Presence of VIM positive *Pseudomonas* species in chickens and their surrounding environment

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Metallo-β-lactamase gene *bla*$_{VIM}$ was identified on the chromosome of four *Pseudomonas* spp. isolates from a chicken farm, including one *P. aeruginosa* from swallow (*Yanornis martini*), one *P. putida* from fly, and two *P. putida* from chickens. The four isolates shared two variants of *bla*$_{VIM}$-carrying genomic contexts, which resemble the corresponding regions of clinical MBL-producing *Pseudomonas* species. Our study suggests the surveillance of carbapenemase-producing bacteria in livestock and their surrounding environment is urgently needed.

Carbapenems are critically important antimicrobials as a last line of defense against multidrug-resistant Gram-negative bacterial infections (1, 2). As such, the increasing prevalence of carbapenemase-producing isolates in the animal husbandry is of great concern. While the metallo-β-lactamase (MBL) producing bacteria has been commonly identified from food animals (3-7), *bla*$_{MBL}$-carrying *Pseudomonas* spp. are very rarely reported in the animal husbandry or the surrounding environment. Although we have reported the high prevalence of NDM in Enterobacteriaceae from poultry production of Shandong province (7), carbapenemase-producing non-Enterobacteriaceae isolates has not been identified from the same region. Here, we report four chromosome-borne VIM-positive *Pseudomonas* isolates: one *P. aeruginosa* isolates from a swallow (*Yanornis martini*), one *P. putida* isolate from a fly, and two *P. putida* isolates from chickens. The *bla*$_{VIM-2}$ was identified in the *P. aeruginosa* isolate, but 27-bp at the 3′-terminal region of *bla*$_{VIM-2}$ was truncated by an

IS*6100* element in three *P. putida* isolates, resulting in a new variant of bla_{VIM} gene, which was designed bla_{VIM-48}, and the bla_{VIM}-carrying genomic regions in these *Pseudomonas* spp. isolates closely resembled the corresponding regions of clinical MBL-producing *Pseudomonas* species.

Ninety-eight non-duplicated samples were randomly collected with an informed consent form from a commercial chicken farm in Shandong Province, China (chicken cloaca swabs, n=30; flies, n=30; dog anal swabs, n=17; swallow fecal swabs, n=10; farmer fecal swabs, n=6; sewage, n=5), the procedures for the collection of all samples were consistent with previous report (7). All samples were plated on CHROMagar *Pseudomonas* (CHROMagar™ Paris, France) containing 8 μg/ml meropenem (Ouhe Technology Company, Beijing, China). Putative *Pseudomonas* colonies with blue color were recovered from 17 samples, and one colony from each sample were identified to the species level by 16S rRNA sequencing (8). Of these, four isolates were bla_{VIM} positive confirmed by PCR and sequencing (9). Further MALDI-TOF MS (BrukerDaltonik GmbH, Bremen, Germany) analysis confirmed the four positive isolates as a *P. aeruginosa* isolate from swallow fecal swabs, including DZ-B1, a *P. putida* isolate from a fly, DZ-F23, and two *P. putida* isolates, DZ-C20 and DZ-C18, from chicken cloaca swabs.

MIC analysis (10, 11) showed that the four *Pseudomonas* spp. isolates were almost resistant to all β-lactam antibiotics tested, including meropenem, imipenem, aztreonam, and ceftazidime, with only isolate DZ-B1 showing susceptibility to aztreonam (4 μg/ml) (Table 1). To further investigate the genetics background of four

isolates, whole genome sequencing was conducted according to previous report (7), and draft assembly sequences were searched against the antibiotic resistance gene database (https://cge.cbs.dtu.dk/services/ResFinder/), which confirmed the presence of *bla*$_{VIM}$ and other antibiotic resistance genes (Table 1). Additionally, in order to investigate the location of the resistance element, the genomics DNA of four VIM-producing isolates were digested with I-Ceu1 and separated by PFGE. The result and Southern blot analysis showed that *bla*$_{VIM}$ was located on the chromosome of all four *Pseudomonas* isolates (Fig. S1). Moreover, the core genome phylogenetic analysis was conducted to reveal the relationship between four isolates and other known *Pseudomonas* isolates carrying *bla*$_{VIM}$ from NCBI database (Table S1) using Parsnp program (12). The phylogenetic tree revealed three *bla*$_{VIM}$-carrying *P. putida* isolates showed distinct genomic heterogeneity, which is consistent with PFGE analysis in SpeI patterns (Fig. S1). The ST385 of *P. aeruginosa* isolate DZ-B1 from our study is confirmed by WGS. This ST type has previously been associated with clinical *P. aeruginosa* isolates from India (13). The genome context of DZ-B1 were closely related to that of seven *bla*$_{VIM-2}$ carrying clinical *P. aeruginosa* from Genebank (Fig. S2).

Four *bla*$_{VIM}$-carrying contigs, were identified from four isolates and confirmed by PCR and sequencing, primers listed in Fig. S3. Analysis of the flanking regions of *bla*$_{VIM-2}$ on the chromosome of DZ-B1 revealed that it was located in a Tn*5090*-like transposon bracketed by two 25-bp inverted repeats, IRi and IRt (Fig. A), suggesting that the whole region could be mobilized using the *tni* machinery (15, 16). This

92 complete transposon shared 92.3% (7296/7907) nucleotide sequence identity to a *P.*
93 *aeruginosa* Tn*5090*-like transposon, also containing *bla*$_{VIM-2}$ gene, isolated from a
94 Chinese patient (accession no. AM993098.1), and even greater sequence identity
95 (99.4%, 4147/4171) was found within the corresponding *tniC-bla*$_{VIM-2}$*-aacA4-dhfr2*
96 gene array located at the 3′ end of the Tn*5090*-like transposon. The Tn*5090*-like
97 transposon contained a conserved segment with an integrase gene, *intl1*, at the 3′ end.
98 *Intl1* is associated with the integration of resistance gene cassette array
99 *bla*$_{VIM-2}$*-aacA4-dhfr2*, which is in the opposite orientation and confers resistance to
100 carbapenems, some aminoglycosides, and trimethoprim, respectively. Another
101 conserved gene cluster, *tniA-tniB-hp-tniC*, was located at the 5′ end of the transposon
102 (15). *TniA* and *tniB* were predicted to be involved in the transposition, while *tniC*
103 codes for a recombination protein that differs from those encoded by *intl1*, *tniA*, and
104 *tniB* (Fig. A) (15). This gene arrangement, in combination with *intl1*, involves in the
105 transfer of a *bla*$_{VIM-2}$-carrying cassette in clinical *Pseudomonas* species (17-20).

106 A 9,875-bp fragment containing the *bla*$_{VIM-2}$ like gene was observed in three *P.*
107 *putida* strains, which sharing 99.9% sequence identity. Comparison analysis revealed
108 that the *bla*$_{VIM-2}$ like gene had a 27-bp deletion at the 3′ terminal region, including its
109 original stop codon, compared with the 801-bp *bla*$_{VIM-2}$ in both DZ-B1 and the
110 previously reported *P. aeruginosa* isolates. Immediately downstream of the 27-bp
111 deletion was an intact insertion element, IS*6100*, and the truncated *bla*$_{VIM-2}$ gene
112 appeared to be terminated at the TAG stop codon located in the right inverted repeat
113 of IS*6100*. This resulted in an 810-bp ORF, designed VIM-48 (KY362199), that was

9-bp longer than the previously reported gene (Fig. B). Further analysis of the flanking regions revealed that it was very similar to the Tn*6217* region in bla_{IMP-9}-carrying plasmid pOZ176 from a clinical *P. aeruginosa* isolate from Guangzhou, China (21). The upstream and downstream regions of bla_{VIM-48} contained the *aacA4-intI1*-Tn*1403*-like and *sul1-hp*-IS*6100* gene clusters, respectively. These two regions shared 99.9% (2792/2794) and 99.9% (5726/5733) nucleotide sequence identity, respectively, to the corresponding regions of Tn*6217*; however, the downstream fragment was inverted, perhaps leading to the missing of 27-bp in the 3'-end of bla_{VIM-2} (Fig. A).

Similar to the Tn*5090*-like transposon in *P. aeruginosa* isolate DZ-B1, a 3,801-bp segment harboring the IS*6100*-bla_{VIM-48}-*aacA4-intI1* gene cluster in the three *P. putida* isolates was bracketed by two 25-bp inverted repeats, IRi and IRt, suggesting that it reached its current location by transposition (Fig. A) (22). Moreover, compared with a 6,942-bp fragment in the 3′ region of the bla_{VIM-2}-carrying segment in *P. aeruginosa* DZ-B1, only a 405-bp segment containing *dhfr2* was absent from the *P. putida* strains. The remaining two nucleotide fragments were highly similar, sharing 100% (1 444/1444) and 99.9% (5091/5097) identity, respectively (Fig. A).

The 810-bp bla_{VIM-48} gene were amplified by PCR and confirmed by sequencing using primers listed in Fig. S3, and then cloned into vector pHSG398 (Takara, Dalian, China), which resulting a recombinant plasmid pHSG398-V01. Both the vector and recombinant plasmid were introduced into *E. coli* Dh5α (Takara, Janan) by electropotation, respectively, resulting in two recombinant *E. coli* strains, designed

HSG398-V01 and HSG398-Dh5α. MIC analysis showed that HSG398-V01 was more active against imipenem and ceftazidime with 4 and 8 fold increase, respectively, when comparing with HSG398-Dh5α (Table 1), which suggested this *bla*$_{VIM-48}$ gene is functional in the three *P. putida* isolates. Phenotypic detection of metallo-β-lactamases were performed on the three *P. putida* according to the previous report (23), and synergy between imipenem and EDTA was observed for all three strains (data not shown).

To date, 47 variants of *bla*$_{VIM}$ gene have been reported (24), and all of them were 801-bp length and differ in several amino acids, while the *bla*$_{VIM48}$ gene in this study was 810-bp length, possibly due to the inverted insertion of IS*6100*-conting containing fragment, revealing a new generation mechanism for the variant of *bla*$_{VIM}$ gene. Similar to the spread of *bla*$_{VIM-2}$ in bacteria of clinical origin, the mobile genetic elements, such as Tn*5090*, Tn*1403*, *intl1* and IS*6100* may play an important role in the dissemination of the *bla*$_{VIM}$ gene and its conserved flanking regions in bacteria among food animals (17, 18, 25). Our study has found not only MBL-producing *Pseudomonas* isolates in the livestock, but also in their surrounding environment, suggesting the surveillance of carbapenemase-producing bacteria in livestock and their surrounding environment is urgently needed.

Nucleotide sequence accession numbers.

All genome assemblies of 4 strains were deposited in GenBank and are registered under BioProject accession number PRJNA381373, and the new VIM enzyme was designed VIM-48 and deposited in GenBank under accession number KY362199.

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255

256 **Figure Legends**

257 Figure 1

258 A: Genetic context of *bla*$_{VIM}$ in *Pseudomonas* species isolates from chickens, a fly,

259 and a swallow, and structural comparison with the corresponding genetic regions in

260 Tn*5090*-like and Tn*6217* transposons. The arrows indicate the positions and directions

261 of transcription of the genes. Different genes are indicated by different types of

262 shading. B The nucleotide sequence of the region encompassing the 3′ ends of IS*6100*

263 and *bla*$_{VIM-48}$. The coding regions of the two open reading frames are marked by gray

264 shading, and the right inverted repeat (IRR) of IS*6100* is underlined.

265

266 **Table 1** Antimicrobial susceptibility profiles of *Pseudomonas putida* isolates DZ-C20, DZ-C18, and DZ-F23, and *Pseudomonas aeruginosa*
267 DZ-B1, and recombinant *E. coli* strains HSG398-V01 and HSG398-DH5α.

Bacterial isolate	Resistance genes	MIC (mg/liter)								
		MEM	IMI	ATM	CAZ	GEN	CIP	COL	TGC	TMP
DZ-C18	bla_{CARB-4}, bla_{VIM48}, *strA, strB, aacA4, aadA13,* QnrVC1, *mph(A), cmlA1, floR, tet(A), tet(G), sul1*	128	128	256	128	64	32	2	8	≥256
DZ-C20	bla_{CARB-4}, $bla_{VIM-48,}$ *strA, strB, aacA4, aphA6,* QnrVC1, *mph(A), floR, catB, tet(G) , sul1*	64	32	128	128	128	32	4	2	≥256
DZ-F23	bla_{CARB-4}, bla_{VIM-48}, *strA, strB, aacA4, aphA6,* QnrVC1, *mph(A), floR, tet(G), sul1*	64	32	32	32	16	32	1	8	≥256
DZ-B1	bla_{VIM-2}, bla_{OXA-50}, *ampC, strA, strB, aacA4, aph(3')-IIa, aph(3')-IIb, aac(6')-IIa, catB7, floR, tet(G), sul1, dfrB1, fosA*	32	32	4	64	256	256	2	8	≥256
HSG398-V01	bla_{VIM-2}-like	0.125	1	0.032	2	-	-	-	-	-
HSG398-DH5α	-	0.0625	0.25	0.032	0.125	-	-	-	-	-

268 The antimicrobial agents are abbreviated as follows: MEM, meropenem; IMI, imipenem; ATM, aztreonam; CAZ, ceftazidime; GEN,
269 gentamicin; CIP, ciprofloxacin; COL, colistin; TGC, tigecycline; TMP, trimethoprim.
270

Fig. A

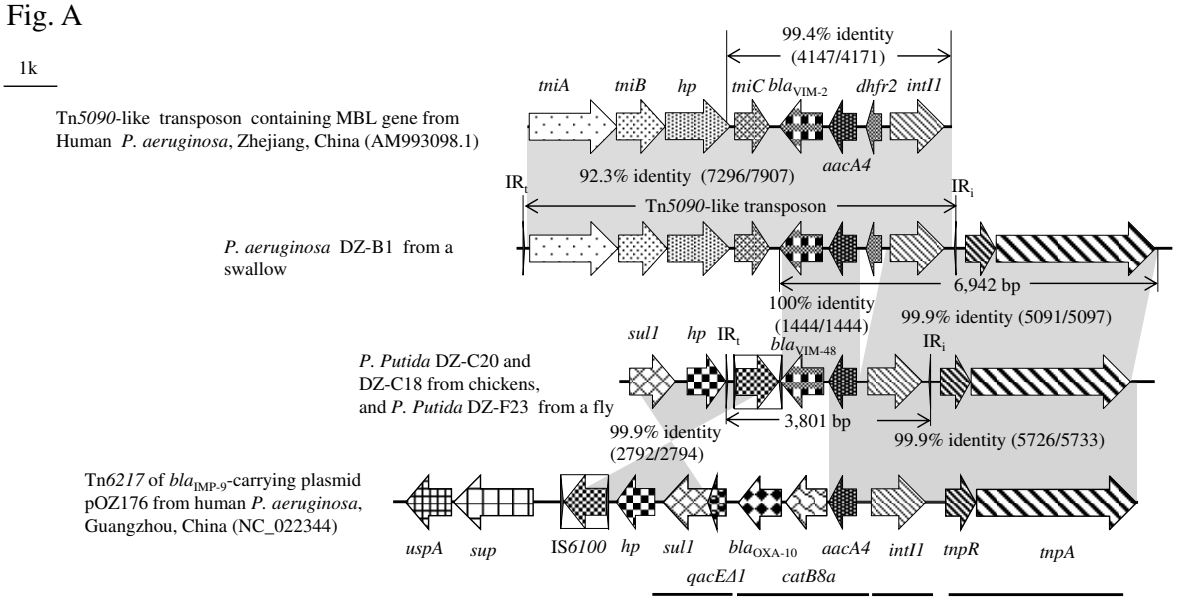

Fig. B