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

3
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21 Running title: VIM-positive *Pseudomonas* spp. from a chicken farm

22
23 **Key words:** chicken, fly, swallow, carbapenem resistance, *Pseudomonas* spp.

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26 Metallo- β -lactamase gene *bla*_{VIM} 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*_{VIM}-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- β -lactamase (MBL) producing bacteria has been
39 commonly identified from food animals (3-7), *bla*_{MBL}-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*_{VIM-2} was identified in the *P.*
47 *aeruginosa* isolate, but 27-bp at the 3'-terminal region of *bla*_{VIM-2} was truncated by an

48 IS6100 element in three *P. putida* isolates, resulting in a new variant of *bla*_{VIM} gene,
49 which was designed *bla*_{VIM-48}, and the *bla*_{VIM}-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*_{VIM} 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

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

87 Four *bla*_{VIM}-carrying contigs, were identified from four isolates and confirmed by
88 PCR and sequencing, primers listed in Fig. S3. Analysis of the flanking regions of
89 *bla*_{VIM-2} on the chromosome of DZ-B1 revealed that it was located in a Tn5090-like
90 transposon bracketed by two 25-bp inverted repeats, IRi and IRt (Fig. A), suggesting
91 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* Tn5090-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 *miC-bla*_{VIM-2}-*aacA4-dhfr2*
96 gene array located at the 3' end of the Tn5090-like transposon. The Tn5090-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, IS6100, 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 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*_{IMP-9}-carrying plasmid pOZ176 from a clinical *P. aeruginosa* isolate from
117 Guangzhou, China (21). The upstream and downstream regions of *bla*_{VIM-48} contained
118 the *aacA4-intI1*-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*_{VIM-2} (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*_{VIM-48}-*aacA4-intI1* 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*_{VIM-2}-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*_{VIM-48} 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 α (Takara, Janan) by
135 electropotation, respectively, resulting in two recombinant *E. coli* strains, designed

136 HSG398-V01 and HSG398-Dh5 α . 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 α (Table 1), which suggested this *bla*_{VIM-48} gene is
139 functional in the three *P. putida* isolates. Phenotypic detection of
140 metallo- β -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*_{VIM} gene have been reported (24), and all of them were
144 801-bp length and differ in several amino acids, while the *bla*_{VIM48} gene in this study
145 was 810-bp length, possibly due to the inverted insertion of IS6100-containing
146 containing fragment, revealing a new generation mechanism for the variant of *bla*_{VIM}
147 gene. Similar to the spread of *bla*_{VIM-2} 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*_{VIM} 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*_{VIM} 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*_{VIM-48}. 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

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-DH5 α .

Bacterial isolate	Resistance genes	MIC (mg/liter)								
		MEM	IMI	ATM	CAZ	GEN	CIP	COL	TGC	TMP
DZ-C18	<i>bla</i> _{CARB-4'} <i>bla</i> _{VIM48'} , <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>sul1</i>	128	128	256	128	64	32	2	8	≥256
DZ-C20	<i>bla</i> _{CARB-4'} <i>bla</i> _{VIM-48} , <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>sul1</i>	64	32	128	128	128	32	4	2	≥256
DZ-F23	<i>bla</i> _{CARB-4'} <i>bla</i> _{VIM-48'} , <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>sul1</i>	64	32	32	32	16	32	1	8	≥256
DZ-B1	<i>bla</i> _{VIM-2} , <i>bla</i> _{OXA-50'} , <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>sul1</i> , <i>dfrB1</i> , <i>fosA</i>	32	32	4	64	256	256	2	8	≥256
HSG398-V01	<i>bla</i> _{VIM-2} -like	0.125	1	0.032	2	-	-	-	-	-
HSG398-DH5 α	-	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

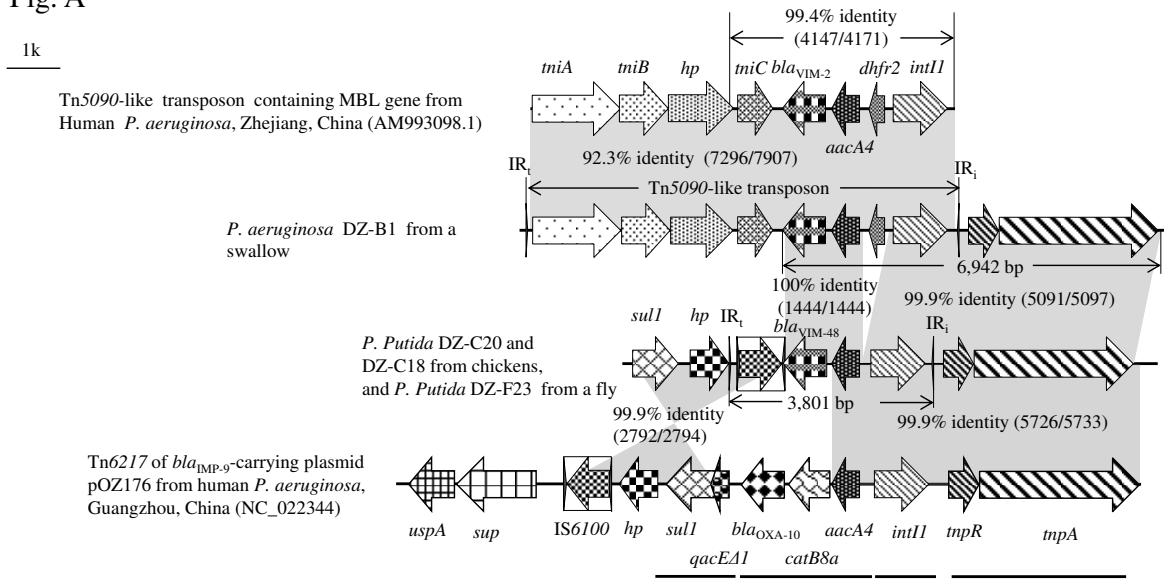


Fig. B

