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Identification and Characterization of Vancomycin-resistant *Enterococcus* species Frequently Isolated from Laboratory Mice

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Abstract: To determine the prevalence of drug resistant bacteria colonizing laboratory mice, we isolated and characterized vancomycin-resistant *Enterococcus* species (VRE) from commercially available mice. A total of 24 VRE isolates were obtained from 19 of 21 mouse strains supplied by 4 commercial breeding companies. Of these, 19 isolates of *E. gallinarum* and 5 isolates of *E. casseliflavus* possessing the *vanC1* and *vanC2/3* genes intrinsically, exhibited intermediate resistance to vancomycin respectively. In addition, these isolates also exhibited diverse resistant patterns to erythromycin, tetracycline, and ciprofloxacin, whereas the use of antibiotics had not been undertaken in mouse strains tested in this study. Although 6 virulence-associated genes (*ace*, *asa*, *cylA*, *efaA*, *esp*, and *gelE*) and secretion of gelatinase and hemolysin were not detected in all isolates, 23 of 24 isolates including the isolates of *E. casseliflavus* secreted ATP into culture supernatants. Since secretion of ATP by bacteria resident in the intestinal tract modulates the local immune responses, the prevalence of ATP-secreting VRE in mice therefore needs to be considered in animal experiments that alter the gut microflora by use of antibiotics.

Key words: ATP, laboratory mice, prevalence, vancomycin-resistant *Enterococcus*, virulence factor

Introduction

Enterococcus species are intestinal bacteria colonizing not only humans, mammals, and birds but also insects. They were regarded as harmless to humans and mammals for a long time. However, they have recently been recognized to be important opportunistic pathogens, causing nosocomial infections of the urinary tract, endocarditis, bacteremia, and central nervous system infections [4, 8]. In addition, *Enterococcus* species are intrinsically resistance to a number of antimicrobial agents such as

β -lactams and aminoglycosides [13], and there are increasingly more acquired antibiotic resistant species to other antimicrobial agents by mutation or acquisition of foreign genetic material via transfer of plasmids and transposons, including the vancomycin-resistant gene [1]. The emergence of vancomycin-resistant *Enterococcus* (VRE) has been linked to an increased use of glycopeptide antibiotics in human medicine and the use of the glycopeptide avoparcin as a growth promoter in livestock [1]. Therefore, the outbreak of VRE in humans is possibly associated with the occurrence of VRE in livestock,

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although the relationship remains unclear.

In laboratory animals, a few reports describing the detection of antibiotic resistant bacteria have been published that indicated a low incidence of drug resistance in laboratory animals, and no correlation between the emergence of the drug resistance in laboratory animals and those in humans or farm animals [11]. The detection of drug resistant *E. faecalis* and *E. faecium* in laboratory mice and rats has also been reported, and these enterococci showed resistance to β -lactams, aminoglycosides, tetracycline, or erythromycin [10, 23, 27]. Therefore, the detailed investigations are needed to clarify the characteristics of VRE currently colonizing commercially produced mice used in biomedical research.

A number of virulence factors of *Enterococcus* species have been identified in the isolates obtained from clinical specimens. These include collagen binding cell wall protein (ace), aggregation substance (asa), endocarditis-specific antigen (efaA) and extracellular surface protein (esp), as well as gelatinase, and cytolysin (hemolysin) that are associated with biofilm formation and cytotoxicity, respectively. In addition, several *Enterococcus* species of intestinal microflora have the ability to secrete a large amount of ATP [12, 16]. High levels of ATP in the gastrointestinal tract derived from intestinal microflora can modulate the balance of helper T cell (Th) immune responses of intestinal mucosa, leading to inflammatory bowel diseases [2, 22].

In the present study, VRE possessing a vancomycin-resistant gene were isolated from laboratory mouse strains (including some immunodeficient strains) obtained from commercial breeding companies. To characterize these VRE isolates, their sensitivity to various antibiotics and several virulence-associated factors including secretion of ATP were investigated. The data provide information on the characteristics of VRE colonizing in intestinal tract of laboratory mice and raises concerns regarding the potential influence of VRE colonization on the results of some animal experiments.

Materials and Methods

Isolation of VRE from laboratory mice

Fresh feces were collected from mice immediately after delivery from four different commercial breeding companies between October 2011 and February 2012 (Table 1). The use of antibiotics had not been under-

taken in the mouse strains tested in this study. Feces were homogenized with 1 ml of PBS by vortexing for 5 min and centrifugation at $6,000 \times g$ for 10 sec. Fifty μl of supernatants were spread on VR-EF plates containing $6 \mu\text{g}/\text{ml}^{-1}$ of vancomycin (Nissui, Tokyo, Japan) and incubated at 37°C for 2 days under aerobic conditions. Single colony isolation was performed using brain-heart infusion agar (Becton Dickinson, MD, USA) containing $6 \mu\text{g}/\text{ml}^{-1}$ of vancomycin (Wako, Osaka, Japan). Animal care and experimental procedures were performed in accordance with the Regulations for Animal Experimentation of Nagasaki University and with approval of the Institutional Animal Care and Use Committee.

Detection of antibiotic resistance and virulence-associated genes

Vancomycin-resistant genes, *vanA*, *vanB*, *vanC1*, and *vanC2/3* were detected by PCR using specific primers [6]. All reactions were performed in a final reaction volume of 20 μl using bacterial cells as template, 10 μl of EmeraldAmpTM PCR Master Mix (TAKARA BIO INC., Japan), and 1 μM of each primer. The PCR reaction was preheated at 95°C for 5 min and then subjected to 30 cycles consisting of denaturation at 95°C for 1 min, primer annealing at 54°C for 1 min, and extension at 72°C for 1 min, with final extension at 72°C for 7 min.

The virulence-associated genes *ace*, *efaA*, *asa*, *cylA*, *esp*, and *gelE* were also detected by PCR with specific primers as described [26]. The PCR reaction was preheated at 95°C for 5 min and then subjected to 30 cycles consisting of denaturation at 95°C for 30 sec, primer annealing at 58°C for 45 sec, and extension at 72°C for 1 min, with final extension at 72°C for 7 min. PCR products were visualized on 1.5% agarose gels.

Identification of Enterococcus species

For identification of bacteria species, gram staining, catalase production and then rapid ID 32 STREP API (bioMérieux, March l'Etoile, France) were performed. If necessary, further identification was performed by detection and sequencing analysis of 16S rRNA genes using a universal primer pair (forward, 5'-AGAGTTT-GATCCTGGCTCAG-3'; reverse, 5'-ACTACCAGGG-TATCTAATC-3'). The PCR products were purified using LaboPassTM Gel kit (COSMO GENETECH CO., Ltd., Korea) according to the manufacturer's instructions. DNA sequences were determined with primers described above using ABI PRISM BigDyeTM Terminator v3.1

Table 1. Isolation and identification of *Enterococcus* species from laboratory mice supplied from 4 commercial breeding companies

Source	Strain ^{a)}	Sex	Age (week)	<i>Enterococcus</i> species Identified (type of <i>van</i> gene)
Company 1	A/J	F	6	<i>E. gallinarum</i> (VanC1)
				<i>E. casseliflavus</i> (VanC2/3)
	C57BL/6N	M/F	4/8	Not detected
	ddY	M	6	<i>E. gallinarum</i> (VanC1)
	BALB/c	M	8	<i>E. gallinarum</i> (VanC1)
				<i>E. casseliflavus</i> (VanC2/3)
	ICR	F	8	<i>E. gallinarum</i> (VanC1)
Company 2	C3H/HeJ	F	8	<i>E. gallinarum</i> (VanC1)
	ddY	F	6	<i>E. gallinarum</i> (VanC1)
Company 3				<i>E. casseliflavus</i> (VanC2/3)
	BALB/c	M/F	≥48	<i>E. gallinarum</i> (VanC1)
				<i>E. casseliflavus</i> (VanC2/3)
Company 3	ICR	M	5	<i>E. gallinarum</i> (VanC1)
	C3H/HeJ	M/F	8	<i>E. gallinarum</i> (VanC1)
	ICR(MCH)	F	4	<i>E. gallinarum</i> (VanC1)
	129+Ter/Sv	M	≥48	<i>E. gallinarum</i> (VanC1)
	BALB/c-nu/nu ^{b)}	F	6	<i>E. gallinarum</i> (VanC1)
Company 4	ICR	F	8	<i>E. gallinarum</i> (VanC1)
	BALB/c	F	8	<i>E. gallinarum</i> (VanC1)
	C57BL/6N	M	8	<i>E. gallinarum</i> (VanC1)
				<i>E. casseliflavus</i> (VanC2/3)
	C57BL/6J	M	5	<i>E. gallinarum</i> (VanC1)
	CBA/J	M	6	<i>E. gallinarum</i> (VanC1)
	SJL/J	M	5	<i>E. gallinarum</i> (VanC1)
	NOD/SCID ^{b)}	M	6	Not detected
	BALB/c-nu/nu ^{b)}	F	5	<i>E. gallinarum</i> (VanC1)

^{a)} The antibiotic treatment had not been undertaken in these mouse strains. ^{b)} These mouse strains are immunodeficiency. F, female; M, male.

Cycle Sequencing Kits (Applied Biosystems, USA). The identity of the 16S rRNA gene sequences was verified by comparison to *Enterococcus* sequences in the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>).

Phenotype characterization of *Enterococcus* species

Gelatinase activity was assessed by inoculation of brain heart infusion broth containing 3% gelatin (Difco Lab., MI, USA), which was then incubated in 37°C for 2 days in aerobic conditions [5]. Positive gelatinase activity was determined by degradation of the gelatin to liquid after 1–1.5 h cooling of the tubes at 4°C. Hemolysin activity was determined by streaking bacterial cultures on a blood agar plate with 5% sheep blood (bioMérieux, March l'Etoile, France) and incubation for 2 days under aerobic conditions. Hemolytic activity was classified as β-hemolysis (a clear zone of hydrolysis around the colonies), α-hemolysis (partial hydrolysis including greenish zones around the colonies), or γ-hemolysis (no reaction). When β-hemolysis was observed, the strain was consid-

ered positive for hemolytic activity.

Antibiotic susceptibility

The minimal inhibitory concentration (MIC) for vancomycin and teicoplanin of each *Enterococcus* isolate was determined using the E-test (bioMérieux, March l'Etoile, France). Antibiotic susceptibilities to ampicillin (AM 10), erythromycin (E 15), chloramphenicol (C 30), tetracycline (TE 30), ciprofloxacin (CIP 5), linezolid (LZD 30), gentamycin (GM 120), and streptomycin (STM 300) were performed using Sensi-Disc™ (Becton Dickinson, MD, USA). These tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) documents M31-A2 (2002) and M100-S22 (2012). Bacterial suspensions in phosphate-buffered saline were adjusted to a turbidity of 0.5 McFarland standard, spread onto Mueller-Hinton agar (Nissui, Tokyo, Japan), and incubated for 24–48 h at 37°C under aerobic conditions. After incubation, MICs were read from where the edge of the inhibition ellipse intersected

the strip and the sensitivities graded as sensitive (S), intermediate (I) or resistant (R) by measuring the diameter of the inhibition zone. *E. faecalis* ATCC 29212 (JCM 7783), *E. casseliflavus* ATCC 25788 (JCM 8723), and *E. gallinarum* ATCC 49573 (JCM 8728) provided by Japan Collection and Microorganisms, RIKEN BRC which is participating in the National BioResource Project of the MEXT, Japan, were used as controls for tests.

Measurement of ATP

Isolates were cultured in serum-free RPMI medium (Gibco, CA, USA) at 37°C for 18 h under aerobic conditions with shaking [2, 16]. After checking growth levels spectrophotometrically at 590 nm, the culture media was centrifuged at 13,000 ×g for 5 min and the supernatants collected. To remove the bacterial cells, supernatants were filtered with a 0.22-µm membrane (Millipore Japan, Tokyo, Japan). ATP concentrations in culture supernatants were measured using an ATP assay kit (Promega, WI, USA) according to the manufacturer's protocols.

Table 2. MICs of 24 *Enterococcus* species isolates to glycopeptides

Isolates (no. of isolates)	Type of <i>van</i> gene	MICs (µg/ml ⁻¹)	
		Vancomycin ^{a)}	Teicoplanin ^{b)}
<i>E. gallinarum</i> (19)	VanC1	6 (1) 8–12 (18)	≤0.75 (19)
<i>E. casseliflavus</i> (5)	VanC2/3	6 (5)	≤0.75 (5)

^{a)} The intermediate breakpoint of MIC for vancomycin is 8–16 µg/ml⁻¹ and resistant breakpoint is ≥32 µg/ml⁻¹ according to CLSI guidelines. ^{b)} The sensitive of MIC for teicoplanin is ≤8 µg/ml⁻¹ according to CLSI guidelines.

Results

Detection of VRE from laboratory mice

Fecal samples were collected from 21 inbred and outbred mouse strains purchased from 4 commercial breeding companies. The tested mouse strains included those commonly used in biomedical research as well as a number of immunodeficient strains. VRE were detected in 19 of 21 mouse strains, excepting C57BL/6N and NOD-SCID mice obtained from Companies 1 and 4, respectively (Table 1). The VRE isolates were identified as *E. gallinarum* or *E. casseliflavus* possessing the vancomycin-resistant gene, *vanC1* or *vanC2/3*, respectively. *E. gallinarum* possessing *vanC1* was present in all 19 positive mouse strains while 5 mouse strains were also colonized with *E. casseliflavus* possessing *vanC2/3*.

Antimicrobial susceptibility of VRE

The susceptibility of 24 isolates to vancomycin and teicoplanin were determined as MICs by the E-test method (Table 2). MICs to vancomycin for 18 of 19 *E. gallinarum* isolates possessing *vanC1* gene were 8–12 µg/ml⁻¹ exhibiting intermediate resistance. The MICs to vancomycin for all 5 *E. casseliflavus* isolates possessing *vanC2/3* gene were 6 µg/ml⁻¹. The MICs of all 24 isolates were less than 0.75 µg/ml⁻¹ indicating sensitivity to teicoplanin.

The susceptibility of the 24 isolates to other antibiotics was evaluated by the disk diffusion method (Table 3). All isolates were susceptible to ampicillin, chloramphenicol, and linezolid, whereas *E. casseliflavus* isolate only exhibited an intermediate resistance to chloramphenicol. No isolates exhibited high-level resistance to

Table 3. Antibiotic resistance of 24 *Enterococcus* species isolates from laboratory mice supplied from 4 breeding companies

Source	Isolates (no. tested)	No. of resistant isolates (no. of intermediate isolates) to:							
		AM 10	E 15	C 30	TE 30	CIP 5	LZD 30	GM 120 ^{a)}	STM 300 ^{a)}
Company 1	<i>E. gallinarum</i> (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (5)	0 (0)	0 (0)	0 (0)
	<i>E. casseliflavus</i> (2)	0 (0)	0 (2)	0 (0)	0 (0)	0 (2)	0 (0)	0 (0)	0 (0)
Company 2	<i>E. gallinarum</i> (2)	0 (0)	0 (0)	0 (0)	1 (0)	0 (2)	0 (0)	0 (0)	0 (0)
	<i>E. casseliflavus</i> (2)	0 (0)	1 (1)	0 (0)	0 (0)	2 (0)	0 (0)	0 (0)	0 (0)
Company 3	<i>E. gallinarum</i> (5)	0 (0)	0 (2)	0 (0)	0 (0)	0 (5)	0 (0)	0 (0)	0 (0)
Company 4	<i>E. gallinarum</i> (7)	0 (0)	0 (0)	0 (0)	1 (0)	1 (6)	0 (0)	0 (0)	0 (0)
	<i>E. casseliflavus</i> (1)	0 (0)	0 (1)	0 (1)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)
<i>E. gallinarum</i> ATCC49573		S	S	S	R	S	S	S	S
<i>E. casseliflavus</i> ATCC25788		S	I	S	S	I	S	S	S

^{a)} These tests are for high-level aminoglycoside resistant *Enterococcus* species. AM, ampicillin; E, erythromycin; C, chloramphenicol; TE, tetracycline; CIP, ciprofloxacin; LZD, linezolid; GM, gentamycin; STM, streptomycin; S, sensitive; I, intermediate; R, resistant.

the aminoglycosides gentamycin (GM 120) or streptomycin (STM 300). However, all *E. gallinarum* and *E. casseliflavus* isolates exhibited either resistance or intermediate resistance to ciprofloxacin, while the two reference strains were sensitive or intermediate resistant, respectively. For erythromycin, 2 out of 5 *E. gallinarum* isolates from mice from company 3 and all *E. casseliflavus* isolates exhibited intermediate resistance except for one *E. casseliflavus* isolate from company 2 that was resistant. Although the reference strain of *E. gallinarum* exhibited resistance to tetracycline, only 2 of 19 isolates from companies 2 and 4 were resistant. No *E. casseliflavus* isolates exhibited resistance to tetracycline, including the reference strain.

Detection of virulence genes and secreting virulence factors

No virulence-associated genes (*ace*, *efaA*, *asa*, *cyla*, *esp*, and *gelE*) or gelatinase activity were detected in any of the 24 *Enterococcus* isolates. Since all 24 isolates exhibited α -hemolysis on 5% sheep blood agar, all isolates were negative for hemolytic activity (data not shown).

ATP in the intestinal tract secreted by intestinal microflora has recently shown to modulate the immune response of the intestinal lamina propria [2, 22]. Therefore, the ability of the *Enterococcus* isolates to secrete ATP was determined. As shown in Fig. 1, higher levels of ATP secretion were detected in each culture medium of all 5 *E. casseliflavus* isolates and 18 of 19 *E. gallinarum* isolates in comparison with that of *E. faecalis* ATCC 29212. Similarly, the two reference strains *E. gallinarum* ATCC 49573 and *E. casseliflavus* ATCC 25788 both secreted ATP. Furthermore, oxygen was dispensable for ATP secretion of these strains, since ATP in culture media under anaerobic condition were comparably detected with those of aerobic condition (data not shown).

Discussion

In the present study, 19 *E. gallinarum* possessing *vanC1*, and 5 *E. casseliflavus* possessing *vanC2/3* were isolated from 19 of 21 mouse strains. These two *Enterococcus* species are generally found in the intestinal tract of mammals along with other *Enterococcus* species such as *E. faecalis* and *E. faecium*. The vancomycin-resistant genes *vanC1* and *vanC2/3* were detected in our isolates.

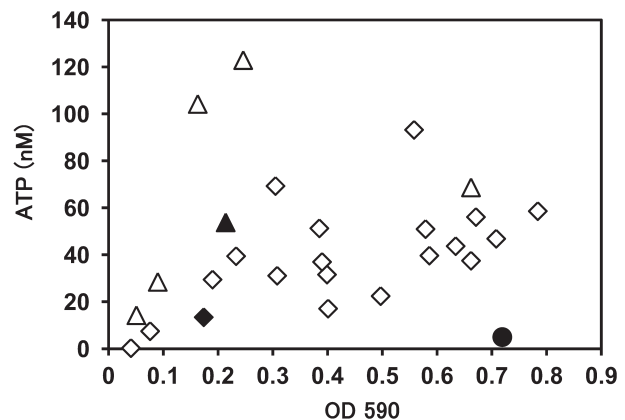


Fig. 1. Detection of ATP in the culture supernatants of vancomycin resistant *Enterococcus* isolates. ATP concentrations in culture supernatants were determined using a luciferase reaction method and the culture density of isolates was determined spectrophotometrically. The 19 *E. gallinarum* isolates (open diamond) and 5 *E. casseliflavus* isolates (open triangle) were tested; each symbol represents the value of an individual isolate. Reference strains of *E. faecalis* (filled circle), *E. gallinarum* (filled diamond), and *E. casseliflavus* (filled triangle) were also tested.

These genes impart low level resistance to vancomycin, and are located chromosomally and so are not readily transferable [8]. In contrast, *Enterococcus* species possessing either the *vanA* or *vanB* gene typically show high-level resistance to glycopeptides, and these genes are present not only on the chromosome but also be able to locate on transposons or plasmids, these are then readily transferred to other microorganisms [19, 31]. Thus, the emergence of VRE possessing the *vanA* or *vanB* gene is clinically important, especially *E. faecalis* and *E. faecium* species since these species are harboring various virulence factors. In this study, neither of these two VRE species nor other *Enterococcus* species carrying the *vanA* or *vanB* gene were detected in commercially bred mice. From the early 1990s, detection of VRE possessing the *vanA* or *vanB* gene has increased in both humans and livestock in Europe and the United States as well as in Japan [1, 9, 24, 25, 32]. However, these VRE have not been detected in livestock in Japan after the use of avoparcin was banned [20, 21]. The present results indicate that the emergences and outbreaks of VRE in humans and farm animals have not extended to commercial laboratory mouse breeding operations.

Isolates of *Enterococcus* from laboratory mouse strains exhibited diverse antibiotic resistant characteristics when tested for resistance to erythromycin, tetracy-

cline, and ciprofloxacin. In addition to intrinsic resistance to a number of antibiotics including β -lactams and aminoglycosides, the occurrence of acquired resistance among *Enterococcus* species to macrolides, tetracyclines, and quinolones by receipt of resistant gene, such as erythromycin (*erm*) or tetracycline (*tet*) resistance genes, and the accumulation of sporadic mutations in DNA gyrase (*gyrA*) or topoisomerase (*parC*) gene have been demonstrated [14, 17, 28]. Furthermore, these antibiotic resistance genes are found in the transferable regions of DNA which can be exchanged among different species [14]. Therefore, the VRE isolates in this study exhibiting resistance to antibiotics might possess transferable antibiotic resistant gene or chromosomal gene with mutations. However, since microflora in laboratory mice used in this study had not undergone a selective pressure by the use of antibiotics, the mechanism to achieve the antibiotic resistance is unclear. The previous study showed the detection of antibiotic resistant in laboratory mice which had not been administered with antibiotics [27]. Therefore, the resistant genes in those mice might be inherited stably in the laboratory mice to this day. In addition to the identifications of resistance genes in our isolates, further studies are required to clarify the source and the prevalence of these transferable resistant genes in laboratory mice.

Although none of 6 virulence-associated genes we tested for were detected in any *Enterococcus* isolates, secretion of ATP at very high levels was detected in supernatants of *E. gallinarum* and *E. casseliflavus* cultures. ATP has been demonstrated to modulate immune cell functions via activation of the ATP receptors P2X and P2Y, leading to the development of excessive immune-mediated and inflammatory disease [7, 15, 18, 30]. Furthermore, ATP in the intestinal tract induces activation of Th17 responses and mast cells in the intestinal mucosa, leading to inflammatory bowel diseases including ulcerative colitis and Crohn's disease [2, 22]. The source of ATP in the intestinal tract has been shown to be intestinal bacteria including several *Enterococcus* species (including *E. gallinarum*) [12, 16]. Our present study showed *E. casseliflavus* may also be a plausible source of ATP in the intestinal tract. Since use of antibiotics can induce abnormal proliferation of VRE via low-induction of an innate immune response to eliminate the Gram-positive bacteria [3, 29], these two VRE species in the gastrointestinal tract may induce inflammatory diseases via accumulation of a large amount of ATP following

use of antibiotics.

In conclusion, the present study has shown that *E. gallinarum* and *E. casseliflavus* possessing *vanC* genes intrinsically were detected frequently in laboratory mice including immunocompromised mouse strain. These VRE isolates exhibited intermediate resistance to vancomycin, and several isolates also showed resistance to erythromycin, tetracycline, and/or ciprofloxacin, while the use of antibiotics had not been undertaken in all mouse strains. Additional studies are needed to clarify the mechanisms of drug-resistances in *Enterococcus* present in laboratory animals. Furthermore, since the present study has firstly elucidated that *E. casseliflavus* has the ability to secrete ATP, this species is consequently considered as a source of ATP in intestinal tracts like several *Enterococcus* species including *E. gallinarum*. Therefore, the potential role of VRE as pathogens and their ability to influence physiological/immunological responses should be considered by researchers using such colonized mice, especially when experiments involve the use antibiotics.

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