Investigation of pathogenicity and antimicrobial resistance of *Acinetobacter* spp. in dogs and cats

犬と猫におけるアシネトバクター属菌の病原性と
薬剤耐性に関する調査

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PREFACE

Acinetobacter spp. is mainly known a nosocomial microbial pathogen in human medicine, because it can persist on the hospital facilities and medical instruments due to its ability to interact with surface (Peleg et al., 2008). This species can cause opportunistic infection to various organs in immune-compromised patient, including pneumonia, urinary tract infection, wound infection and bacteremia in humans (Francey et al., 2000). Similarly, *Acinetobacter* species have been identified from various site of animals having diseases such as pneumonia, cellulitis and sepsis. (Kuzi et al., 2016), but the pathogenicity to animals is incompletely revealed.

Antimicrobial drugs have an essential role to treat for bacterial infectious disease of humans and animals. On the other hand, for the use of antimicrobial drug, there is often a risk of emergence of antimicrobial resistance. Since the discovery of penicillin-resistant staphylococci in 1940, bacteria have acquired tolerance to various antimicrobial drugs as humans develop new type antibiotics. In animals as well as humans, antimicrobial resistant have been increasing and attracting attention.

Acinetobacter spp. naturally possess intrinsic resistance mechanisms, and even acquire rapidly the extrinsic resistance mechanism due to the possession of high number of mobile genetic elements (Müller et al., 2014). In addition, the strains isolated in hospital environment tend to have acquired numerous antimicrobial resistant and nosocomial infection often cause a disease with a high mortality rate (Bergogne-Béréin and Towner., 1996). Multidrug resistant (MDR) *Acinetobacter* is the great concern in humans medical and public health, and it is designated as Category V Infectious Diseases and needed notifiable disease surveillance in Japan (the Infectious Diseases Control Raw, 2014). Notably, carbapenem-resistant *Acinetobacter baumannii* is designated as one of the priority considered pathogens by World Health Organization (WHO, 2017). Today, companion animals become close to their human owners and share the living space. On the other hands, the spread of pathogenic bacteria has been reported from companion animals to humans, and vice versa (Weese, 2008; Zinsstag et al., 2011). The strain belonging to the human clonal lineages as a fact is reported (Müller et al., 2014). However, it remains unidentified whether companion animals can become the reservoir of antimicrobial resistant *Acinetobacter*.

My research was conducted in following two part with the aim to clarify the pathogenicity

of *Acinetobacter* and prevent the expansion of antimicrobial resistance in companion animals. In chapter 1, I isolated metallo-β-lactamase producing *A. radioresistens* from diseased dogs and cats for the first time in the world, and defined the molecular biological properties. In chapter 2, I investigated *Acinetobacter* spp. isolated from dogs and cats in various region of Japan and characterized the pathogenicity and antimicrobial resistance of them.

CHAPTER 1

Analysis of IMP-1 type metallo-β-lactamase–producing *Acinetobacter radioresistens* isolated from companion animals

1. Introduction

Resistance of *Acinetobacter* spp. to carbapenems is increasing. Among *Acinetobacter* spp. that have been isolated from humans, carbapenem resistance in *A. baumannii* is conferred mainly by carbapenemases (carbapenem-hydrolyzing β-lactamases of Ambler class D: CHDLs) such as OXA-51 (Ambler, 1980; Kouyama et al., 2012). Among *Acinetobacter* spp. other than *A. baumannii*, metallo-β-lactamases (MBLs) (Ambler class B) are the main carbapenem resistance factors (Kouyama et al., 2012).

MBLs are characteristically capable of hydrolyzing almost all β-lactams including carbapenems (Palzkill, 2013). MBL genes are known to exist in special genetic structures called integrons that efficiently accumulate resistance factors and propagate their gene cassettes (Partridge et al., 2013).

A diverse range of MBL-producing bacteria have been isolated in hospitals (non-veterinary) since the 1990s. These include *Acinetobacter* spp., *Citrobacter freundii*, *Klebsiella pneumoniae*, *Morganella morganii*, *Providencia rettgeri*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (Palzkill, 2013). However, reports of the isolation of MBL-producing bacteria from dogs and cats are rare (Shimada et al., 2012; Sun et al., 2015; Shaheen et al., 2013). With regard to *Acinetobacter* spp., Sun *et al*. reported the isolation of New Delhi metallo-β-lactamase-1 (NDM-1) -producing *Acinetobacter lwoffii*

from companion animals (Sun et al., 2015). Shimada *et al*. also reported isolating MBL-producing *A*. *lwoffii* from dogs and a cat, but the MBL genotypes were not determined (Shimada et al., 2012).

Various types of pathogenic bacteria are reported to spread from companion animals to humans (Weese, 2008; Zinsstag et al., 2011). Companion animals are close to their human owners and share the same living space. Furthermore, as companion animals' infections are frequently treated with antibiotics developed for humans, the appearance of resistant bacteria can have a direct effect on the health of humans (Weese, 2008; Zinsstag et al., 2011). Therefore, the National Action Plan on Antimicrobial Resistance announced by the Japanese government on April 5, 2016, includes "the establishment of a system of investigation and monitoring of trends in antibiotic resistance in companion animals" and "implementation of data communications regarding investigation and monitoring of trends in antibiotic resistance in humans, animals, and food." (http://www.mhlw.go.jp/file/06-Seisakujouhou-10900000-Kenkoukyoku/0000120769.pdf). It is therefore important from the standpoint of public health as well as for the treatment of infectious diseases to know about the emergence of resistant bacteria in dogs and cats.

In 2014, I observed a case of cystitis in a dog and a case of conjunctivitis in a cat caused by MBL-producing *Acinetobacter radioresistens.* This report describes these cases and the molecular biological properties of the isolates.

2. Case 1

This was a dog (Yorkshire terrier), neutered male, 9 years old, weight 2.4 kg. The dog was a companion animal living in Yamaguchi city, and the owner brought the dog to the Miyamoto Animal Hospital on April 20, 2014 because of pollakuria. The dog was diagnosed as diabetic on July 26, 2011, and its blood glucose was being controlled by insulin. Six months before (November 2013) presenting with pollakuria, it had shown vomiting, and diarrhea with an increased blood leukocyte count (31,300 cells/μL), and had been treated with fosfomycin 20 mg/kg, PO, BID for 20 days. This dog had no history of hospitalization. On April 20, 2014, urine was collected by means of a sterile catheter, centrifuged at 1500 × *g* for 10 minutes and the urinary sediment was collected with a sterile cotton swab. Isolation and identification of bacteria from this specimen, antibiotic susceptibility testing and detection of MBL were consigned to Japan Clinical Laboratories, Inc. (Kyoto, Japan). In the urinary bacteria culture tests, only MBL-producing *Acinetobacter* spp. ($\geq 10^5$ CFU/mL) was isolated. The isolated MBL-producing *Acinetobacter* spp. were resistant to all the β-lactams that I studied, but were susceptible to minocycline, gentamicin, and amikacin (Table 1). After the administration of doxycycline (5 mg/kg, PO, BID), the symptoms improved, and cure was achieved after 20 days at which point the administration was stopped.

3. Case 2

This was a cat (Munchkin), male, 8 months old, weight 5.2 kg. The cat had the complaint of dacryorrhea. This case had been diagnosed as conjunctivitis and was treated with an antibiotic drug (unknown) at a previous veterinary hospital in Hagi city, but the symptoms did not improve and the case was referred to Miyamoto Animal Hospital. Both eyes had purulent secretions containing blood and swelling of the conjunctiva. This cat had no history of hospitalization. On July 16, 2014, a sample of the purulent secretion was collected on a sterile cotton swab. Isolation and identification of bacteria from this specimen, antibiotic susceptibility testing and detection of MBL were consigned to Japan Clinical Laboratories, Inc. The isolated MBL-producing *Acinetobacter* spp. were resistant to all the β-lactams that I studied, but showed sensitivity to minocycline, aminoglycosides, and fluoroquinolones (Table 1), so I began administration of doxycycline (5 mg/kg, PO, BID) and ofloxacin eye drops (0.3% fluid, TID). Subsequently, the symptoms improved and cure was achieved after 20 days at which point the administration was stopped.

4. Results of genomic analysis

In order to identify the bacteria type and to conduct a comprehensive search of all drug resistance factors of the MBL-producing *Acinetobacter* spp. isolated from the dog with cystitis and the cat with conjunctivitis, I carried out a total genomic analysis by next-generation sequencing (300-bp paired-end sequencing on a MiSeq system; Illumina, San Diego, CA, USA). For assembly of the 300-bp short reads I used CLC Genomics Workbench (Qiagen, Chatsworth, CA, USA). For identification of bacteria type I used the full-length sequence of the *rpoB* gene which codes the RNA polymerase subunit B. Also, using the Quick BioInformatic Phylogeny of Prokaryotes v 1.1 web tool (https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi) I conducted similarity searches against the *rpoB* sequences of *Acinetobacter* spp. type strains. For the comprehensive search of drug resistance factors, I used ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/). The bacterial strains isolated from Case 1 and Case 2 were respectively named TUM13966 and TUM14624. The full-length *rpoB* sequences of TUM13966 (4064 bp) and TUM14624 (4065 bp) showed a high level of homology (each 99.4%) with the 4089-bp full-length *rpoB* sequence (DQ207489) of the *Acinetobacter radioresistens* type strain. The second highest homology was found with *Acinetobacter ursingii*, but they were both 85.0%, which is clearly distinguishable, so both bacterial strains were identified as *A. radioresistens*. Antibiotic-resistance genes

from the two *A. radioresistens* strains are shown in Table 2. Both strains yielded the MBL gene $bla_{\text{IMP - 1}}$. The CHDL gene $bla_{\text{oxa - 23}}$ was also detected in both strains, but insertion sequences such as IS*Aba1* containing promoter regions needed for expression of $bla_{\text{oxa - 23}}$ could not be found in the upstream position.

5. Discussion

In recent years, carbapenem-resistant *Acinetobacter* spp. in humans has presented a serious social problem (Kouyama et al., 2012; Palzkill, 2013). In companion animals as well, isolation of carbapenem-resistant *Acinetobacter* spp. have recently come to be reported (Shimada et al., 2012; Sun et al., 2015; Endimiani et al., 2011; Pomba et al., 2014). Endimiani et al. report that carbapenem resistance in *Acinetobacter baumannii* from companion animals is related to the overexpression of OXA-type carbapenemase genes that are specific to bacterial types (Endimiani et al., 2011), and Pomba et al. report that carbapenem resistance of *A. baumannii* isolated from cats is related to production of OXA-23 as a carbapenemase (Pomba et al., 2014). Resulting of environment screening for *Acinetobacter* species in the Miyamoto Animal Hospital, a $bla_{\text{OXA-23}}$ positive *A. lwoffii* were isolated from the examination table only (data not shown). Furthermore, Sun *et al*. report that carbapenem resistance of *A. lwoffii* isolated from cats is related to the production of NDM-1 (Sun et al., 2015). Other than NDM-1, no other MBL genotypes from companion animals have been recognized. The MBL genotype that I isolated is $bla_{\text{IMP - 1}}$, which is carried by *A. radioresistens*; this is the first report of the isolation of an *Acinetobacter* species carrying $bla_{\text{IMP - 1}}$ from a dog or cat. In our two cases, both strains of *A. radioresistens* also yielded the $bla_{\text{OXA - 23}}$ gene. However, there were no upstream sequences with promoter regions such as IS*Aba1* (Hiiggins et al., 2013), so the

imipenem resistance is considered to be due to IMP-1 production. Since *A. radioresistens* may cause infections in immunocompromised human patients, it should be careful in defending infection from companion animals and their owners (Visca et al., 2001).

Because *Acinetobacter* spp. easily becomes resistant to various types of antibiotics, treatment of *Acinetobacter* infections can be difficult (Fishbain and Peleg, 2010; Maragakis and Perl, 2008; Peleg et al., 2008). Treatment of human *Acinetobacter* infections is based on carbapenems, fluoroquinolones, aminoglycosides, β-lactamase inhibitors, and rifampicin, which can be useful in combination therapies. However, ultimately it is important to isolate the bacterial strain, conduct antibiotic susceptibility testing and select an antibiotic that promises to be effective based on the test results (Fishbain and Peleg, 2010; Maragakis and Perl, 2008; Peleg et al., 2008). In earlier report by a member of my hospital, MBL-producing *A. lwoffii* isolates were almost all resistant to β-lactams, but were susceptible to minocycline and aminoglycosides (Shimada et al., 2012). Half were susceptible to fluoroquinolones, and as I have reported in the current study, those cases were all cured by administration of antibiotics that showed sensitivity (Shimada et al., 2012). I have therefore confirmed that in the case of *Acinetobacter* spp. infections in dogs and cats, it is also important to always isolate the

bacterial strains, test them for drug sensitivity, and use the results to select the antibiotics to be administered.

The increased use of carbapenems in humans has led to the increase of carbapenem-resistant *Acinetobacter* spp., especially in the West (Fishbain and Peleg, 2010). Lu *et al*. reported that 47 carbapenem-resistant *Acinetobacter* spp. were isolated from 33 human patients and one of these was *A. radioresistens* having IMP-1 genes (Lu et al., 2008). In my hospital, on the other hand, carbapenems were never used, so the isolates of MBLs-producing *Acinetobacter* spp. did not receive any selective pressure from carbapenems. Where the *A. radioresistens* isolated in the Miyamoto hospital obtained the $bla_{\text{IMP-1}}$ is therefore unknown. Both cases in this report had no history of hospitalization. Case 2 had lived in Hagi city next to Yamaguchi city where Miyamoto Animal Hospital locates in, and this coming to Miyamoto Animal Hospital was the first time. Therefore, it was considered that there was no relationship between these two cases. Furthermore, 3,569 single nucleotide polymorphisms were identified between *A. radioresistens* TUM13966 and TUM14624 in genomic region shared by the core genome (data no shown). If synonymous nucleotide substitution rate of *A. radioresistens* shows assuming that is as the same of *Escherichia coli*, a genetic distance between both isolates presume

approximately 150 years (Wielgoss et al., 2011). From now on I need to study the details of the infection source and infection pathway.

Reports of MBL-producing bacterial isolates from dogs and cats are extremely rare. I was able to discover that IMP-1 type MBL-producing bacteria could be isolated from dogs and cats. To prevent the proliferation of MBL-producing bacteria, veterinary staff at veterinary hospitals should be concerned about possible infections of dogs and cats with MBL-producing organisms, and continuous surveillance and drastic contact precautions in veterinary hospitals should be needed.

Table 1

Results of antibiotic susceptibility testing of *Acinetobacter radioresistens*.

Groups	Antibiotics	TUM13966 (Case 1)		TUM14624 (Case 2)	
		MIC (μg/mL)	Interpretation[a]	MIC (μg/mL)	Interpretation
Penicillins	Piperacillin	>128	R	>128	R
Cephalosporins	Ceftazidime	>64	R	>64	R
Cephalosporins	Cefepime	>64	R	>64	R
Carbapenems	Imipenem	>32	R	>32	R
Carbapenems	Meropenem	>32	R	>32	R
Aminoglycosides	Gentamicin	≤2	S	≤2	S
Aminoglycosides	Amikacin	8	S	16	S
Tetracyclines	Minocycline	≤1	S	≤1	S
Fluoroquinolones	Ciprofloxacin	16	R	≤0.5	S
Fluoroquinolones	Levofloxacin	8	R	≤1	S

Microbroth dilution method and interpretation of MIC were according to documents M07-A10 and M100-S25 published by the Clinical and Laboratory Standards Institute, respectively.

[a] S: susceptible, R: resistant.

Table 2

Drug-resistance genes of isolated metallo-β-lactamase-producing *Acinetobacter radioresistens*.

Case 1 TUM13966	Case 2 TUM14624
bla$_{\text{IMP-1}}$	*bla*$_{\text{IMP-1}}$
bla$_{\text{OXA-23}}$[a]	*bla*$_{\text{OXA-23}}$[a]
sul1	*sul1*
aadA1	*sul2*
aac(6')-31	*aadA1*
	aac(6')-31
	aph(3')-VIa
	strB
	strA

[a] Promote sequences such as ISA*ba1* were not detected upstream, and so *bla*$_{\text{OXA-23}}$ is not expressed.

CHAPTER 2

Species distribution, virulence factors, and antimicrobial resistance of *Acinetobacter* spp. isolates from dogs and cats: a preliminary study

1. Introduction

The genus *Acinetobacter* comprises gram-negative coccobacilli that are strictly aerobic, non-motile, catalase-positive, and oxidase-negative (Foster and Daschner, 1998). *Acinetobacter baumannii*, the most medically important species in the genus, is responsible for nosocomial infections in humans (Foster and Daschner, 1998). In companion animals, this species is an opportunistic pathogen that can cause pneumonia, urinary tract infections, cellulitis, and sepsis (Kuzi et al., 2016). Virulence factors have been identified in *A. baumannii* isolates from humans, the main factors that contribute to infection of hosts being adhesion molecules, outer membrane protein, and iron acquisition (McConnell et al., 2013; Momtaz et al., 2015). However, these virulence factors have not been fully elucidated in *Acinetobacter* spp. isolates from companion animals.

Antimicrobial-resistant *Acinetobacter* spp. emergence in both companion animals (Kuzi et al., 2016; Zordan et al., 2011; Ewers et al., 2017) and humans (McConnell et al., 2013; Lee et al., 2017) is of great concern. *Acinetobacter* spp. possess both intrinsic and extrinsic resistance mechanisms (Müller et al., 2014). For example, resistance to fluoroquinolone is acquired mainly by both chromosomal mutations in target enzymes (DNA gyrase and topoisomerase IV) and plasmid-mediated quinolone resistance (PMQR)

(Vila et al., 1995; Vila et al., 1997; Yang et al., 2016). The prevalence of antimicrobial-resistant *Acinetobacter* spp. in companion animals in Japan is currently unknown.

The aim of the present study was to preliminarily investigate the prevalence of virulence factors and antimicrobial resistance among *Acinetobacter* spp. isolates from dogs and cats in Japan.

2. Isolation of bacteria from dogs and cats

In this retrospective study, I investigated 67 *Acinetobacter* spp. isolates collected from

dogs (n = 54) and cats (n = 13) that had been taken to veterinary hospitals between 2012 and 2016. These hospitals are located in the following prefectures in Japan: Hokkaido, Iwate, Ibaraki, Chiba, Tokyo, Kanagawa, Shizuoka, Aichi, Niigata, Fukui, Nara, Tottori, Yamaguchi, and Kumamoto. All animals were diagnosed clinically as having bacterial infections and the specimens were obtained from various infection sites, including the skin (n = 20), pus from unspecified organs (n = 19), nasal secretions (n = 11), urine (n = 5), mouth mucosa (n = 4), the eye (n = 2), the vagina (n = 2), the ear (n = 1), feces (n = 1), the respiratory tract (n = 1), and an unknown site (n = 1). *Acinetobacter* spp. isolates used in this study are listed in the Table S1. No information was available regarding previous antimicrobial treatment received by the dogs and cats. In this study, we focused on bacterial aspects; thus, ethical approval was not needed according to the guidelines for epidemiological research set by the Japanese government.

3. Bacterial identification

I identified bacteria using CHROMagar orientation medium (Ohkusu, 2000), Kanto

Chemical Co., Inc.,Tokyo, Japan, an API 20E kit (Sysmex bioMérieux, Tokyo, Japan), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry with a Bruker MALDI Biotyper system (Bruker Daltonik, Bremen, Germany). Strains identified as *A. baumannii* were confirmed positive for a unique carbapenem-resistant gene, *blaOXA-51-like*, by PCR (Turton et al., 2006). I stored all confirmed *Acinetobacter* spp. isolates at −80°C in 10% skim milk. Seifert et al. have reported identifying 72.9% of 584 *Acinetobacter* isolates from humans as *A. baumannii* (Seifert et al., 1993). However, there are few reports on the species distribution of *Acinetobacter* isolates from companion animals worldwide. In this study, I classified 61/67 *Acinetobacter* isolates into the following 10 species (Table 1). I was unable to identify the species of five isolates (7.5%). Overall, non-*baumannii Acinetobacter* isolates were more prevalent than *A. baumannii* isolates, which is consistent with Rafei et al's. report of a higher prevalence of non-*baumannii Acinetobacter* isolates in companion animals in Lebanon (Rafei et al., 2014). In contrast, Zordan *et al*. reported identifying most *Acinetobacter* spp. isolates from mainly companion animals in Germany as *A. baumannii* (Zordan et al., 2011). These findings imply that the distribution of *Acinetobacter* species in companion animals is geographically diverse; however, the species distribution is yet to be investigated in many other countries. The diversity of *Acinetobacter* species that can be isolated from

companion animals warrants greater attention.

4. Pathogenicity

To assess the significance of *Acinetobacter* spp. as a veterinary pathogen, I examined the prevalence of known virulence factors in gram-negative bacteria. I extracted genomic

DNA from each isolate by suspending several colonies in 0.5 ml of water and boiling for 10 min and investigated the following 15 genes' coding virulence factors by multiplex PCR (Momtaz et al., 2015) on each isolate: *afa/draBC* (afimbrial adhesin), *papC*, *papG II-III* (P fimbriae), *sfa/foc* (S/F1C fimbriae), *fimH* (type 1 fimbriae), *cvaC* (microcin V), *cnf1* (cytotoxic necrotizing factor), *cnf2*, *kpsMT II* (group II capsule), *fyuA* (yersiniabactin), *csgA* (curli fiber), *ibeA* (invasion brain endothelium), *PAI* (pathogenicity-associated island marker), *iutA* (aerobactin), and *traT* (serum resistance). Positive results were confirmed by individual gene PCRs. Through this process, I found that 68.7% of the isolates had one or more virulence factors. The virulence factor *afa/draBC* was the most prevalent (29.9%), followed by *papC* (22.4%), *cvaC* (20.9%), and *cnf2* (10.4%) (Table 2). The genes *afa/draBC*, *papC*, *sfa/focDE*, *cvaC*, *cnf2*, and *fyuA* were significantly more prevalent in *A. baumannii* strains than non-*baumannii* *Acinetobacter* strains ($P < 0.05$ by the Fisher's exact test). Such a high prevalence of virulence factors among *A. baumannii* strains indicates that this species is the most clinically important among *Acinetobacter* species from companion animals. My finding are consistent with the fact that *A. baumannii* has caused life-threatening hospital-acquired infections for companion animals (Francey et al., 2000). Momtaz *et al*. have reported a lower prevalence of the *afa/draBC* and *papC* genes and a higher prevalence of

sfa/focDE, *fimH*, and *cnf1* genes were confirmed in *A. baumannii* isolates from human patients in hospitals (Momtaz et al., 2015). In contrast, Braun and Vidotto reported that these virulence factors were not detected in *A. baumannii* isolates from urine of human patients (Braun and Vidotto, 2004). Therefore, the prevalence of virulence factors of *A. baumannii* isolates is likely to be highly diverse, although relationships between these virulence factors and clinical features remain to be fully investigated.

5. Antimicrobial resistance

I evaluated susceptibilities to piperacillin (PIP), ceftazidime (CAZ), cefotaxime (CTX), meropenem (MEM), gentamicin (GEN), tetracycline (TET), ciprofloxacin (CIP), enrofloxacin (ENR), and trimethoprim-sulfamethoxazole (TMS) using the agar dilution

method (Clinical and Laboratory Standards Institute VET01-A4, 2013) and interpreted the results according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI VET01-S2 and M100-S20, 2013). I used *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 as quality control strains. I found that 17 isolates (25%) exhibited resistance to one or more antimicrobials. Resistance to GEN was the most prevalent (n = 10, 14.9%), followed by TET (n = 8, 11.9%), CIP (n = 8, 11.9%), and TMS (n = 5, 7.5%) (Table 2), whereas all strains were susceptible to PIP, CAZ, CTX, MEM, and ENR. I did not identify any significant differences were confirmed in antimicrobial resistance prevalence between *A. baumannii* and non-*baumannii* *Acinetobacter* isolates ($P > 0.05$), nor did I detect any multidrug-resistant (MDR) *Acinetobacter* spp., which are simultaneously resistant to aminoglycosides, fluoroquinolones, and carbapenems (Ikeda et al., 2011). My results suggest that companion animals in Japan are unlikely to be a reservoir of MDR *Acinetobacter* spp. However, I detected resistance to GEN and/or CIP were detected in 5/21 (23.8%) of my *A. baumannii* isolates. I subjected the five resistant *A. baumannii* strains (i.e. strains AC8, AC31, AC41, AC79, and AC84) to multilocus sequence typing with seven genes (i.e. *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*) according to the Pasteur scheme (Diancourt et al., 2010), followed by eBURST analysis (Feil et al., 2004). This process

resulted in these five strains being assigned to ST203, ST149, ST164, ST25, and ST1198 (novel ST), respectively. According to eBURST analysis, none of the five STs found in this study belonged to the four multidrug-resistant international clones (CC1 to CC3 and ST15) (22). ST25, ST164, and ST203 have been observed in both humans (Gogou et al., 2011; PubMLST) and companion animals (Rafei et al., 2014; Belmonte et al., 2014; Pailhoriés et al., 2015), and ST149 has been isolated from human patients in Japan (PubMLST). In addition, I reported carbapenem-resistant *Acinetobacter* strains from dogs and cats in Japan (chapter 1), as has been reported for other countries (Ewers et al., 2017; Hérivaux et al., 2016). Therefore, attention should be paid to the emergence of MDR *Acinetobacter* spp. isolates from companion animals in Japan. Further studies are needed to clarify the significance as a zoonotic pathogen of *A. baumannii* isolates from companion animals.

6. Genes contributing to antimicrobial resistance

I screened all *Acinetobacter* spp. isolates for eight PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *oqxAB*, *qepA*, and *aac(6')-Ib-cr*) using multiplex PCR (Ciesielczuk et al., 2013) and subjected the isolates with minimum inhibitory concentrations (MICs) for CIP of ≥ 2

μg/mL, quinolone resistance-determining regions (QRDRs) in *gyrA* and *parC* to amplification by PCR with primer sets described elsewhere (Vila et al., 1997; Park et al., 2011). However, original primer sets were used for *A. radioresistens*, for QRDRs in *gyrA* and *parC* (forward 5′-AAGTCTGCCCGTGTGGTCG-3′ and reverse 5′.GCCATACCGACTGCAATACCG-3′ for *gyrA*, and forward 5′-ATGAGTGAACTTGGTCTGAAACAC-3′ and reverse 5′-TCAAAGTTATCCTGCCATTCTACTG-3′ for *parC*). The PCR products were bi-directionally sequenced with the same primers. Genetic analysis showed that all four CIP-resistant (MIC of 16 or 64 μg/mL) strains of *A. baumannii* had a S83L mutation in GyrA; three of the four also had a S80L mutation in ParC (Table 3). These mutations are identical to those reported elsewhere in human isolates (Vila et al., 1997; Valentine et al., 2008). In addition, my findings support those of Ardebili *et al*., who reported that, in *A. baumannii*, simultaneous mutations in GyrA and ParC contribute to high-level fluoroquinolone resistance, whereas a single mutation is associated with intermediate resistance (Ardebili et al., 2015). I also found CIP-intermediate-susceptible and -resistant strains of *A. radioresistens* (n = 3 and 2, respectively) and *A. pitti* (n = 1 and 2, respectively) in my sample. Of these strains, I found that the four strains with intermediate susceptibility have a mutation at position 83 of GyrA, whereas the resistant strains additionally have a

mutation at position 80 of ParC. Thus, QRDR mutations of GyrA and ParC are likely to occur in non-*baumannii Acinetobacter* in the same manner with *A. baumannii*. In addition to QRDR mutations, PMQRs reportedly contribute to the occurrence and spread of fluoroquinolone resistance among *Acinetobacter* spp. (Yang et al., 2016). In this study, I detected no PMQR genes in *Acinetobacter* spp. isolates with/without QRDR mutations, indicating that PMQRs are uncommon among *Acinetobacter* spp. isolates from companion animals in Japan. However, PMQRs are reportedly prevalent among other Gram-negative bacteria (Harada et al., 2014: Harada et al., 2016; Harada et al., 2017).

7. Conclusion of chapter 2

Because this study was a preliminary study of a small number of *Acinetobacter* spp. isolates, the present results cannot comprehensively characterize virulence factors and antimicrobial resistance of the bacteria from companion animals in Japan. Nevertheless, the present findings emphasize the significance of *A. baumannii* as a virulent pathogen in

companion animals. Although resistance to the tested anti-*Acinetobacter* drugs is relatively infrequent, I identified the emergence of aminoglycoside- and/or fluoroquinolone-resistant strains belonging human-related ST. Continued surveillance of animal-origin *Acinetobacter* spp. in Japan may be warranted to predict virulent and/or MDR clones of this bacterial species becoming a threat to both animal and human health.

Table 1

Species distribution of Acinetobacter spp. isolates from dogs and cats.

Species	No. of isolates (%)		
	Dog (n=54)	Cats (n=13)	Total (n=67)
A. baumannii	18 (33.3)	3 (23.1)	21 (31.3)
A. radioresistens	9 (16.7)	2 (15.4)	11 (16.4)
A. pitti	7 (13.0)	3 (23.1)	10 (14.9)

A. ursingii	6 (11.1)	1 (7.7)	7 (10.4)
A. lwoffii	4 (7.4)	0 (0)	4 (6.0)
A. baylyi	3 (5.6)	0 (0)	3 (4.5)
A. junii	2 (3.7)	0 (0)	2 (3.0)
A. nosocomialis	2 (3.7)	0 (0)	2 (3.0)
A. calcoaceticus	1 (1.9)	0 (0)	1 (1.5)
A. johnsonii	0 (0)	1 (7.7)	1 (1.5)
Others	2 (3.7)	3 (23.1)	5 (7.5)

Table 2

Prevalence of virulence factors and antimicrobial resistance among *Acinetobacter baumannii* and non-*baumannii Acinetobacter* isolates.

		No. of isolates (%)		
		A. baumannii (n = 21)	Non-*baumannii Acinetobacter* (n = 46)	Total (n = 67)
Virulence factor†	*afa/draBC*	14 (66.7)*	6 (13.0)	20 (29.9)
	papC	14 (66.7)*	1 (2.2)	15 (22.4)
	sfa/focDE	5 (23.8)*	1 (2.2)	6 (9.0)
	papGII, III	2 (9.5)	0 (0)	2 (3.0)
	cvaC	8 (38.1)*	6 (13.0)	14 (20.9)
	cnf1	1 (4.8)	4 (8.7)	5 (7.5)
	cnf2	7 (33.3)*	0 (0)	7 (10.4)
	kpsMTII	0 (0)	6 (13.0)	6 (9.0)
	fyuA	4 (19.0)*	1 (2.2)	5 (7.5)
	csgA	0 (0)	3 (6.5)	3 (4.5)
	ibeA	0 (0)	2 (4.3)	2 (3.0)
	PAI	1 (4.8)	1 (2.2)	2 (3.0)
	iutA	0 (0)	1 (2.2)	1 (1.5)
Antimicrobial resistance‡	Gentamicin	4 (19.0)	6 (13.0)	10 (14.9)
	Tetracycline	2 (9.5)	6 (13.0)	8 (11.9)
	Ciprofloxacin	4 (19.0)	4 (8.7)	8 (11.9)
	Trimethoprim-sulfamethoxazole	3 (14.3)	2 (4.3)	5 (7.5)

† The *fimH* and *traT* genes were not detected among our sample.

‡ Resistance to piperacillin, ceftazidime, cefotaxime, meropenem, and enrofloxacin was not detected in our sample.

*Significantly higher rates in *A. baumannii* isolates than non-*baumannii Acinetobacter* isolates ($P < 0.05$).

Table 3

Quinolone resistance-determining region mutations in Acinetobacter spp. isolates with a minimum inhibitory concentration for ciprofloxacin of ≥ 2 μg/mL (*n* = 12).

Species	Strain	MIC of ciprofloxacin (μg/mL)	QRDR mutation	
			GyrA	ParC
A. baumannii (n = 4)	AC31	64	Ser83Leu	Ser80Leu
	AC41	16	Ser83Leu	Ser80Leu
	AC79	64	Ser83Leu	Ser80Leu
	AC84	16	Ser83Leu	None
A. radioresistens (n = 5)	AC6	16	Ser83Tyr	Ser80Leu
	AC9	2	Ser83Tyr	None
	AC11	16	Ser83Tyr	Ser80Leu
	AC13	2	Ser83Tyr	None
	AC86	2	Ser83Tyr	None
A. pitti (n = 3)	AC1	16	Ser83Leu	Ser80Leu
	AC37	2	Ser83Leu	None
	AC43	64	Ser83Leu	Ser80Leu

CONCLUSION

The genus *Acinetobacter* is consisted by more than 50 validly named species, and it has been recognized as pathogens in hospital-acquired infection. Furthermore, *Acinetobacter* spp. have become an important pathogen increasingly because of the threat of multidrug resistance: the intrinsic resistance and the ability to acquire new resistant mechanisms (Clark NM, 2016). In veterinary medicine, reports about *Acinetobacter* infections case are increasing these days. However, the number of reports are limited and the study about molecular mechanisms of pathogenicity and antimicrobial resistance is still not adequate. In recent year, veterinary medicine has been advanced as high as human medicine, nosocomial infections via medical device such as catheters or ventilation systems to animals hospitalized in intensive care can be a serious problem. To prevent the emergence of infection by *Acinetobacter* spp. and spreading of MDR *Acinetobacter* spp., it is necessary to understand the characteristics of strains isolated from companion animals: the frequency of antimicrobial resistance and the preservation of pathogenic factors to companion animals.

In chapter 1, I reported that the isolation and analysis of genes contributing to

antimicrobial resistance of two IMP-1 type metallo-β-lactamase producing *A. radioresitens* strains. Unfortunately, infection routes of these strains were not defined, because these two strains were phylogenetically different each other. However IMP-1 type metallo-β-lactamase possessed in these two strains is a common genotype in *Acinetobacter* spp. isolated from humans (Kouyama et al., 2012). These results suggest that these antimicrobial resistant strains or antimicrobial resistant genes can be easily transmitted from human strains to animal strains, and vice versa. In addition, these strains possessed resistance genes against various class of antimicrobial drugs such as aminoglycoside or sulfanilamide, although the expression of drug resistance was not observed. These results indicated the risk of acquiring MDR and spreading of multidrug resistance under the condition of antibiotics abuse in both of human and animals.

In chapter 2, I collected *Acinetobacter* spp. isolated from diseased dogs and cats in various region of Japan and investigated the frequency of antimicrobial resistance and prevalence of pathogenic factors of *Acinetobacter* spp.. In human, most common pathogenic species of *Acinetobacter* is *A. baumannii* and it accounts for more than 70% of isolated *Acientobacter*. In dogs and cats, *A. baumannii* was also most common species, but the frequency was only approximately 30% and more than ten species were isolated. The

frequency of antimicrobial resistance of isolates *Acinetobacter* to at least one antibiotics was more than 25%. The antimicrobial resistance was equally observed in *A. baumannii* and non-*baumannii* species. The MDR *Acinetobacter* spp. defined as simultaneous resistance to aminoglycosides, fluoroquinolones, and carbapenems were not detected at present. The prevalence of pathogenic factors were mainly observed in *A. baumannii*, suggesting that *A. baumannii* was also most virulent strain in companion animals.

Because the numbers of sample in my research were limited, it is difficult to describe the characteristics of *Acinetobacter* species comprehensively. However, my present findings revealed that antimicrobial resistance of *Acinetobacter* spp. had already spread in wide range of Japan, and we are faced on emergence of multidrug resistance of *Acinetobacter* spp.. Continuous surveillance of antimicrobial resistance in companion animal-derived *Acinetobacter* may help to prevent spreading of multidrug resistance not only in companion animals but also in human. I believe that my research will contribute to enhance the further study about pathogenicity and antimicrobial resistance of *Acinetobacter* spp. in both of veterinary and human medicine.

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