by the IS*1* element resulted in *qacED1* and *sul1* genes loss. This recombination in *P. mirabilis* was mediated by surrounding IS26 elements resulting in the acquisition of *bla*$_{VIM-1}$ [28]. Reports of the *bla*$_{VIM-1}$ carriage in *P. mirabilis* isolates by a class 1 integron containing *aac(6')-I*, *dhfrA1*, and *ant(3")-Ia* gene cassettes are available from Bulgaria in 2016 [35].

It seems that the IncA/C plasmids (e.g., plasmid 120-kb, pPM91) carrying *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* gene cassettes are transferable between different Enterobacterales species [36]. Our data showed that these plasmids harbor intact conjugative elements, including the origin of transfer site *oriT*, T4CP, and T4SS. Thus, it can be concluded that these plasmids are effectively being transferred among bacteria. Moreover, the plasmid analysis indicated that IS elements including IS*1*, IS*26*-4, IS*26*-3, and IS*6100* are frequently found in association of integron class 1 harboring *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* array. The results of plasmid sequence alignment indicated that plasmids harboring *bla*$_{VIM-1}$-*aacA7-dhfrA1-aadA1* have high genetic similarities implying their high levels of conservation. Transferable plasmids harboring *bla*$_{VIM-1}$ in ICREs pose a severe threat to public health. Further and continuous investigations are needed to detect CRE in clinical settings. Our epidemiological understanding requires to prevent the expansion of carbapenem-resistant Enterobacteriaceae in clinical settings.

ACKNOWLEDGMENT

We thank the laboratory team of the Bacteriology Department of Pasteur Institute of Iran and the Hospitals of Torbat Heydariyeh.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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Cite this article:
Bolourchi N, Azizi O, Rohani M, Seyyedi Abhari S, Soltani Shirazi A, Badmasti F. Identification of Carbapenemase within Class 1 Integron Structure in Intrinsically Colistin-resistant Enterobacteriaceae. J Med Microbiol Infect Dis, 2021; 9 (4): 203-209. DOI: 10.52547/JoMMID.9.4.203.

[DOI: 10.52547/JoMMID.9.4.203]