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Isolation of VanA-Type Vancomycin-Resistant *Enterococcus* Strains from Domestic Poultry Products with Enrichment by Incubation in Buffered Peptone Water at 42°C▽

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Eight VanA-type enterococcal strains were isolated from 8 of 171 domestic poultry products by using enrichment by incubation in buffered peptone water at 35°C and 42°C. The pulsed-field gel electrophoresis patterns of all six VanA-type *Enterococcus faecalis* isolates were nearly indistinguishable, indicating the presence of a specific clone in Japan.

The use of avoparcin as a growth promoter and prophylactic agent in feed has been associated with the occurrence of vancomycin-resistant enterococci (VRE) in farm animals in the European Union (EU) (2, 3, 4, 11). In Japan, avoparcin was also used as a food additive on animal farms for approximately 7 years until it was banned in March 1997 (10, 22). However, there has been no documentation in the literature on the isolation of VanA-type VRE from domestic poultry products in Japan (8, 10). Several traditional methods for detecting enterococci, including VRE, in poultry products include an enrichment step using buffered peptone water (BPW) (7, 12, 14, 21). Although BPW is typically incubated at 35 to 37°C, based on the reported optimal growth temperature for enterococci of 35°C (19, 20), the growth of VRE at this temperature can be inhibited by antagonistic activities of background microflora in samples contaminated with low levels of VRE, as 35 to 37°C is also the optimal growth temperature for most mesophilic microorganisms. Several investigators have demonstrated that incubation at an elevated temperature using selective enrichment medium was useful in the isolation of enterococcal strains from retail meat (9, 17, 18). To our knowledge, however, no study has evaluated cultivation temperature during the enrichment process in the isolation of VanA-type VRE from poultry products to examine the influence of background microorganisms on VanA-type VRE growth in BPW. In this study, we carried out isolation of VanA-type VRE from retail Japanese domestic chicken products by using two different enrichment temperatures (35°C and 42°C) and characterized VanA-type VRE isolates by antimicrobial susceptibility testing and pulsed-field gel electrophoresis (PFGE). We also assessed the effects of incubation temperature on the growth of VanA-type VRE under pure culture conditions, on the rates of recovery of inoculated VanA-type VRE strains from artificially contaminated poultry samples, and on the inhibition of suspected enterococcal strains by background microorganisms in poultry samples.

A total of 171 retail domestic poultry products obtained in Osaka, Japan, between September 2008 and April 2009 were

TABLE 1. Characteristics of vancomycin-resistant enterococcal strains isolated from domestic poultry samples in this study

Strain no.	*Enterococcus* species	Vancomycin resistance gene	MIC (μg/ml)		Enrichment temp[a] (°C)	Isolation date
			Vancomycin	Teicoplanin		
Ha8	*E. faecalis*	*vanA*	>256	>256	42	October 2008
Ha16	*E. faecalis*	*vanA*	>256	>256	42	October 2008
Ha36	*E. faecalis*	*vanA*	>256	>256	42	March 2009
Ha55	*E. faecalis*	*vanA*	>256	128	42	April 2009
Ha58	*E. faecalis*	*vanA*	>256	32	35	April 2009
Ha61	*E. faecalis*	*vanA*	>256	256	35/42	April 2009
Ha20	*E. faecium*	*vanA*	>256	32	42	November 2008
Ha26	*E. faecium*	*vanA*	>256	32	35/42	December 2008

[a] Abbreviations: 35, a strain isolated only from BPW enrichment culture incubated at 35°C; 42, a strain isolated only from BPW enrichment culture incubated at 42°C; 35/42, a strain isolated from BPW enrichment culture incubated at 35°C and 42°C.

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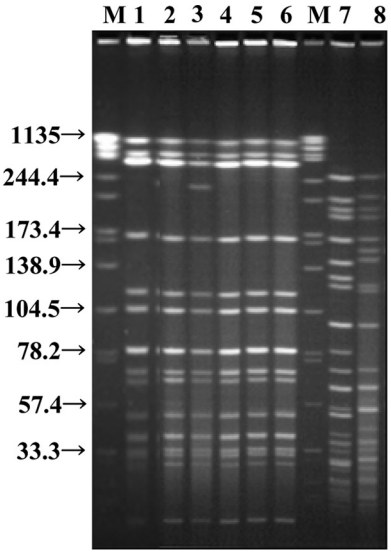

FIG. 1. DNA fingerprinting of VanA-type VRE (*E. faecalis* and *E. faecium* strains) isolated from retail domestic poultry samples. Isolated genomic DNA of each isolate was digested with SmaI and subjected to PFGE. Lane 1, *E. faecalis* Ha8; lane 2, *E. faecalis* Ha16; lane 3, *E. faecalis* Ha36; lane 4, *E. faecalis* Ha55; lane 5, *E. faecalis* Ha58; lane 6, *E. faecalis* Ha61; lane 7, *E. faecium* Ha20; lane 8, *E. faecium* Ha26; lanes M, XbaI-digested PFGE patterns of DNA size standard *Salmonella enterica* serovar Braenderup H9812. Numbers to the left of the gel image indicate the molecular size in kilobase pairs.

examined for the presence of VanA-type VRE. Twenty-five grams of each sample was removed aseptically and placed in a sterile plastic bag (30 by 19 cm), to which 225 ml of BPW (Eiken Chemical Co., Ltd., Tokyo, Japan) was added. The sample was then incubated for 24 h at either 35°C or 42°C. Ten microliters of the culture supernatants was spread plated on Enterococcosel agar (Becton, Dickinson and Company, Sparks, MD) plates containing 4 μg/ml vancomycin (Wako Pure Chemical Industries, Ltd., Hyogo, Japan) and incubated at 36°C for 48 h. Screening for the *vanA* gene was done using the colony-sweep PCR method with phosphate-buffered saline (PBS) washed-cell suspension, Z *Taq* polymerase (Takara Biochemicals, Shiga, Japan), and previously described primers (1, 16). PCR-positive sweeps were streaked for isolated VanA-type VRE colonies on an Enterococcosel agar plate containing 32 μg/ml vancomycin. The plates were incubated at 36°C for 48 h, and suspected VRE colonies were confirmed as enterococci by conventional biochemical methods (8). In addition, identification of *Enterococcus faecalis* and *Enterococcus faecium* harboring the *vanA* gene was performed by a multiple PCR assay previously described by Depardieu et al. (6). For the five VanA-positive poultry product samples obtained in October 2008 and April 2009, the number of VanA-type VRE was determined by plating 1-ml volumes of samples diluted 10-fold in sterile peptone saline onto Enterococcosel agar plates containing 32 μg/ml vancomycin. Colonies were counted after incubation at 36°C for 48 h. MICs of *vanA*-genotype VRE isolates to vancomycin and teicoplanin were determined using the Etest method (AB Biodisk, Solna, Sweden) in accordance with the criteria of the Clinical and Laboratory Standards Institute (5). Subtyping of VanA-type VRE isolates was performed by PFGE with SmaI-digested chromosomal DNA (8, 15). PFGE was performed on a 1.0% SeaKem Gold agarose gel (Cambrex Bio Science, Rockland, ME) and a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, CA). The PFGE standard strain *Salmonella enterica* serovar Braenderup H9812 was obtained from the PulseNet program, Enteric Diseases Laboratory Branch, Centers for Disease Control and Prevention, through the National Institute of Infectious Diseases (Japan).

The two representative VanA-type VRE strains were precultured overnight in Trypticase soy broth (Becton, Dickinson and Company) at 36°C. Each culture medium was diluted in BPW to obtain 2 log CFU/ml, and 100 μl of the culture dilution was inoculated into 40 ml of brain heart infusion (BHI) broth (Becton, Dickinson and Company) in a 200-ml Erlenmeyer flask. The flask was incubated at 35°C or 42°C with constant shaking (160 rpm) for 24 h, and the optical density at 600 nm (OD_{600}) of the cultures was monitored every 30 min using an OD-Monitor A & S (Taitec Co., Ltd., Saitama, Ja-

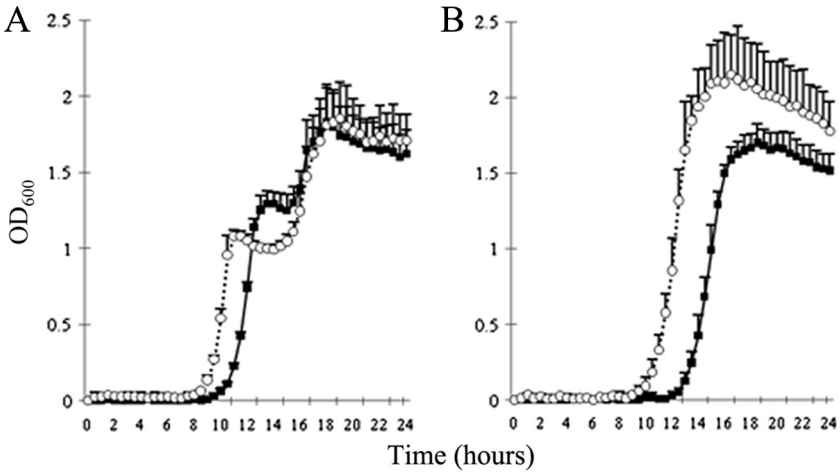

FIG. 2. Growth curves of *E. faecalis* Ha8 (A) and *E. faecium* Ha20 (B) cultured in BHI broth incubated at either 35°C (■) or 42°C (○). Results obtained from three independent experiments are presented as means ± standard deviations.

TABLE 2. Comparison of *vanA* gene detection by colony-sweep PCR after enrichment incubation with BPW at 35°C and 42°C

Inoculum strain	Inoculum dose (CFU/25 g)	No. of positive samples/no. of samples tested with incubation temp:	
		35°C	42°C
E. faecalis Ha8	300–310	2/3	3/3
E. faecalis Ha16	130–600	2/3	3/3
E. faecalis Ha36	100–290	1/3	2/3
E. faecalis Ha55	630–1,400	2/3	3/3
E. faecalis Ha58	260–660	2/3	3/3
E. faecalis Ha61	780–1,700	2/3	3/3
E. faecium Ha20	260–450	0/3	2/3
E. faecium Ha26	350–510	0/3	0/3
Total		11/24	19/24

pan). Each strain was examined in triplicate. All VanA-type isolates in this study were inoculated onto the poultry samples to compare the effect of BPW incubation at 42°C on the isolation of VanA-type VRE with the effect of incubation at 35°C. The strains were precultured at 36°C for 24 h under static conditions and then serially diluted in BPW to obtain a cell concentration of approximately 3 log CFU/ml. One hundred microliters of each bacterial cell suspension was individually inoculated onto two 25-g portions of each poultry sample in sterile plastic bags. Each inoculated poultry sample was added to 225 ml of BPW and homogenized for 1 min. One sample was incubated at 35°C for 24 h, and the second was incubated at 42°C for 24 h. Colony-sweep PCR analyses with Enterococcosel agar containing 4 μg/ml vancomycin for the presence of the *vanA* gene were then performed as described above. The artificially contaminated poultry sample experiment was performed in triplicate. To evaluate the success of cultivation using the different enrichment temperatures, data regarding the identification of the *vanA* gene from the colony-sweep PCR were analyzed using Fisher's exact test. To examine the effect of enrichment temperature on inhibition of suspected enterococcal strains by background microflora, growth kinetics were compared among these microorganisms in three different poultry samples. For each sample, two 25-g portions were placed into sterile plastic bags to which 225 ml of BPW was added. After homogenization, the samples were incubated at 35°C and 42°C. Each culture was sampled every 3 h for the initial 15 h and after incubation for 24 h. The number of background microorganisms was determined by plating 1-ml volumes of samples serially diluted in PBS onto standard agar (Eiken) plates. Colonies were counted after incubation at 36°C for 24 h. The growth of enterococcal strains was monitored by plating 100-μl volumes of appropriate dilutions onto EF agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and characteristic enterococcal colonies were counted after incubation at 36°C for 48 h.

Colony-sweep PCR analyses revealed that 8 of 171 domestic poultry samples were positive for the *vanA* gene. Isolation of VRE strains from the PCR-positive sweeps resulted in the identification of six *vanA*-genotype *E. faecalis* strains and two *vanA*-genotype *E. faecium* strains. Of the eight *vanA*-genotype VRE-positive samples, five were identified from only the 42°C BPW enrichment culture, while only a single isolate was uniquely identified from incubation at 35°C (Table 1). The number of VanA-type VRE was beneath the detection limit (i.e., less than 10 CFU/g) in each of the five VRE-positive poultry samples obtained in October 2008 and April 2009, suggesting that VanA-type VRE levels might be markedly low in domestic chickens in Japan. All *vanA*-genotype *E. faecalis* and *E. faecium* strains had vancomycin and teicoplanin MICs of >256 μg/ml and ≥32 μg/ml, respectively, confirming that these strains corresponded to the VanA phenotype (Table 1). Although the PFGE patterns of two VanA-type *E. faecium* strains were distinct (Fig. 1, lanes 7 and 8), the patterns of all *E. faecalis* isolates were almost indistinguishable (lanes 1 to 6). Given that the six VanA-type *E. faecalis* strains were isolated from distinct poultry samples that were obtained from 5 different retail shops, these results indicated that a specific *E. faecalis* clone harboring the *vanA* gene may be widely disseminated in Japan. The dominant species of VanA-type VRE isolated from poultry sources has been shown to differ depending on region. In New Zealand, greater than 80% of VRE isolates from broiler fecal samples were identified as *vanA*-genotype *E. faecalis* strains with very similar PFGE patterns,

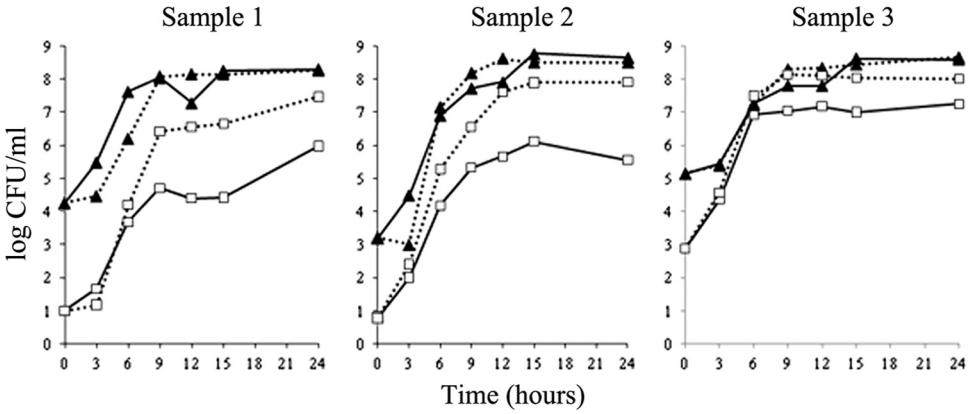

FIG. 3. Growth curves of background microflora and suspected enterococcal strains in three different poultry samples cultured in BPW incubated at either 35°C or 42°C. ▲, background microflora; □, suspected enterococcal strains. Solid lines show the data for incubation at 35°C, and dotted lines show those for incubation at 42°C.

indicating that once established, a single, dominant clonal strain is capable of widespread contamination, and supporting the idea of clonal VRE dissemination rather than horizontal transfer of vancomycin resistance elements (13). Our findings also suggest that the epidemiological features of VanA-type VRE strains associated with poultry production environments in Japan may be similar to those observed in New Zealand. To our knowledge, this is the first report of the isolation of VanA-type VRE from retail domestic poultry products in Japan.

The growth kinetics of *E. faecalis* Ha8 and *E. faecium* Ha20 cultured under pure culture conditions in BHI broth incubated at 35°C or 42°C are shown in Fig. 2. For both isolates, initiation of the exponential growth phase was shortened by approximately 2 to 3 h at the 42°C incubation temperature. In poultry samples artificially contaminated with VanA-type VRE isolates, a significant difference in the detection of the *vanA* gene using colony-sweep PCR with BPW was observed between incubation at 35°C and incubation at 42°C, with detection rates of 11/24 and 19/24, respectively ($P < 0.05$) (Table 2), indicating that the use of the 42°C enrichment step increased the recovery rate of the VanA-type VRE strains, which was significantly higher than that at 35°C. Figure 3 illustrates the growth kinetics of background microflora and the suspected enterococcal strains isolated from three independent retail poultry products in BPW enrichment cultures incubated at 35°C and 42°C. The initial levels of background microflora and suspected enterococcal strains in samples 1, 2, and 3 were 4.3 and 1.0 log CFU/ml, 3.2 and 0.7 log CFU/ml, and 5.1 and 2.9 log CFU/ml, respectively. After the BPW enrichment step, the maximum densities of background microflora in cultures incubated at 35°C were similar to those at 42°C. In contrast, the maximum levels of suspected enterococcal strains in BPW enrichment cultures incubated at 35°C and 42°C in samples 1, 2, and 3 were 6.0 and 7.5 log CFU/ml, 5.6 and 7.9 log CFU/ml, and 7.3 and 8.0 log CFU/ml, respectively (Fig. 3). These results suggested that the impact of elevated incubation temperature on the enrichment of VRE may be greater in poultry with low levels of enterococcal contamination, such as the VanA-positive samples obtained in this study. We conclude that BPW incubation at 42°C is superior to incubation at 35°C for the enrichment and detection of VanA-type VRE in poultry samples.

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