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**Key words:** chicken, fly, swallow, carbapenem resistance, *Pseudomonas* spp.

26 Metallo- $\beta$ -lactamase gene *bla*<sub>VIM</sub> was identified on the chromosome of four  
27 *Pseudomonas* spp. isolates from a chicken farm, including one *P. aeruginosa* from  
28 swallow (*Yanornis martini*), one *P. putida* from fly, and two *P. putida* from  
29 chickens. The four isolates shared two variants of *bla*<sub>VIM</sub>-carrying genomic  
30 contexts, which resemble the corresponding regions of clinical MBL-producing  
31 *Pseudomonas* species. Our study suggests the surveillance of  
32 carbapenemase-producing bacteria in livestock and their surrounding  
33 environment is urgently needed.

34

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

48 IS6100 element in three *P. putida* isolates, resulting in a new variant of *bla*<sub>VIM</sub> gene,  
49 which was designed *bla*<sub>VIM-48</sub>, and the *bla*<sub>VIM</sub>-carrying genomic regions in these  
50 *Pseudomonas* spp. isolates closely resembled the corresponding regions of clinical  
51 MBL-producing *Pseudomonas* species.

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

66 MIC analysis (10, 11) showed that the four *Pseudomonas* spp. isolates were  
67 almost resistant to all β-lactam antibiotics tested, including meropenem, imipenem,  
68 aztreonam, and ceftazidime, with only isolate DZ-B1 showing susceptibility to  
69 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*<sub>VIM</sub> and other antibiotic resistance genes (Table 1). Additionally, in order to investigate the location of the resistance element, the genomic DNA of four VIM-producing isolates were digested with I-CeuI and separated by PFGE. The result and Southern blot analysis showed that *bla*<sub>VIM</sub> 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*<sub>VIM</sub> from NCBI database (Table S1) using Parsnp program (12). The phylogenetic tree revealed three *bla*<sub>VIM</sub>-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*<sub>VIM-2</sub> carrying clinical *P. aeruginosa* from Genebank (Fig. S2).

Four *bla*<sub>VIM</sub>-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*<sub>VIM-2</sub> on the chromosome of DZ-B1 revealed that it was located in a Tn5090-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

complete transposon shared 92.3% (7296/7907) nucleotide sequence identity to a *P. aeruginosa* Tn5090-like transposon, also containing *bla*<sub>VIM-2</sub> gene, isolated from a Chinese patient (accession no. AM993098.1), and even greater sequence identity (99.4%, 4147/4171) was found within the corresponding *tniC*-*bla*<sub>VIM-2</sub>-*aacA4*-*dhfr2* gene array located at the 3' end of the Tn5090-like transposon. The Tn5090-like transposon contained a conserved segment with an integrase gene, *intI1*, at the 3' end. *IntI1* is associated with the integration of resistance gene cassette array *bla*<sub>VIM-2</sub>-*aacA4*-*dhfr2*, which is in the opposite orientation and confers resistance to carbapenems, some aminoglycosides, and trimethoprim, respectively. Another conserved gene cluster, *tniA*-*tniB*-*hp*-*tniC*, was located at the 5' end of the transposon (15). *TniA* and *tniB* were predicted to be involved in the transposition, while *tniC* codes for a recombination protein that differs from those encoded by *intI1*, *tniA*, and *tniB* (Fig. A) (15). This gene arrangement, in combination with *intI1*, involves in the transfer of a *bla*<sub>VIM-2</sub>-carrying cassette in clinical *Pseudomonas* species (17-20).

A 9,875-bp fragment containing the *bla*<sub>VIM-2</sub> like gene was observed in three *P. putida* strains, which sharing 99.9% sequence identity. Comparison analysis revealed that the *bla*<sub>VIM-2</sub> like gene had a 27-bp deletion at the 3' terminal region, including its original stop codon, compared with the 801-bp *bla*<sub>VIM-2</sub> in both DZ-B1 and the previously reported *P. aeruginosa* isolates. Immediately downstream of the 27-bp deletion was an intact insertion element, IS6100, and the truncated *bla*<sub>VIM-2</sub> gene appeared to be terminated at the TAG stop codon located in the right inverted repeat of IS6100. This resulted in an 810-bp ORF, designed VIM-48 (KY362199), that was



114 9-bp longer than the previously reported gene (Fig. B). Further analysis of the  
115 flanking regions revealed that it was very similar to the Tn6217 region in  
116 *bla*<sub>IMP-9</sub>-carrying plasmid pOZ176 from a clinical *P. aeruginosa* isolate from  
117 Guangzhou, China (21). The upstream and downstream regions of *bla*<sub>VIM-48</sub> contained  
118 the *aacA4-int11*-Tn1403-like and *sul1-hp-IS6100* gene clusters, respectively. These  
119 two regions shared 99.9% (2792/2794) and 99.9% (5726/5733) nucleotide sequence  
120 identity, respectively, to the corresponding regions of Tn6217; however, the  
121 downstream fragment was inverted, perhaps leading to the missing of 27-bp in the  
122 3'-end of *bla*<sub>VIM-2</sub> (Fig. A).

123 Similar to the Tn5090-like transposon in *P. aeruginosa* isolate DZ-B1, a 3,801-bp  
124 segment harboring the *IS6100-bla*<sub>VIM-48</sub>-*aacA4-int11* gene cluster in the three *P. putida*  
125 isolates was bracketed by two 25-bp inverted repeats, IRi and IRt, suggesting that it  
126 reached its current location by transposition (Fig. A) (22). Moreover, compared with a  
127 6,942-bp fragment in the 3' region of the *bla*<sub>VIM-2</sub>-carrying segment in *P. aeruginosa*  
128 DZ-B1, only a 405-bp segment containing *dhfr2* was absent from the *P. putida* strains.  
129 The remaining two nucleotide fragments were highly similar, sharing 100% (1  
130 444/1444) and 99.9% (5091/5097) identity, respectively (Fig. A).

131 The 810-bp *bla*<sub>VIM-48</sub> gene were amplified by PCR and confirmed by sequencing  
132 using primers listed in Fig. S3, and then cloned into vector pHSG398 (Takara, Dalian,  
133 China), which resulting a recombinant plasmid pHSG398-V01. Both the vector and  
134 recombinant plasmid were introduced into *E. coli* Dh5 $\alpha$  (Takara, Janan) by  
135 electropotation, respectively, resulting in two recombinant *E. coli* strains, designed

136 HSG398-V01 and HSG398-Dh5 $\alpha$ . MIC analysis showed that HSG398-V01 was more  
137 active against imipenem and ceftazidime with 4 and 8 fold increase, respectively,  
138 when comparing with HSG398-Dh5 $\alpha$  (Table 1), which suggested this *bla*<sub>VIM-48</sub> gene is  
139 functional in the three *P. putida* isolates. Phenotypic detection of  
140 metallo- $\beta$ -lactamases were performed on the three *P. putida* according to the previous  
141 report (23), and synergy between imipenem and EDTA was observed for all three  
142 strains (data not shown).

143 To date, 47 variants of *bla*<sub>VIM</sub> gene have been reported (24), and all of them were  
144 801-bp length and differ in several amino acids, while the *bla*<sub>VIM48</sub> gene in this study  
145 was 810-bp length, possibly due to the inverted insertion of IS6100-containing  
146 fragment, revealing a new generation mechanism for the variant of *bla*<sub>VIM</sub>  
147 gene. Similar to the spread of *bla*<sub>VIM-2</sub> in bacteria of clinical origin, the mobile genetic  
148 elements, such as Tn5090, Tn1403, *int11* and IS6100 may play an important role in  
149 the dissemination of the *bla*<sub>VIM</sub> gene and its conserved flanking regions in bacteria  
150 among food animals (17, 18, 25). Our study has found not only MBL-producing  
151 *Pseudomonas* isolates in the livestock, but also in their surrounding environment,  
152 suggesting the surveillance of carbapenemase-producing bacteria in livestock and  
153 their surrounding environment is urgently needed.

154

#### 155 **Nucleotide sequence accession numbers.**

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



159

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255

256 **Figure Legends**

257 Figure 1

258 A: Genetic context of *bla*<sub>VIM</sub> in *Pseudomonas* species isolates from chickens, a fly,  
259 and a swallow, and structural comparison with the corresponding genetic regions in  
260 Tn5090-like and Tn6217 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 IS6100  
263 and *bla*<sub>VIM-48</sub>. The coding regions of the two open reading frames are marked by gray  
264 shading, and the right inverted repeat (IRR) of IS6100 is underlined.

265

**Table 1** Antimicrobial susceptibility profiles of *Pseudomonas putida* isolates DZ-C20, DZ-C18, and DZ-F23, and *Pseudomonas aeruginosa* DZ-B1, and recombinant *E. coli* strains HSG398-V01 and HSG398-DH5 $\alpha$ .

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

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



Fig. A

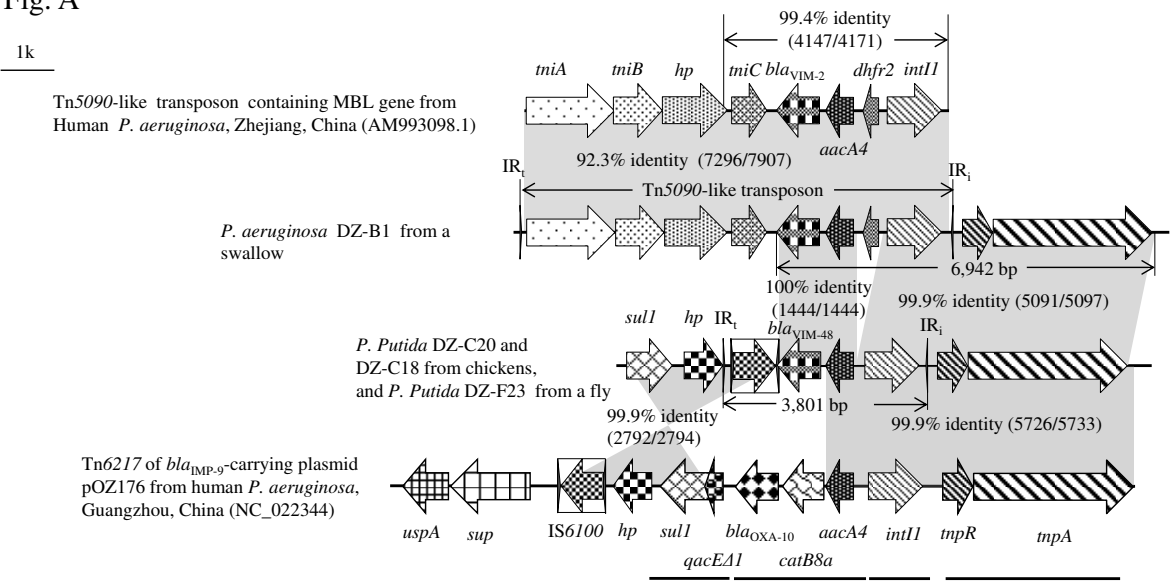


Fig. B

