1 **Title**

2 Characterization of a Novel Plasmid in *Serratia marcescens* Harboring *bla*_{GES-5} Isolated from
3 a Nosocomial Outbreak in Japan

4 **Running title**

5 Plasmid in *S. marcescens* Confers Carbapenem Resistance

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Abstract

Serratia marcescens is a nosocomial pathogen with carbapenem resistance, limiting the availability of effective treatment options. In this study, we performed molecular characterization of GES-5 carbapenemase-producing *S. marcescens* isolated from an outbreak in Japan. Comparative genetic analysis revealed that the *bla*_{GES-5}-encoding plasmid p2020-O-9 is a unique plasmid contributing towards carbapenem resistance. Furthermore, this study highlights the necessity of surveillance programs for monitoring novel, along with commonly occurring carbapenemases in clinical settings.

KEYWORDS: *Serratia marcescens*, carbapenemase, *bla*_{GES-5}, whole-genome sequencing

Abbreviations: WGS: whole-genome sequencing

28 *Serratia marcescens* is a major opportunistic pathogen known to cause nosocomial
 29 infections associated with high morbidity and mortality (1, 2). As this bacterium shows
 30 resistance towards widely used carbapenem class of antibiotics, treatment options available
 31 for nosocomial infections becomes limited (3-5). Carbapenemases (a class of β -lactamases)
 32 identified in Japan comprise KPC, GES, NDM, IMP, VIM, and OXA-48 variants, with IMP
 33 being the most prevalent in the country (6). GES type Amber class A β -lactamases, in
 34 addition to exhibiting β -lactam resistance, also confer carbapenemase activity, such as the
 35 GES-5 variant (7, 8). In recent times, nosocomial outbreaks have largely been attributed to
 36 GES-5 carbapenemase-producing bacterial strains in various countries including Japan,
 37 wherein the first outbreak of *P. aeruginosa* harboring the *bla*_{GES-5} gene occurred in the year
 38 2014 (9-13). This study reports the genetic characteristics of a different GES-5 producing

bacterium, *S. marcescens* isolated from an ICU outbreak in Japan in 2020.

A total of six carbapenem-resistant *S. marcescens* strains were isolated from the samples from three ICU patients collected during May-October 2020 (Table 1). Strain was identified using MALDI Biotyper (Bruker Daltonics K.K., Yokohama, Japan), and the minimal inhibitory concentrations (MICs) of various antimicrobial agents were determined by E-test (bioMérieux Japan Ltd., Tokyo, Japan) following the manufacturer's guidelines. As indicated in Table 1, all of the six *S. marcescens* strains were resistant to imipenem, meropenem, and ceftazidime. Modified carbapenem inactivation method (mCIM) indicated the absence of carbapenemase genes in all the strains. This was supported by the negative results of PCR screening conducted for the major carbapenemase genes *bla*_{IMP}, *bla*_{NDM}, *bla*_{KPC}, and *bla*_{OXA-48-like}; however, a positive result was obtained for *bla*_{GES}. The MICs of antimicrobial agents (except ampicillin (AM) and cefotaxime (CTX)) in 2020-O-14-2 strain lacking the *bla*_{GES-5} gene, isolated from patient B, were significantly lower than all the other

isolates. Whole-genome sequencing of the six isolates harboring *bla*_{GES-5} gene and the one lacking the gene was done for analyzing single nucleotide variations and deriving genetic relationships among the seven isolates to understand the mechanisms of antimicrobial resistance. A survey for analyzing environmental contamination in the ICU was conducted during the outbreak period; however, the presence of carbapenem-resistant *S. marcescens* strains was not detected.

Genomic libraries of all seven isolates were prepared using the Nextera XT DNA library prep kit (Illumina, San Diego, CA, USA) and sequenced on the iSeq 100 system (Illumina). Additionally, Oxford Nanopore Technologies (ONT) were used for constructing a sequencing library of the strain 2020-O-9 with the help of Rapid Barcoding Kit (SQK-RBK004), and sequenced on a MinION device using flow cell type R9.4.1 (FLO-MIN106D). Hybrid assembly of 2020-O-9 genome was performed using Unicycler

65 v0.4.8 (14), and a circular chromosome and plasmid (p2020-O-9) sequence was obtained.

66 The genome sequences were annotated using DFAST (<https://dfast.nig.ac.jp/>) and are

67 available in DDBJ (DNA Data Bank of Japan) under the accession numbers AP024847

68 (chromosome) and AP024848 (plasmid). SNP analysis of the remaining 6 strains was

69 performed taking the genome sequence of 2020-O-9 as a reference, using bwa version 0.7.17,

70 samtools version 1.9, and VarScan v2.4.4 (15-17). The analysis revealed high degree of

71 genetic homogeneity with 0 to 5 SNPs consistent among the seven strains isolated, including

72 GES-5 negative strain 2020-O-14-2. The whole-genome sequencing reads are available in

73 DDBJ Sequence Read Archive under the accession number listed in Table 1. Since MICs of

74 carbapenems in 2020-O-14-2 strain lacking the *bla*_{GES-5} gene were significantly decreased, it

75 is evident that the newly detected *bla*_{GES-5} containing plasmid contributes to carbapenem

76 resistance.

77 Further genetic characterization of p2020-O-9 was done using ResFinder 3.2 to identify
78 resistance genes, and plasmid incompatibility replicon typing was performed using
79 PlasmidFinder 2.0 developed by the Center for Genomic Epidemiology
80 (<http://www.genomicepidemiology.org/>). The chromosomal sequence of 2020-O-9
81 consisted of a single antimicrobial resistance gene *bla*_{SRT-2}, as identified by existing
82 databases. p2020-O-9 is a novel 23,921-bp circular untypeable plasmid, with the *bla*_{GES-5}
83 gene located between *intI1* of class 1 integron and the *qacEΔ1* and *sulI*, together with a gene
84 of GNAT-family N-acetyltransferase, *aac(6)-29a*. It has a GC-content of 61% and carries 26
85 protein-coding genes (Fig.1). Sequence analysis using NCBI revealed that the plasmid
86 backbone of p2020-O-9 showed highest similarity with plasmid pCAV1374-16 from

87 *Klebsiella oxytoca* with a query coverage of 59%; however, there was no *bla*_{GES-5} in

88 pCAV1374-16 (Accession no. CP011628). The class 1 integron cassette around the *bla*_{GES-5}

89 gene in p2020-O-9 is similar to that of pMRY16-414SMA_2 from *S. marcescens* (Accession

90 no. LC486677) and the plasmid from *Aeromonas hydrophila* strain WCHAH 01-derived

91 plasmids pGES5 (Accession no. KR014105) (Fig. 1). Plasmid sequences similar to the

92 backbone of p2020-O-9 such as pCAV1374-16, have been registered in databases from a

93 wide range of bacterial species. It is believed that *bla*_{GES-5} was integrated into the class 1

94 integron cassette of a plasmid like pCAV1374-16 to construct the novel p2020-O-9 plasmid.

95 This suggests that p2020-O-9 could be an untypeable plasmid with the potential to distribute

96 itself widely in *Enterobacteriaceae*.

In summary, we reported the molecular characterization of GES-5 producing *S. marcescens* strain harboring a novel *bla*_{GES-5} gene-encoding plasmid, p2020-O-9 isolated from the ICU outbreak in Japan. The prevalence and transmission of GES-producing bacteria is largely underestimated due to their rare presence in clinical isolates. However, through this study, we highlight the necessity of surveillance programs for monitoring novel, as well as commonly occurring carbapenemases in clinical settings.

Data availability

Sequence data that support the findings of this study have been deposited in DDBJ (<https://www.ddbj.nig.ac.jp/>) with the accession numbers AP024847 and AP024848.

108 **Author contributions**

109 NN, RN designed the study methods and wrote the first draft of the manuscript. NN, RN,
110 SK collected the data. NN, RN analyzed the data. TI contributed to the writing of the
111 manuscript.

112

113 **Ethical approval**

114 This study was approved by the Ethical Review Committee of the Kobe Institute of Health
115 (approval No. SenR3-9).

116

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123 We declare no conflicts of interest.

124

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Figure legends

Figure 1.

Circular representation of p2020-O-9 was generated using BLAST Ring Image Generator

0.95 (18). A comparison of the plasmid with pMRY16-414SMA_2 from *Serratia marcescens*

MRY-414SMA (LC486677), pGES5 from *Aeromonas hydrophila* WCHAH 01

(KR014105), and pCAV1374-16 from *Klebsiella oxytoca* (CP011628). The outermost circle

indicates the coding sequence of p2020-O-9. Red, carbapenemase genes; blue, other

antimicrobial resistance genes; purple, IS6100; orange, transposase, and recombinase genes;

green, integrase genes; grey, other genes or coding sequences.

TABLE 1. Information and the antibiotic susceptibility of the *Serratia marcescens* isolated from three patients involved in the ICU outbreaks

Patients	Age (yr), sex ^a	No. of isolate	Date of isolation (year/month/day)	Specimens	MICs (μg/ml) for ^b													<i>bla</i> _{GES-5}	DDBJ accession no. of read data
					AM	CTX	CAZ	FEP	ATM	IMP	MPM	DOR	ETP	AK	MC	CIP	PMB		
A	76, F	2020-O-9	2020/5/25	Sputum	>256	>32	>32	6	6	>32	>32	>32	>32	>256	4	0.5	>1024	+	DRR308222
		2020-O-12	2020/6/23	Blood	>256	>32	>32	3	4	>32	>32	>32	>32	>256	3	1	>1024	+	DRR308223
		2020-O-21-2	2020/8/3	Sputum	>256	>32	>32	2	3	6	>32	>32	8	64	3	1.5	64	+	DRR308224
		2020-O-21-3	2020/8/4	Blood	>256	>32	>32	2	8	>32	>32	24	>32	96	3	1.5	128	+	DRR308225
B	70, M	2020-O-14-1	2020/6/19	Blood	>256	>32	>32	4	6	>32	>32	>32	>32	128	12	>32	96	+	DRR308226
		2020-O-14-2			>256	>32	2	3	4	1	1.5	1.5	4	16	3	3	64	-	DRR308227
C	59, M	2020-O-25	2020/10/15	Ascites	>256	>32	>32	3	8	>32	>32	>32	>32	>256	3	0.38	>1024	+	DRR308228

^a F, feamale, M, male.

^b ampicillin (AM), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), imipenem (IMP), meropenem (MPM), doripenem (DOR), ertapenem (ETP), amikacin (AK), minocycline (MC), ciprofloxacin (CIP), polymyxin B (PMB)

Figure 1. Circular representation of p2020-O-9 was generated using BLAST Ring Image Generator 0.95 (18). A comparison with the pMRY16-414SMA_2 from *Serratia marcescens* MRY-414SMA (LC486677), pGES5 from *Aeromonas hydrophila* WCHAH 01 (KR014105), and pCAV1374-16 from *Klebsiella oxytoca* (CP011628). The outermost circle shows the coding sequence of p2020-O-9. Red, carbapenemase genes; blue, other antimicrobial resistance genes; purple, IS6100; orange, transposase and recombinase genes; green, integrase genes; grey, other genes or coding sequences.