Antimicrobial Resistance and Type III Secretion System Virulotypes of *Pseudomonas aeruginosa* Isolates from Dogs and Cats in Primary Veterinary Hospitals in Japan: Identification of the International High-Risk Clone Sequence Type 235

Wataru Hayashi, a Katsutoshi Izumi, b Satoshi Yoshida, b Shino Takizawa, b Kanae Sakaguchi, b Keita Iyori, c Ken-ichi Minoshima, c Shinya Takano, d Maki Kitagawa, d Yukiko Nagano, e Noriyuki Nagano a,b

aDepartment of Medical Sciences, Shinshu University Graduate School of Medicine, Science and Technology, Nagano, Japan
bDepartment of Health and Medical Sciences, Shinshu University Graduate School of Medicine, Nagano, Japan
cVDT Co., Ltd., Tokyo, Japan
dResearch and Development Division, Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan
eNagoya University Graduate School of Medicine, Nagoya, Japan

**ABSTRACT** This study aimed to investigate the current trends in antimicrobial resistance among *Pseudomonas aeruginosa* clinical isolates of canine and feline origin and the prevalence of their sequence types (STs) and type III secretion system (T3SS) virulotypes, which remains unknown in Japan. A total of 240 nonduplicate clinical isolates of *P. aeruginosa* from dogs (*n* = 206) and cats (*n* = 34) collected from 152 primary care animal hospitals between August 2017 and October 2019 were examined. PCR detection of T3SS genes (*exoU* and *exoS*) and carbapenemase genes, multilocus sequence typing, and whole-genome sequencing of the representative carbapenem-resistant isolates were performed. Resistance rates to imipenem and meropenem were 6.67% and 2.08%, respectively. A high resistance rate (17.92%) was encountered with ciprofloxacin. The *exoU*2/*exoS*1 was the predominant T3SS virulotype (195 isolates, 81.3%), followed by *exoU*1/*exoS*2 (35 isolates, 14.6%), *exoU*2/*exoS*2 (7 isolates, 2.9%), and *exoU*1/*exoS*1 (3 isolates, 1.3%). A high frequency of the high-risk clones ST235 and clonal complex 235 (CC 235) (28.9%), followed by ST357 (21.1%), were noted among these 38 *exoU*1 isolates. Seventeen carbapenem-resistant isolates comprising 2 *exoU* isolates, including an ST235 isolate, and 15 *exoU*/*exoS* isolates belonging to non-ST235/CC235 were detected, of which all were carbapenemase negative. Different combinations of mutations among oprD, efflux pump regulatory genes, and AmpC β-lactamase regulatory genes were identified among representative isolates with high-level resistance to imipenem. This study emphasizes the occurrence of ST235 isolates among companion animals, which may represent a threat to public health because of the ability of this clone to acquire and spread resistance elements, including carbapenemase genes.

**IMPORTANCE** *Pseudomonas aeruginosa* is an environmentally ubiquitous and important opportunistic human pathogen responsible for life-threatening health care-associated infections. Because of its extensive repertoire of virulence determinants and intrinsic and acquired resistance mechanisms, the organism could be one of the most clinically and epidemiologically important causes of morbidity and mortality. In recent years, worldwide spreading of multidrug-resistant high-risk clones, particularly sequence type 235 (ST235), has become a serious public health threat. Companion animals which share much of their living environment with humans could be important reservoirs and spreaders of antimicrobial-resistant bacteria and resistance genes of clinical importance in humans, such as extended-spectrum β-lactamase-producing *Enterobacteriales* and methicillin-resistant *Staphylococcus aureus*. However, antimicrobial resistance, virulence, and genotyping of
*P. aeruginosa* in companion animals remain largely unknown. This work sheds light on the potential spread of high-risk clones in companion animals.

**KEYWORDS** *Pseudomonas aeruginosa*, companion animal, T3SS, ST235

*Pseudomonas aeruginosa* is an important opportunistic human pathogen capable of causing a wide variety of life-threatening acute and chronic infections and is also a major cause of health care-associated infections. The bacterium is environmentally ubiquitous, inhabiting soil, water, plants, and animals, and can be isolated from the skin, throat, and stool of healthy persons. *P. aeruginosa* is equipped with an extensive repertoire of virulence determinants implicated in pathogenesis and intrinsic and acquired resistance mechanisms, which makes the organism one of the most clinically and epidemiologically important causes of morbidity and mortality (1).

Recent studies have raised attention to the increasing worldwide prevalence of *P. aeruginosa* high-risk clones that are multidrug resistant (MDR) or extensively drug resistant (XDR) in hospitals. Those internationally recognized high-risk clones include sequence type 235 (ST235), ST111, ST233, ST244, ST357, ST308, ST175, ST277, ST654, and ST298 (2). Carbapenem resistance in *P. aeruginosa* can be mediated by several mechanisms, including the interaction of OprD inactivation, Mex efflux system overexpression, and intrinsic AmpC \(\beta\)-lactamase overexpression and the production of acquired carbapenemases. VIM- and IMP-type metallo-\(\beta\)-lactamases are the most commonly encountered carbapenemases in *P. aeruginosa*, including international high-risk clones, although other carbapenemases, such as KPC, GES, NDM, and SPM, have also been reported globally (2). In Japan, GES, IMP, and VIM types have so far been identified among carbapenemase-producing high-risk clones, including ST235, ST244, ST357, ST308, ST175, and ST277 (2). Particularly, outbreaks of ST235 producing IMP type or GES 5 have been reported in clinical settings in Japan (3, 4).

One of the most important virulence factors resulting in a poor prognosis of infections is the type III secretion system (T3SS) (5). The system injects bacterial effector exotoxins, including ExoU (phospholipase A2), ExoS (Rho GTPase-activating protein [RhoGAP]/ADP-ribosyltransferase [ADPRT]), ExoT (RhoGAP/ADPRT), and ExoY (adenyl cyclase) identified so far, into host cells (6). ExoU and ExoS are responsible for the cytotoxic phenotype and invasive phenotype, respectively, contributing more significantly to pathogenesis than the other exotoxins (7, 8). ExoU is located in pathogenicity island 2 (PAPI-2), and ExoS shows mostly mutually exclusive distributions, while ExoT and ExoY are carried by most of the strains (9). The *exoU*1 genotype has been frequently associated with multidrug resistance, fluoroquinolone resistance, and highly virulent phenotypes, as represented by the ST235 high-risk clone, resulting in increased mortality in bloodstream infections (10–12).

Companion animals have been recognized as potential reservoirs of antimicrobial resistance genes and resistant bacteria that can be transmitted to humans (13, 14). However, studies on *P. aeruginosa* virulence and antimicrobial resistance in companion animals are scarce and have focused mostly on human isolates. In France, ST233 and potential high-risk clones ST395 and ST253 have been found among 29 carbapenemase-negative carbapenem-nonsusceptible *P. aeruginosa* clinical isolates of canine and feline origin (15). VIM-2-positive ST233 and IMP-45-positive ST308 *P. aeruginosa* have been reported to be associated with possible human-to-dog transmission in Brazil and China (16, 17). In a US canine study, *exoU*--/exoS+, *exoU*+/exoS+, and *exoU*-/exoS-- T3SS virulotypes have been identified in 15, 3, and 1 isolates, respectively, among 19 isolates from ocular infections, while *exoU*--/exoS+ and *exoU*+/exoS+ virulotypes have been detected in 5 and 1 isolates, respectively, among 6 normal conjunctival microflora isolates (18).

In Japan, only a few epidemiological studies are available to date on the antimicrobial resistance of *P. aeruginosa* clinical isolates of companion animal origin (19, 20). A recent study has documented the low rates of resistance to ceftazidime (0.5%), ciprofloxacin (9%), and amikacin (2.5%), and neither imipenem-resistant isolates nor metallo-\(\beta\)-lactamase producers have been identified (20). This study investigates the current trends in antimicrobial resistance among *P. aeruginosa* clinical isolates of canine and feline origin and the prevalence of...
TABLE 1 The MICs and the antimicrobial resistance rates for the 240 *P. aeruginosa* isolates from dogs and cats

| Antimicrobial agent(s) | MIC range (mg/liter) | \(\text{MIC}_{50}\) (mg/liter) | \(\text{MIC}_{90}\) (mg/liter) | Resistance (%) | 2019 JANIS data\(^a\) on human clinical isolates (resistance %) from: |
|-----------------------|----------------------|-------------------------------|-------------------------------|----------------|------------------------------------------------|
|                       | Inpatients           | Outpatients                   |                               |                |                                               |
| Piperacillin           | ≤4 to >128           | 8                             | 16                            | 0.83           | 10.3                                         |
| Piperacillin-tazobactam| ≤8/4 to 128/4        | ≤8/4                          | 16/4                          | 0.83           | 8.4                                          |
| Ceftazidime            | ≤2 to 64             | ≤2                            | 8                             | 0.83           | 8.7                                          |
| Cefepime               | ≤2 to 32             | 4                             | 8                             | 0.42           | 5.9                                          |
| Aztreonam              | ≤2 to 64             | 8                             | 32                            | 11.25          | 13.3                                         |
| Imipenem               | ≤1 to >64            | 2                             | 4                             | 6.67           | 16.2                                         |
| Meropenem              | 0.5 to 16            | 0.5                           | 2                             | 2.08           | 10.6                                         |
| Gentamicin             | ≤1 to >32            | 2                             | 4                             | 2.08           | 3.1                                          |
| Amikacin               | ≤2 to >64            | 4                             | 8                             | 0.42           | 0.9                                          |
| Tobramycin             | ≤2 to >32            | ≤2                            | ≤2                            | 1.67           | NA\(^b\)                                    |
| Ciprofloxacin          | ≤0.5 to >8           | ≤0.5                          | 4                             | 17.92          | 9.8 (levofloxacin)                           |

\(^a\)JANIS, Japan Nosocomial Infections Surveillance.  
\(^b\)NA, not available.

their STs and T3SS virulotypes, which remains unknown in Japan, to better understand their epidemiological aspects.

**RESULTS**

**Prevalence of antibiotic resistance and T3SS virulotypes among *P. aeruginosa* isolates.** The ranges of MICs for 240 isolates of *P. aeruginosa* against imipenem and meropenem were ≤1 to >64 mg/liter and ≤0.5 to 16 mg/liter, respectively. The rates of resistance were 6.67% (16 isolates) for imipenem and 2.08% (5 isolates) for meropenem (Table 1). Notably, those results are comparable to the corresponding carbapenem resistance rates, namely, 5.7% for imipenem and 2.8% for meropenem in *P. aeruginosa* isolates among outpatients in 2019 obtained from the Japan Nosocomial Infections Surveillance (JANIS) database (Table 1) ([https://janis.mhlw.go.jp/report/open_report/2019/3/1/ken_Open_Report_201900_Outpatient.pdf](https://janis.mhlw.go.jp/report/open_report/2019/3/1/ken_Open_Report_201900_Outpatient.pdf)). In contrast, the resistance rates against piperacillin, piperacillin-tazobactam, ceftazidime, and cefepime were 0.83% (2 isolates), 0.83% (2 isolates), 0.83% (2 isolates), and 0.42%, (1 isolate), respectively, which were lower than those from JANIS data of outpatient isolates. The resistance levels of aminoglycosides were 0.42% (1 isolate) for amikacin, 2.08% (5 isolates) for gentamicin, and 1.67% (4 isolates) for tobramycin. A high resistance rate was encountered with ciprofloxacin (43 isolates, 17.92%), surpassing the resistance rate against levofloxacin among clinical isolates from both inpatients (9.8%) and outpatients (6.0%). Individual MIC values for each isolate are listed in Table S1 in the supplemental material.

The presence of 40 mg/liter phenylalanine-arginine β-naphthylamide (PaβN) did not inhibit the growth of 43 ciprofloxacin-resistant *P. aeruginosa* isolates except 1, for which the PAβN concentration not affecting its growth (20 mg/liter) was used for the efflux pump inhibition. All 43 isolates, including 7 carbapenem-resistant isolates, showed more than or equal to a 4-fold reduction in the MIC of ciprofloxacin in the presence of PAβN (data not shown).

Overall, the frequency of exoU and exoS genes was 15.8% (38/240 isolates) and 82.5% (198/240 isolates), respectively. PCR revealed 4 T3SS virulotypes among 240 isolates, namely, 35 exoU+/exoS− isolates (14.6%), 195 exoU−/exoS+ isolates (81.3%), 3 exoU+/exoS+ isolates (1.3%), and 7 exoU−/exoS− isolates (2.9%). Table 2 shows the distribution of T3SS virulotypes according to antimicrobial resistance. The exoU+/exoS− isolates, compared with the exoU−/exoS+ isolates, were significantly associated with resistance to piperacillin (*P* = 0.02843), piperacillin-tazobactam (*P* = 0.03787), gentamicin (*P* = 0.009844), and tobramycin (*P* = 0.04685). Alternatively, no statistically significant association was found between these virulotypes and resistance to imipenem, meropenem, and ciprofloxacin.

**Multilocus sequence typing (MLST) of exoU+ isolates.** The 35 exoU+/exoS− isolates were assigned to 20 STs from which 2 new STs, namely, ST235-like and ST3654, were identified (Table 3). The 3 exoU+/exoS+ isolates belonged to different STs, including a new ST, ST3653. The high frequency of high-risk clone ST235 (8, 21.1%) and ST141...
TABLE 2 Distribution of T3SS virulotypes among *P. aeruginosa* clinical isolates from companion animals according to antimicrobial resistance

| Antimicrobial(s)       | exoU+/exoS− | exoU−/exoS+ | exoU+/exoS+ | exoU−/exoS+ |
|------------------------|-------------|-------------|-------------|-------------|
|                        | (n = 35)    | (n = 195)   | (n = 3)     | (n = 7)     |
| Piperacillin           | 6 (17)      | 11 (6)      | 0           | 0           | 0.02843  |
| Piperacillin-tazobactam| 6 (17)      | 12 (6)      | 0           | 0           | 0.03787  |
| Ceftazidime            | 1 (3)       | 5 (3)       | 0           | 0           |          |
| Cefepime               | 1 (3)       | 12 (6)      | 0           | 0           |          |
| Aztreonam              | 8 (23)      | 49 (25)     | 0           | 0           |          |
| Imipenem               | 4 (11)      | 41 (21)     | 0           | 0           |          |
| Meropenem              | 2 (6)       | 11 (6)      | 0           | 0           |          |
| Gentamicin             | 6 (17)      | 8 (4)       | 0           | 0           | 0.009844 |
| Amikacin               | 0 (0)       | 2 (1)       | 0           | 0           |          |
| Tobramycin             | 3 (9)       | 3 (2)       | 0           | 0           | 0.04685  |
| Ciprofloxacin          | 6 (17)      | 60 (31)     | 0           | 1           |          |
| Colistin               | 0 (0)       | 1 (1)       | 0           | 0           |          |

(1, 2.6%), ST235-like (1, 2.6%), and ST3653 (1, 2.6%) belonging to CC235 was noted among those 38 exoU+ isolates (11, 28.9%). Other high-risk clones and clonal complexes identified were ST357 (8, 21.1%), ST2644 (CC274) (3, 7.9%), ST308 (2, 5.3%), ST253 (1, 2.6%), and ST446 (CC298) (1, 2.6%).

**Phenotypic characteristics of carbapenem-resistant *P. aeruginosa* isolates.** Among the 240 isolates, 17 (7.1%) were resistant to imipenem and/or meropenem. Detection of carbapenemase production by a modified carbapenem inactivation method using Tris-HCl (CIMTris) assay yielded negative results for all 17 isolates. In those isolates, the major carbapenemase genes *bla*\_IMP, *bla*\_NDM, *bla*\_VIM, and *bla*\_GES were not detected (Table 4).

**TABLE 3** STs identified among the 38 exoU+ *P. aeruginosa* clinical isolates from companion animals

| MLST sequence type | Clonal complex | No. of clinical isolates by virulotype and animal | Total no. of isolates |
|-------------------|----------------|-----------------------------------------------|----------------------|
| ST235             | CC235          | 8                                            | 32                   |
| ST141             | 1              | 1                                            | 1                    |
| ST235-like\(^a\)  | 1              | 1                                            | 1                    |
| ST3653\(^b\)     | 1              | 1                                            | 1                    |
| ST357             | CC357          | 8                                            | 8                    |
| ST2644            | CC274          | 3                                            | 3                    |
| ST308             | CC308          | 2                                            | 2                    |
| ST671             | CC560          | 2                                            | 2                    |
| ST253             | CC253          | 1                                            | 1                    |
| ST313             | CC313          | 1                                            | 1                    |
| ST316             | CC316          | 1                                            | 1                    |
| ST2555            | 1              | 1                                            | 1                    |
| ST446             | CC298          | 1                                            | 1                    |
| ST606             | NA\(^c\)       | 1                                            | 1                    |
| ST773             | NA             | 1                                            | 1                    |
| ST829             | NA             | 1                                            | 1                    |
| ST1121            | NA             | 1                                            | 1                    |
| ST1248            | NA             | 1                                            | 1                    |
| ST1334            | NA             | 1                                            | 1                    |
| ST3654\(^b\)     | NA             | 1                                            | 1                    |

\(^a\)Novel *rpoE* allele was associated with ST235-like.

\(^b\)Sequence types newly assigned in this study.

\(^c\)NA, not assigned.
### TABLE 4 Features of the 17 carbapenem-resistant *P. aeruginosa* isolates

| Isolate | T3SS virulotype | Carbapenemase detection | MIC (mg/liter) of: |
|---------|-----------------|--------------------------|--------------------|
|         |                 |                          | IPM               |
|         |                 |                          | IPM + PAβN        |
|         |                 |                          | IPM + APB         |
|         |                 |                          | IPM + PaβN + APB  |
|         |                 |                          | MEPM + PAβN       |
|         |                 |                          | MEPM + APB        |
|         |                 |                          | MEPM + PaβN + APB |
|         |                 |                          | IPM + PAβN + APB  |
|         |                 |                          |                   |
| No.     | Name            | Animal species | Clinical sample  | ST3014 (CC3014) | - | + | - | - | 8 | 1 | 0.5 | ≤0.25 |
| 1       | CA10562         | Dog            | Ear discharge   | ST1600          | - | + | - | - | 64 | 4 | 8 | 1 |
| 2       | CA12133         | Dog            | Ear discharge   | ST198 (CC198)   | - | + | - | - | 16 | 16 | 1 | 1 |
| 3       | CA12482         | Cat            | Skin            | ST209 (CC274)   | - | + | - | - | 8 | 8 | 1 | 1 |
| 4       | CA13227         | Dog            | Ear discharge   | ST277 (CC277)   | - | + | - | - | 4 | 2 | 0.5 | ≤0.25 |
| 5       | CA13620         | Dog            | Ear discharge   | ST1097          | - | + | - | - | 8 | 4 | 0.5 | ≤0.25 |
| 6       | CA14241         | Dog            | Ear discharge   | ST3045          | - | + | - | - | 16 | 16 | 1 | 2 |
| 7       | CA16138         | Dog            | Ear discharge   | ST3574*(CC262) | - | + | - | - | 8 | 2 | 4 | 1 |
| 8       | CA16209         | Dog            | Ear discharge   | ST606           | + | - | - | - | 8 | 1 | 0.5 | ≤0.25 |
| 9       | CA17343         | Dog            | Uterine pus     | ST348 (CC348)   | - | + | - | - | 128 | 8 | 4 | 0.5 |
| 10      | CA17462         | Dog            | Uterine pus     | ST270           | - | + | - | - | 4 | 1 | 0.5 | ≤0.25 |
| 11      | CA17502         | Dog            | Uterine pus     | ST155 (CC155)   | - | + | - | - | 16 | 4 | 8 | 4 |
| 12      | CA19603         | Cat            | Ear discharge   | ST235 (CC235)   | + | - | - | - | 32 | 16 | 2 | NG |
| 13      | CA19802         | Dog            | Urine           | ST399 (CC399)   | - | + | - | - | 32 | 16 | 2 | 2 |
| 14      | CA19818         | Dog            | Urine           | ST266           | - | + | - | - | 4 | 2 | ≤0.25 | ≤0.25 |
| 15      | CA20091         | Dog            | Ear discharge   | ST348 (CC348)   | - | + | - | - | 4 | 4 | 0.5 | 0.5 |
| 16      | CA20115         | Dog            | Urine           | ST3135          | - | + | - | - | 4 | 8 | 0.5 | 0.5 |

*A decrease of more than 4-fold in MIC values are indicated in bold.

*Sequence type newly assigned in this study.

*PAβN concentration of 20 mg/liter was used.

*No visible growth was observed in the presence of imipenem or meropenem plus 20- or 40-mg/liter PAβN and 300-mg/liter APB.
The presence of 40-mg/liter PAβN did not inhibit the growth of 17 carbapenem-resistant \textit{P. aeruginosa}, except for 2 isolates (no. 8 and 10), for which PAβN concentration not affecting their growth (20 mg/liter) was used for the efflux pump inhibition. A 4-fold or greater decrease in the MICs of imipenem and/or meropenem with the addition of PAβN was observed in 8 of 17 isolates (47.1%) (Table 4). In the presence of 3-aminophenylboronic acid (APB), an inhibitor of AmpC \(\beta\)-lactamase, most isolates (15/16, 93.8%) exhibited a greater than or equal to an 8-fold reduction in the MICs of imipenem, while MICs of meropenem for 5 isolates were not affected. It was noticed that APB and PAβN plus APB effectively change interpretive categories from imipenem resistant/intermediate (MIC, \(\geq 8\)/4 mg/liter) to susceptible (MIC, \(\leq 2\) mg/liter) in 13 (81.3%) and 15 isolates (93.8%), respectively. Meropenem MIC of an isolate (no. 12) with the imipenem-susceptible, meropenem-resistant phenotype was affected by PAβN (4-fold reduction) but was not affected by APB.

**Genotypic characteristics of carbapenem-resistant \textit{P. aeruginosa} isolates.** MLST sequence types and T3SS virulotypes of 17 carbapenem-resistant isolates are shown in Table 4. These isolates were assigned to 16 STs, including a newly identified ST3574 (CC262). They contained 2 \(exoU^-/exoS^+\) isolates and 15 \(exoU^-/exoS^+\) isolates. It was of note that high-risk international clones ST235 with an \(exoU^-/exoS^-\) virulence type (no. 13) and ST277 with an \(exoU^-/exoS^+\) virulence type (no. 5) were each identified in 1 isolate. The remaining 1 \(exoU^-/exoS^-\) isolate belonged to ST606 (no. 9). ST348 with an \(exoU^-/exoS^-\) virulence type was detected in 2 isolates, namely, no. 10 and 16 (11.8%), of which 1 exhibited high-level resistance to imipenem (MIC, 128 mg/liter).

**Genomic features of carbapenem-resistant isolates.** Three representative \textit{P. aeruginosa} isolates, including 2 showing high-level resistance to imipenem (no. 2 and 10; MICs, \(\geq 64\) mg/liter) and 1 isolate of the ST235 international high-risk clone (no. 13), were subjected to whole-genome sequencing (WGS). The assembled genomes of isolate no. 2, 10, and 13 contained 67, 118, and 143 contig sequences, with a total length of 6,270,234 bp, 6,831,356 bp, and 6,646,878 bp, respectively, with a GC content ranging from 66.1% to 66.5%.

Multiple antimicrobial resistance genes, including species-intrinsic \(bla\), \(aph(3')\)-Ib, \(catB7\), \(aadA1\), \(aadB\), \(dfrA14\), \(catB7\), and \(exoS\), were found in the 3 isolates, where ST1600/O3 (isolate no. 2) harbored newly identified \(bla\) (Table 5). In addition, ST348/O2 (isolate no. 10) with a ciprofloxacin MIC of 0.5 mg/liter harbored \(cpR\) (ciprofloxacin-modifying enzyme) that was recently identified in a \textit{P. aeruginosa} clinical isolate in Mexico (21). ST235/O11 (isolate no. 13) also contained 2 different class 1 integron-associated gene cassettes, and 1 contained \(qnrVC1\) (\(\beta\)-lactamase, trimethoprim, chloramphenicol, and sulfonamide. Different amino acid substitutions in AmpR were observed in the ST348/O2 and ST235/O11 isolates. The ST1600/O3 isolate with a ciprofloxacin MIC of 32 mg/liter had Ser466Phe in the quinolone resistance-determining region (QRDR) of GyrB.

A mutated OprD porin by amino acid substitutions Thr103Ser, Lys115Thr, and Phe170Leu (ST1600/O3); a premature stop codon (ST348/O2); and a 75-bp deletion leading to a frameshift (ST235/O11) were identified (Table 5). These isolates had amino acid substitutions in the MexAB-OprM repressors MexR and NanC, Gly71Glu and Ser209Arg in NanC (ST1600/O3), and Val126Glu in MexR and Glu153Lys in NanC (ST235/O11). The substitutions of Arg21His, Glu153Lys, and Gly133Ser in NalC (ST235/O11). Also, three isolates shared \(qnr\) (H2-T6SS effector encoding gene), whereas only one ST235/O11 isolate also carried \(qnr\) (H2-T6SS effector encoding gene) (24). Heavy metal resistance genes, including those associated with copper (\(copR\), \(copAB\), and \(copD\)), cobalt/zinc/cadmium (\(czcABCD\)), chromate (\(chr\)), cadmium (\(sodA\)), and colR regulating multimer metal resistance, were identified among them (25).
**TABLE 5 Genetic characteristics of P. aeruginosa isolates no. 2, no. 10, and no. 13**

| Characteristic           | Results for:                                                                 |
|--------------------------|-----------------------------------------------------------------------------|
| Sequence type/serotype   | ST1600/O3, blaOXA-48, apr(3’)-Iib, lasA, lasB, plcH, plcN, plcB, prpL, pshA, lipA, lipC, lsoX, toxA, cbrD, dmpA, pmpA, T3SS (exoX, exoY, exoZ, exoY), H2-T6SS (tssB2, pldB), H3-T6SS (tssB3), Q5 (lasI, lasR, rhlI, rhlII), others (licC, rhlIAB) | ST348/O2, blaOXA-48, apr(3’)-Iib, lasA, lasB, plcH, plcN, plcB, prpL, pshA, lipA, lipC, lsoX, toxA, cbrD, dmpA, pmpA, T3SS (exoX, exoY, exoY), H2-T6SS (tssB2, pldB), H3-T6SS (tssB3), Q5 (lasI, lasR, rhlI, rhlII), others (licC, rhlIAB) | ST235/O11, blaOXA-48, apr(3’)-Iib, lasA, lasB, plcH, plcN, plcB, prpL, pshA, lipA, lipC, lsoX, toxA, cbrD, dmpA, pmpA, T3SS (exoX, exoY, exoY), H2-T6SS (tssB2, pldB), H3-T6SS (tssB3), Q5 (lasI, lasR, rhlI, rhlII), others (licC, rhlIAB) |
| Antimicrobial resistance genes |                                                                              |                                                                              |                                                                              |
| Amino acid substitutionsa |                                                                              |                                                                              |                                                                              |
| AmpC regulators          |                                                                              |                                                                              |                                                                              |
| AmpR                    | Ser5Phe, Gly148Ala, Asp183Tyr                                              | Arg244Trp, Gly273Glu                                                        | Gly283Glu, Met288Arg                                                         |
| AmpD                    | Ile67Thr                                                                   | Ala219Thr                                                                  | Ala208Val                                                                   |
| AmpDh2                  |                                                                              |                                                                              |                                                                              |
| MexAB-OprM regulators   | MexR                                                                        |                                                                              |                                                                              |
| NaIC                    | Gly71Glu, Ser209Arg                                                        |                                                                              |                                                                              |
| NaID                    |                                                                              |                                                                              |                                                                              |
| MexCD-OprJ regulator    | NxB                                                                         |                                                                              |                                                                              |
| MexEF-OprN regulators   | MexS                                                                        |                                                                              |                                                                              |
| MexT                    |                                                                              |                                                                              |                                                                              |
| MexXY-OprA regulator    | MexZ                                                                        |                                                                              |                                                                              |
| Whole-genome multilocus sequence typing (wgMLST). The genomic diversity of the ST235 isolates was assessed by wgMLST gene-by-gene comparison. The animal-derived ST235/O11 isolate with the ST235 isolates of human origin characterized by the exoU+ genotype (Fig. 1). They shared mostly important virulence-associated genes, including pldB and pldA. It is worth noting that the class I integron-associated gene cassettes that confer antimicrobial resistance, i.e., qnrVC1-aac(6’)-Ib-blaOXA-10-aadA1-dfrA14 and aadB-cmlA10-aadA2-sul1, were identified in isolate 13.**

**DISCUSSION**

The perspective of One Health emphasizes the importance of AMR monitoring and integrated actions across human, food, animal, and environmental health. However, there are few studies on the epidemiology of antimicrobial resistance in P. aeruginosa clinical isolates from companion animals that share a living environment with humans. The strength of our study is that we included 240 P. aeruginosa clinical isolates collected from 152 primary care animal hospitals across the country, allowing us to understand the current situation of their antimicrobial resistance in companion animals as well as to investigate the resistance trends over time in Japan by comparing the results with those in earlier studies (19, 20). Namely, a trend toward a progressive increase in the frequency of imipenem resistance, from 0% in 2003 to 2010 (19), to 0.5% in 2014 to 2015 (20), to 6.67% in 2017 to 2019 (this study) is noted according to the breakpoints of the Clinical and Laboratory Standards Institute (CLSI) M100 30th edition guidelines (26).

The carbapenem resistance rates of isolates from companion animals were found to be similar to those from human outpatients in Japan, leading to concerns about the
transmission of carbapenem-resistant *P. aeruginosa* between humans and companion animals in community settings. The ciprofloxacin resistance rate of 17.92% in our study is nearly flat from the previous years; 23.39% was found in 2003 to 2010 and 14.87% in 2014 to 2015 (19, 20). Constant amounts of veterinary fluoroquinolones such as enrofloxacin and orbifloxacin, at 0.90 tons per year during 2013 and 2018, have been used for dogs and cats as described in the Nippon AMR One Health Report (NAOR) 2020 (in Japanese, https://www.mhlw.go.jp/content/10900000/000715546.pdf). However, consumption of fluoroquinolones which are approved for human use such as levofloxacin and ciprofloxacin in companion animals cannot be traced because their prescription is left to the discretion of veterinarians in Japan. Thus, attention is necessary to monitor for high levels of resistance to ciprofloxacin, which is an active metabolite of enrofloxacin in companion animals, because ciprofloxacin is an effective treatment option in many human diseases. In contrast, piperacillin, piperacillin-tazobactam, ceftazidime, and cefepime retained activity against >99% of animal isolates, which is greater than the activity against isolates from hospital inpatients and outpatients (Table 1). Interestingly, in companion animals, none of the carbapenem-resistant isolates produced carbapenemases, and no multidrug-resistant *P. aeruginosa* isolates (MDRP) as defined by the Infectious Diseases Control Law in Japan (resistant to imipenem [MIC, $\geq 16$ mg/liter], amikacin [MIC, $\geq 32$ mg/liter], and ciprofloxacin [MIC, $\geq 4$ mg/liter]) were identified, which is consistent with observations in previous animal studies (19, 20). However, our study detected four pre-MDRP isolates with imipenem MICs of $\geq 16$ mg/liter and ciprofloxacin MICs of $\geq 4$ mg/liter, emphasizing the need for continuous surveillance of resistance trends of this important pathogen.

To our knowledge, only one study has analyzed the T3SS virulotypes in a limited number of *P. aeruginosa* isolates from canine, reporting that 3 of 19 isolates from ocular infections were exoU+/ genotypes (18). This study revealed that the prevalence of T3SS virulence genes among 240 clinical isolates of canine and feline origin (15.8% for exoU and 82.5% for exoS) is more similar to that of 90 environmental isolates (17.8% for exoU and 82.2% for exoS) than that of 243 human isolates from bloodstream infections (20.6% for exoU and 76.1% for exoS) (12, 27). There was no significant association between the exoU+/exoS− virulotype and ciprofloxacin and carbapenem resistance, contrary to some other studies reporting such an association in human isolates (11, 28). However, the exoU+ genotype has been an independent risk factor for early mortality of human bloodstream infections (11, 12), and our results draw attention to the prevalence of exoU+ isolates in a greater number of animal isolates.
Among global high-risk clones with MDR/XDR profiles, ST235 is highly associated with \textit{exoU}, and this combination has been a predictor of a highly unfavorable prognosis (29). Thus, MLST analysis of \textit{exoU+} isolates is of clinical importance, as ST235 has not been confirmed among \textit{P. aeruginosa} isolates from companion animals. MLST revealed clonal heterogeneity in the 38 \textit{exoU+} isolates, including \textit{exoU+/exoS−} and \textit{exoU+/exoS+} virulotypes, with the presence of 20 STs, including 3 newly identified STs. Notably, the global high-risk clone ST235 was detected in 7 \textit{exoU+/exoS−} isolates and in 1 \textit{exoU+/exoS+} isolate. Moreover, 3 CC235 isolates were found in 2 \textit{exoU+/exoS−} isolates and in 1 \textit{exoU+/exoS+} isolate. Thus, 11 ST235/CC235 isolates were detected in 38 \textit{exoU+} isolates (28.9%), resulting in an overall ST235/CC235 frequency of 20.8% in 53 isolates with the \textit{exoU+} genotype and/or carbapenem resistance that were typed by MLST. In contrast, ST235/CC235 isolates were not found in 15 \textit{exoU+/exoS−} isolates resistant to carbapenems. The CC235 has been linked with metallo-\beta-lactamase genes and, hence, is responsible for their dissemination worldwide, including to Japan (4, 30). Our findings provide important insights into the role of companion animals as the potential reservoirs of \textit{P. aeruginosa} high-risk clones, although they did not harbor metallo-\beta-lactamase genes.

In \textit{P. aeruginosa}, mutational overexpression of a multidrug efflux pump, MexAB-OprM, leads to reduced susceptibility to fluoroquinolones and most \beta-lactams, including meropenem, while imipenem is not affected (31). MexAB-OprM overexpression combined with OprD inactivation produces high-level meropenem resistance (MIC, >32 mg/liter) (32). Mutational inactivation of OprD contributes mainly to imipenem resistance, even achieving high-level resistance (MIC, >32 mg/liter), but drives only moderate resistance to meropenem (32). In addition, OprD inactivation, in combination with AmpC \beta-lactamase overexpression, confers resistance to carbapenems (33). In the present study, 7.1% (17/240) of isolates from companion animals were found to be carbapenem resistant but carbapenemase non-IMP producers. MIC assays performed in the presence of an efflux pump inhibitor PA\textit{b}N and/or AmpC \beta-lactamase inhibitor APB revealed the contribution of AmpC \beta-lactamase production to imipenem resistance in 10 of 12 imipenem-resistant meropenem-susceptible isolates. In the remaining 2 isolates, the active role of efflux pumps (isolate 8) or the synergistic role of AmpC \beta-lactamase production and efflux pumps (isolate 2, imipenem MIC of 64 \mu g/ml) in mediating imipenem resistance is estimated. Among 4 imipenem- and meropenem-resistant isolates, AmpC \beta-lactamase-mediated imipenem resistance is considered in 3 isolates, including 1 also exhibiting efflux pump-mediated meropenem resistance (isolate 13, imipenem MIC of 32 mg/liter, meropenem MIC of 8 mg/liter). The involvement of the synergistic effect of AmpC \beta-lactamase production and efflux pumps in imipenem resistance and that of the efflux pump mechanism in meropenem resistance could be considered in the remaining 1 isolate (isolate 10, imipenem MIC of 128 mg/ml, meropenem MIC of 16 mg/liter). Efflux pump-mediated meropenem resistance is suggested for a meropenem-resistant imipenem-susceptible isolate.

Representative isolates analyzed by WGS contained defective \textit{oprD} mutations in ST348/O2 (isolate 10) and ST235/O11 (isolate 13), while 3 amino acid substitutions observed in ST1600/O3 (isolate 2) may not be involved in carbapenem resistance because they have been frequently detected among carbapenem-susceptible isolates, including \textit{P. aeruginosa} PA14 (34). Also, substitutions in regulators of AmpC \beta-lactamases (PDC variants) are found in these isolates, i.e., AmpR (Gly283Glu and Met288Arg), AmpD (Gly148Ala and Asp183Tyr), and AmpDh3 (Ala208Val and Ala219Thr), which have frequently been identified in wild-type isolates and \textit{P. aeruginosa} PA14, and are considered nonsignificant (35, 36). All substitutions in MexAB-OprM regulators, namely, Arg21His and Asp56Gly in NfxB repressor of MexCD-OprJ and Asp249Asn in MexS repressor of the MexEF-OprN, have been found in susceptible isolates, including \textit{P. aeruginosa} PA14, and thus may not be related to the overexpression of those efflux pumps (37–39). In consequence, carbapenem resistance may arise from AmpC derepression due to Ser5Phe in AmpD and/or Ile67Thr in AmpDh3 (40) combined with MexCD-OprJ overexpression due to Gly133Ser in NfxB in the ST1600/O3 isolate. A significant association between MexCD-OprJ overexpression and meropenem and imipenem resistance has been described (41). Carbapenem resistance in ST348/O2 and ST235/O11 isolates may be attributed to mutational inactivation of OprD combined with MexEF-OprN overexpression due to Gly78Ser or a frameshift mutation in MexS (39). MexEF-OprN overexpression, with a
concomitant loss of OprD caused by mutated MexS, has been linked to carbapenem resistance (42). Besides, the involvement of AmpC (PDC) variants in decreased imipenem susceptibility cannot be excluded (43).

In conclusion, this study confirms the presence of high-risk clones in canine and feline isolates with the \textit{exoU} \textit{genotype}; ST235/CC235 isolates were the most predominant, followed by ST357 isolates. These animal isolates belonging to high-risk clones were found to be carbapenemase nonproducers, although international high-risk clones have been associated frequently with carbapenemase production. Nonetheless, the predominant occurrence of ST235 among companion animals may represent a threat to public health because of the ability of this clone to acquire and spread resistance elements, including carbapenemase genes. A comprehensive survey of \textit{P. aeruginosa} needs to be conducted to better understand the spread of antimicrobial resistance elements, STs, and T3SS virulotypes at the human-animal-environment interface and to assess their clinical implications on humans and animals.

**MATERIALS AND METHODS**

**Bacterial strains.** A total of 240 nonduplicate clinical isolates of \textit{P. aeruginosa} from dogs (\(n = 206\)) and cats (\(n = 34\)) were collected from 152 primary care animal hospitals across Japan between August 2017 and October 2019. Those isolates were received from different geographic regions, as follows: Kanto (\(n = 117\) from 71 hospitals), Chubu (\(n = 32\) from 25 hospitals), Kinki (\(n = 24\) from 19 hospitals), Chugoku (\(n = 21\) from 11 hospitals), Tohoku (\(n = 19\) from 9 hospitals), Kyushu (\(n = 13\) from 10 hospitals), Hokkaido (\(n = 9\) from 4 hospitals), and Shikoku (\(n = 5\) from 3 hospitals). Clinical sources of the isolates were ear (\(n = 149\)), urine (\(n = 9\)), skin (\(n = 28\)), nasopharynx (\(n = 16\)), uterus (\(n = 3\)), cornea (\(n = 2\)), and others (\(n = 9\)), including 3 isolates of unknown origin. \textit{P. aeruginosa} was confirmed with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics Japan, Yokohama, Japan) using \(\geq 2,000\) score cutoffs for species-level identification.

**Antimicrobial susceptibility testing.** MICs were determined by the broth microdilution method recommended by the CLSI using a custom-designed microtiter panel (Kyokuto Optopanel MP; Kyokuto Pharmaceutical, Tokyo, Japan), and the results were interpreted according to CLSI M100 30th edition guidelines for human (26). \textit{Escherichia coli} ATCC 25922 and \textit{P. aeruginosa} ATCC 27853 were used as quality-control strains.

**TABLE 6 Primers used for PCR amplification in this study**

| Gene by type          | Primer sequence* (5’ to 3’)              | Amplicon size (bp) |
|-----------------------|------------------------------------------|--------------------|
| **T3SS virulence genes** |                                          |                    |
| \textit{exoU}         | F; ATGCATATCCAAATCGTTG                  | 2,000              |
|                       | R; TCATGTGAACCTTATT                     |                    |
| \textit{exoS}         | F; CTGAAAGGAACTCGACAAGG                 | 504                |
|                       | R; TTCAGGTCCCGGTAATGTAAT                |                    |
| **Carbapenemase genes** |                                          |                    |
| \textit{bla}\textit{cep} | F; ACGCAGCAAGTCTTTGCC                   | 587                |
|                       | R; ACAACAGGTTTGCGCTTACC                 |                    |
| \textit{bla}\textit{NDM} | F; GGGCGCTATAGGGTA                     | 758                |
|                       | R; GAAGCTGACCCAGCGACCTTAG               |                    |
| \textit{bla}\textit{VIM} | F; ATGTCAAACCTTTGGAGTAAG               | 801                |
|                       | R; TCACTCAACGACTGACGCC                  |                    |
| \textit{bla}\textit{GES} | F; ATGGCCTTCATTACCAAGCAC               | 864                |
|                       | R; CTATTCTGGCCTGCTCAGGA                 |                    |
| **MLST**              |                                          |                    |
| \textit{acsA}         | F; ACCGTTTGTCAGCCTCGTGAC                | 842                |
|                       | R; GACATAGATGCCTCGCCCTTGAT             |                    |
| \textit{aroE}         | F; TGGGCTATAGCCTGGAAACC                 | 825                |
|                       | R; TAAACCGTTTGTTGATGATCCTACA           |                    |
| \textit{guaA}         | F; CGGCCCTGAGCTGGGATG                  | 940                |
|                       | R; GAAGCCCTGCGCTGCTGGCTGTTGGTA        |                    |
| \textit{nuoD}         | F; ACGGCCACCGCAGCTG                  | 1,042              |
|                       | R; TCTCGCCCATCTTGACCAA                 |                    |
| \textit{ppsA}         | F; GGTGTCCTGCTCAGAAGGTAGTGG            | 989                |
|                       | R; GGTGCCTCTCTCCCGGCTCGTAG             |                    |
| \textit{trpE}         | F; GCCGCCAAGGTCTGAG                    | 811                |
|                       | R; CCCGCCCTGTGGATGTTGT                  |                    |
| \textit{mutL}         | F; AGGTGGCGAGGACCTGTTCTT               | 688                |
|                       | R; GGACTCTCCAGGGACGCTCT                |                    |

*F, forward primer; R, reverse primer.
Detection of T3SS virulence genes (exoU and exoS). All isolates were subjected to PCR detection of T3SS effector toxin genes exoU and exoS using specific primers as shown in Table 6 (44, 45). The amplification products from some arbitrarily selected isolates were sequenced to confirm the reliability of the results.

Analysis of carbapenem-resistant P. aeruginosa isolates. Detection of carbapenemase activity in P. aeruginosa isolates exhibiting resistance to imipenem and/or meropenem was performed using the modified carbapenem inactivation method CIMTris (46). Carbapenemase genes (blaIMP, blaAP, and blaOXA) were detected by PCR assay using specific primers as shown in Table 6.

The imipenem and meropenem MICs were determined using microdilution panels prepared in-house in the absence and in the presence of 20- or 40-mg/liter efflux pump inhibitor PaβN (MP Biomedicals, Santa Ana, CA), 20- or 40-mg/liter PAβN plus 300-mg/liter APB (Sigma-Aldrich Japan, Tokyo, Japan), and 300-mg/liter APB in parallel. F. aeruginosa ATCC 27853 was used as the control strain.

MLST analysis. MLST for carbapenem-resistant isolates and exoU+ isolates was performed according to the MLST scheme described by Curran et al, except that Tks Gflex DNA polymerase (Takara Bio, Otsu, Japan) and alternative PCR primers for mutL shown in Table 6 were used (47, 48). Briefly, seven housekeeping genes (acsA, araE, gusA, mutL, nuxO, ppsA, and trpE) were amplified and sequenced. Those sequences were submitted to the PubMLST database (https://pubmlst.org/organisms/pseudomonas-aeruginosa) for the assignment of allelic numbers and STs. Those STs were assigned to clonal complexes, as defined by closely related ST groups within the single-locus variant or double-locus variant linkages (49).

WGS analysis of high-level imipenem-resistant P. aeruginosa. WGS and de novo assembly of three representative carbapenem-resistant isolates were conducted in a manner like that described previously (30). Briefly, the genome was sequenced using the 150-bp paired-end method with the NovaSeq6000 platform (Illumina, San Diego, CA), and the resulting raw reads were assembled de novo into scaffolds using the SPAdes v.3.14.1. The assembled scaffolds were queried with ResFinder 4.1 and PAst 1.0 that are available from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org) for the prediction of antibiotic resistance genes and in silico serotyping, respectively. A WgMLST tree was constructed using PGAdb-builder (https://wgmlstdb.imst.nsysu.edu.tw), with the addition of the genomes of 21 P. aeruginosa strains belonging to ST235/O11 obtained from the NCBI database (Table 7). The phylogenetic tree was visualized using iTOL v5 (http://itol.embl.de/). An in-depth exploration of heavy metal resistance and virulence genes was performed manually on WGS data.

The nucleotide sequences of AmpC β-lactamase regulator genes (ampR, ampD, ampDh2, and ampDh3); oprD porin genes; efflux system regulator genes (mexR, nacC, nacD, mexZ, ndaB, mexS, and mexY); and QRDR of gyrA, gyrB, parC, and parE β-lactams were compared with those of the reference strain P. aeruginosa PAO1 (GenBank accession number AE504091).

Analysis of ciprofloxacin-resistant P. aeruginosa isolates. Efflux pump-mediated fluoroquinolone resistance was investigated in ciprofloxacin-resistant isolates using efflux pump inhibitor PaβN as described above. A decrease of more than 4-fold in MIC in the presence of PaβN was considered the participation of efflux pumps in the resistance to ciprofloxacin.

Statistical analysis. The Fisher’s exact test was performed to assess the association between T3SS virulotypes (exoU+/exoU− and exoU−/exoU−) and antimicrobial resistance by using R v.4.0.5. Resistant

| Strain          | Isolation source       | Country | GenBank assembly accession no. |
|-----------------|------------------------|---------|--------------------------------|
| ST235           | Homo sapiens           | Italy   | GCA_011634585.1                |
| UNIBA_ST235PA   | Blood                  | Italy   | GCA_013276295.1                |
| TRN6637         | Homo sapiens           | Russia  | GCA_001921165.1                |
| PA_ST235        | Blood                  | Spain   | GCA_000737795.1                |
| MAL             | Homo sapiens           | France  | GCA_901035125.1                |
| 24Pae112        | Blood                  | Colombia| GCA_003433235.1                |
| WH-SGI-V-07626  | Homo sapiens           | USA     | GCA_001452025.1                |
| RNS_PA46        | Burn wound             | Australia| GCA_001623955.1               |
| RNS_PA46S       | Hand sanitizer         | Australia| GCA_001623985.1               |
| TRN6594         | Homo sapiens           | Argentina| GCA_001921015.1               |
| TRN6598         | Homo sapiens           | South Africa| GCA_001921025.1            |
| TRN6622         | Homo sapiens           | Thailand| GCA_001921095.1                |
| TRN6635         | Homo sapiens           | Russia  | GCA_001921125.1                |
| TRN6649         | Homo sapiens           | Nigeria | GCA_001921175.1                |
| NCGM 1900       | Homo sapiens           | Japan   | GCA_000829275.1                |
| AZP13880        | Homo sapiens           | Mexico  | GCA_000795725.1                |
| AZP14887        | Intra-abdominal tract infection | Croatia   | GCA_000790725.1 |
| AZP14690        | Urinary tract infection | Romania   | GCA_000794555.1 |
| AZP14702        | Respiratory tract infection | Philippines | GCA_000794785.1 |
| AZP14705        | Urinary tract infection | Greece   | GCA_000795805.1                |
| AZP15063        | Respiratory tract infection | Brazil     | GCA_000793315.1 |
SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.

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