

Species Distribution and Resistance Phenotypes of Vancomycin-Resistant *Enterococcus* Isolated from Pigs in Pulau Pinang, Malaysia

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ABSTRACT

Vancomycin-resistant enterococci (VRE) are important nosocomial pathogens. The extensive use of avoparcin as a growth promoter in poultry and pigs is the hypothesized factor for the emergence of vancomycin resistance in enterococci in animals. As pork is one of the major protein sources for 30% of Malaysians, the present study was conducted to elucidate the role of pigs in the epidemiology of VRE. In this study, 220 rectal swabs were collected from pigs at 12 pig farms in Pulau Pinang. The study found 10 of 12 farms (83.3%) and 92 (41.8%) of the sampled pigs were positive for VRE. Of the 92 isolates examined by PCR, *E. faecium* (14%), *E. casseliflavus* (21.7%), *E. gallinarum* (1.1%) and other *Enterococcus* species (63.0%) were identified. *VanA* was detected in *E. faecium* and *E. gallinarum*. Questionnaire survey indicated that none of the sampled farms had used glycopeptides, either for growth promotion or for therapy. Tylosin, which has also been associated with vancomycin cross-resistance, was used in 41.8% of the sampled farms; however, there was no significant difference ($P>0.05$) between the proportion of VRE detected in the farms which used tylosin to those farms which did not. E-test on selected 49 isolates showed 16.0% of the isolates had $MIC\leq 8$ and 22.0% had $MIC\geq 32$. Single isolates of *E. faecium* and *E. gallinarum*, both possessed the resistance gene *vanA*, showed very high resistance ($MIC>256$). About 10.0% of the isolates, in which *van* genes was not detected, had $MIC>32$. In conclusion *E. faecium* and *E. faecalis* were found to be present at a low rate in the pigs sampled in this study. However, detection of *vanA* with high level of vancomycin resistance ($MIC>256$) highlights the potential public health threat associated with the pigs.

Keywords: *Enterococcus*, Malaysia, vancomycin-resistant, pigs, resistance phenotypes

INTRODUCTION

Vancomycin-resistant *Enterococcus* (VRE) is one of the major organisms causing nosocomial infections in humans (Simjee *et al.*, 2006). Vancomycin is a glycopeptide antibiotic, and is the drug of choice for *Enterococcus* infections. In the recent years, an increasing resistance towards the antibiotic has been seen; therefore, treatment

of the infection can be difficult. Molecular evidence suggests animals as the likely reservoir for VRE (Depardieu and Courvalin, 2005). Avoparcin, which is also of the glycopeptide antibiotic group, was used extensively in feed as a growth promoter for pigs and poultry. Such use has resulted in the ensuing cross-resistance which may be transmitted to enterococcal strains infecting humans (Centinkaya *et al.*, 2000).

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The use of vancomycin in the local hospitals in Malaysia was common prior to 1994 (Cheong *et al.*, 1994; Cheong *et al.*, 1996). Nevertheless, limited reports are available about VRE in humans. Only one report by Raja *et al.* (2005) was encountered on the community-acquired high vancomycin-resistant *Enterococcus faecium* infection which was presented at the University Malaya Medical Centre, Selangor. This could signify that the infection is rare, under reported or under diagnosed. In contrast, detection of VRE in animal and animal products has been reported by a few authors. Among other, Ong *et al.* (2002) detected low rates (2%) of VRE in the Malaysian poultry wet markets, while Dahlia *et al.* (2005) reported five VRE isolates from a total of 172 *Enterococcus* from ducks. An epidemiological study by Hassan *et al.* (2006) documented that 43.8 % of the sampled broilers, including day-old-chicks of six poultry farms, were colonised by VRE. Radu *et al.* (2001) recorded the occurrence of *vanA* and *vanC2/C3* genes in *Enterococcus* species isolated from poultry source. In addition, Radu *et al.* (1999) also molecularly characterised vancomycin-resistant *E. faecium* from the imported beef samples in Malaysia.

Pork serves as a major protein source for 7.5 million (30%) of Malaysians (DVS Perak, 2007). In other countries, the role of pigs in VRE dissemination, reservoir and human infection was suggested as VRE has been isolated from pork, pigs, pig farms and the environment (Klein *et al.*, 1998; Kariyama *et al.*, 2001; Manero *et al.*, 2006). In addition, Lu *et al.* (2002) reported cases of VRE infection in humans which were linked to an outbreak of VRE infection in pigs (Manero *et al.*, 2006). Yet, no studies have been conducted on VRE in pigs in Malaysia to elucidate its role in the epidemiology of VRE and the risk of transmission to humans. The present study was conducted to detect the occurrence of VRE at selected pig populations in Pulau Pinang, Malaysia, as well as to describe the distribution of *Enterococcus* species and their resistance phenotypes. The study employed a multiplex polymerase chain reaction (M-PCR)

to simultaneously detect the species of VRE and resistance determining genes.

MATERIALS AND METHODS

Study Design

A cross-sectional study was conducted between October and November 2006 at selected pig farms in Pulau Pinang. Farms were chosen based on the farm owners' willingness to participate in the study. The State Department of Veterinary Services (DVS) was contacted via a formal letter and was informed about the study. The farmers were first contacted via the regional public health officer. They were briefed about the project and were invited to participate in the study. Once they agreed, an appointment was arranged and the researchers proceeded with the study.

Study Population

The study population was the finishing pigs from 12 farms, and for this purpose, 15 to 20 pigs were sampled from each of the 12 pig farms.

Sample Size

At the time of the study, there were a total of 219 farms and a standing pig population of 296, 232 in Pulau Pinang (DVS, 2006). Using the assumption of an overall animal-level prevalence of 18% (Butaye *et al.*, 1999) and a farm-level prevalence of 30% at a confidence level of 95%, the sample size calculation was performed as suggested by Thrusfield (2005) and Dohoo *et al.* (2006).

DATA AND SAMPLE COLLECTION

Questionnaire Survey

Information related to pig herd and the management was obtained by interviewing the farm managers using a structured pre-tested questionnaire. The information included size of the farm, number of finishing pigs, antibiotics used for therapy and supplements, use of growth promoters, type of feed, source of piglets,

possibility of contact with other animals and farms' bio-security measures.

Sample Collection

Using a sterile swab, a rectal swab was performed on each pig, following the procedure described by Garcia-Migura *et al.* (2005).

MICROBIOLOGICAL ANALYSIS

Isolation of VRE and Biochemical Characterization

Each rectal swab was streaked onto a plate containing Slanetz and Bartley agar (Merck Inc., Germany), supplemented with 32 µg ml⁻¹ vancomycin (Sigma, USA). From each plate, all red-maroon colonies were purified. Confirmation of suspected enterococci colonies was done using biochemical tests described for the genus *Enterococcus* (Simjee *et al.*, 2006). Those confirmed as *Enterococcus* were stored in 20% glycerol (Ameresco) Brain Heart Infusion (BHI; Pronadisa, Spain) broth at -20 °C until further use.

Identification of VRE: Molecular Characterization

DNA extraction

Briefly, overnight culture was made to turbidity level of McFarland 0.5 standard and the cells were digested using enzymatic lysis buffer (20mM Tris.Cl, pH 8.0; 2mM Sodium EDTA, 1.2% Triton X-100% and 20mg/ml lysozyme). DNease® Blood and Tissue DNA extraction kit (Qiagen®, Germany) was used to extract genomic DNA according to the protocol described for Gram-positive bacteria by the manufacturer.

Species and *van* gene determination

Multiplex polymerase chain reaction (M-PCR) assay developed by Kariyama *et al.* (2000) was used with a few modifications (Table 1). Briefly, seven pairs of *E. faecalis*, *E. faecium*, *E. gallinarum* (*vanC1*) and *E. casseliflavus* (*vanC2/C3*), *vanA*, *vanB*, and *rrs* 16S rRNA specific primers were used in a single reaction for a simultaneous determination of the common and clinically important VRE species and resistance genes (Dutka-Malen *et al.*, 1995; Kariyama *et*

TABLE 1
Multiplex PCR primers used for species identification and vancomycin- resistance gene detection

Primer specificity	Primer	Primer pair sequences	Product size (bp)
<i>vanA</i> gene	<i>vanA</i>	5'-CATGAATAGAATAAAAGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	1,030
<i>vanB</i> gene	<i>vanB</i>	5'- AAG CTA TGC AAG AAG CCA TG -3' 5'- CCG ACA ATC AAA TCA TCC TC-3'	536
<i>E. gallinarum</i>	<i>vanC1</i>	5'-GGTATCAAGGAAACCTC-3' 5'-CTTCCGCCATCATAGCT-3'	822
<i>E. casseliflavus</i> / <i>E. flavescens</i>	<i>vanC2/C3</i>	5'-CGGGGAAGATGGCAGTAT-3' 5'-CGCAGGGACGGTGATTTT-3'	484
<i>E. faecalis</i>	ddIE. faecalis	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	941
<i>E. faecium</i>	ddIE. faecium	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	658
PCR internal control	<i>rrs</i> (16S rRNA)	5'-GGA TTA GAT ACC CTG GTA GTC C-3' 5'- TCG TTG CGG GAC TTA ACC CAA C-3'	320

al., 2000). Primer for *vanB* was adopted from Elsayed *et al.* (2001). All the primers were synthesised by Research Biolabs, Singapore.

Species identification and *van* gene determination by multiplex PCR were repeatedly checked against ATCC *Enterococcus faecalis vanB* strain (ATCC 51299), *E. faecium vanA* strain (ATCC 51559), *E. gallinarum* (ATCC 49573), and *E. casseliflavus* (ATCC 25788) to evaluate and optimize the PCR conditions in reference to the expected PCR product band sizes (Fig. 1).

Agarose Gel-Electrophoresis Analysis

The PCR product was analysed using gel electrophoresis on 1.5% agarose gel at 75 volts for 1 hr. The gel was stained in ethidium bromide (5µg/ml) and gel photo was taken under UV using the AlphaImager®. Meanwhile, the analysis was made through visual inspection

for the presence of expected band size and by comparing with standard controls and DNA markers.

E-test

The level of resistance to vancomycin was determined using E-test kit for vancomycin (AB Biodisk, Sweden) according to the manufacturer's guideline. Brief, overnight culture was used to make a suspension of McFarland 2 standard. A suspension of 150µl was evenly streaked on a plate containing BHI agar. Vancomycin strip was placed at the centre of the plate. After 48 hr of incubation at 37 °C, the MIC value was determined by reading the edge of the inhibition zone ellipse intersecting the side of the strip. The E-test was done on 49 isolates consisting most of the isolates identified to the species level, and 21 isolates which were not identified to the species level (Table 3).

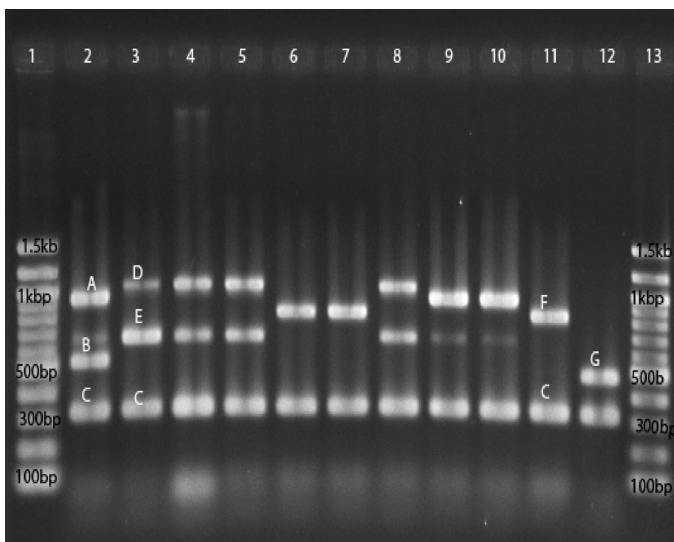


Fig. 1: Multiplex PCR products gel-electrophoresis photo Lane [L]1 & 13: 100bp DNA marker [BioLabs], L2: *E. faecalis* ATCC 51299, L3: *E. faecium* ATCC51559, L11: *E. gallinarum* ATCC 49573 and L12: *E. casseliflavus* ATCC 25788 are positive controls. Lanes 4-to-10 are samples. Alphabet A indicates a band as result of amplification of *E. faecalis* specific gene [941bp], B: *vanB* specific [536bp], C: *rrs* specific [320bp], D: *vanA* specific [1,030bp], E: *E. faecium* specific [658bp], F: *E. gallinarum/vanC1* specific [822bp] and G: *E. casseliflavus/ vanC2/3* specific gene [484bp]

Data analysis

Farm data were managed, collated, and analysed using SPSS version-15 statistical software (SPSS Inc. Chicago). A descriptive analysis was used to describe the sampled population in the study. The differences between the proportions were tested using the Chi-square (χ^2) analysis at the significance level of $\alpha = 0.05$.

Results

A total of 220 pigs were sampled throughout the period of study, from which 92 (41.8%) animals were found to be VRE positive. The organism

was detected in 10 of the 12 sampled farms (83.3%) with a detection rate ranging between 0% and 85% ($\chi^2=65.3, p<0.05$) (Table 2).

Description of the Herds

Seventy-five percent of the sampled farms supply pigs for the local, state, and nationwide markets. Meanwhile, only farms 4 and 7 supply to limited local markets in Penang. The swine population in the sampled farms ranged from 1000 to 12,000 (mean= 3205, sd = 3139.4). Each pig farm had a mean of 409 finishers with 8 to 20 pigs per pen. The age range of the farms visited was between

TABLE 2
Demography and VRE status of sampled pig farms in Pulau Pinang, Malaysia

Farm	Standing pig population	Finisher pigs	No. pigs sampled	VRE positives (%)	No. VRE isolated
1	4,369	500	20	17 (85.0)	22
2	1,400	250	15	9 (60.0)	9
3	1,200	130	15	9 (60.0)	12
4	2,500	100	20	12 (60.0)	16
5	12,000	2,000	20	0	0
6	1,000	100	15	2 (13.3)	2
7	2,000	220	20	0	0
8	2,000	200	15	7 (46.6)	9
9	2,000	110	20	11 (55.0)	13
10	6,000	700	20	10 (50.0)	15
11	3,000	300	20	10 (50.0)	13
12	1,000	300	20	5 (25.0)	6
Total (Mean)	38,469 (3205.75)	4,910 (409)	220	92 (41.8)	117

TABLE 3
The minimum inhibitory concentration (MIC) of vancomycin-resistant *Enterococcus* species isolated from pigs

VRE species	Minimum inhibition concentration [$\mu\text{g/ml}$]			
	MIC = 8	MIC 9-31	MIC 32-128	MIC >256
<i>E. faecium</i> (n=10)	-	60.0%	30.0%	10.0%
<i>E. gallinarum</i> (n=1)	-	-	-	100%
<i>E. casseliflavus</i> (n=17)	11.8%	64.7%	23.5%	-
<i>Enterococcus sp.</i> (n=21)	28.6%	71.4%	-	-
Total (N=49)	16.0%	62.0%	18.0%	4.0%

6 and 50 years (mean = 34, sd = 12.8). For ease of analysis, the farms were categorised into new (<25 years) and old (≥ 25 years), respectively. Based on this categorization, three of the farms were deemed new while nine of them were categorised as old. However, the Pearson Chi-square analysis showed no significant difference ($\chi^2 = 0.900$, $p > 0.05$) between farm age and the proportion of VRE detected in the farm.

Commercial or self-mixed pigs' meals and well water were used at all farms. None of the farmers reported using avoparcin. Glycopeptides, including vancomycin, appeared to have never been used in any of the farms for therapeutic or as a feed supplement. For therapeutic purposes, wide ranges of antibiotics obtained from the pharmaceutical company sales representative were also used. Tylosin was the commonly used antibiotic at five of the 12 farms (41.6%) which reported to use it. However, the statistical analysis did not show any significant association ($\chi^2 = 1.888$, $p > 0.05$) between the use of tylosin and the proportion of VRE positive pigs in the farms. All except two farms (farms 9 and 12) used disinfectant in footbath and vehicle-dip at the entrance, and restrict visitors from entering the farms. All the farms were located far from residential areas (judged by the absence of housing development at least 15 km from the farms), and they are separated from poultry and other livestock farms. All the farms are at least 500 meters away from each other.

Detection of VRE Species and Resistance Phenotypes

A total number of 117 VRE were isolated from 92 pigs (Table 2). The DNA extraction and purification were performed on 92 of the isolates and preserved for PCR.

From the 92 isolates, PCR identified three species, namely *E. faecium* (14.0%), *E. casseliflavus* (21.7%), *E. gallinarum* (1.1%), and other *Enterococcus* species (63%).

Vancomycin resistance gene *vanA* was detected in *E. faecium* (1 of 13) and *E. gallinarum* (1 of 1) isolates. However, Vancomycin resistant gene *vanB* was not observed in any of the

isolates. *VanC1* and *vanC2/3*, which are intrinsic to *E. gallinarum* and *E. casseliflavus*, were also detected. Interestingly, the resistance gene for 70 of the 92 (76.1%) PCR tested isolates was not detected.

The E-test on 49 isolates showed that 16% of the isolates have $MIC \leq 8$, whereas 64% had MIC between 8 and 31 and 22% had $MIC \geq 32$. All *E. faecium* isolates tested had $MIC > 8$. In fact, a single isolate of *E. faecium* and *E. gallinarum*, which both possess the resistance gene *vanA*, showed very high resistance to vancomycin ($MIC > 256$). Moreover, from 17 *E. casseliflavus* isolates tested, with natural resistance gene *vanC2/3*, vancomycin resistance $MIC \geq 32$ was observed in 23% of the species, while a majority (64.7%) of them exhibited intermediate resistance of MIC ranging from 8 to 31 (Tables 3 and 4). As presented in Table 4, 10% of the isolates, where *van* genes was not detected, were resistant to $MIC > 32$ (Table 4).

DISCUSSION

Farm owners interviewed in this study reported that they had not used avoparcin or any glycopeptide drugs, either as a growth promoter or for therapeutic purposes. Bager *et al.* (1997) were the first to establish the association between avoparcin and the presence of VRE in pig farms. The findings from the study of Bager *et al.* (1997) were followed by several others who had prompted European countries to ban the use of avoparcin in their livestock. Nevertheless, research in other countries, where avoparcin is banned, was able to detect VRE in animals and their products albeit at a lower rate (Boerlin *et al.*, 2001; Manero *et al.*, 2006). VRE has also been observed in farms where no antibiotics or growth promoters were used (Garcia-Migura *et al.*, 2005). This led these authors to suggest that vancomycin resistance is a spontaneous defensive response by enterococci, and thus the use of avoparcin might have not been the exclusive factor for the development of vancomycin resistance in animals. Manero *et al.* (2006) proposed that the extensive use of feed additives such as tylosin could create a

TABLE 4
Resistance phenotypes and the minimum inhibitory concentrations (MIC) of
vancomycin-resistant *Enterococcus* species isolated from pigs

van genes	Minimum inhibition concentration ($\mu\text{g/ml}$)			
	MIC = 8	MIC 9-31	MIC 32-128	MIC >256
vanA (n=1)	0 %	0 %	0 %	100 %
vanC1/A (n=1)	0 %	0 %	0 %	100 %
vanC2/C3 (n=17)	12.5 %	62.5 %	25 %	0 %
ND (n=30)	19.4 %	71 %	9.7 %	0 %

ND: No van gene detected

co-linkage between the vancomycin resistance genes and resistance determinants to other antibiotics used as growth promoters, and thus farms using tylosin could be positive for VRE in the absence of avoparcin. In the present study, the researchers found that 41.6% of the sampled farms were using tylosin as a feed additive and/or for therapy. Nevertheless, no significant difference was observed in the VRE detection rate between the farms which were using tylosin and those which were not. However, due to the small number of farms sampled, this observation might not be conclusive. Other authors have also suggested the existence of selective pressures other than glycopeptide antibiotic usage (Bahirathan *et al.*, 1998; Klein *et al.*, 1998) which resulted in the development of resistance to vancomycin.

Enterococci are intrinsically resistant to a broad range of antimicrobial agents and the use of antibiotics, to which enterococci are naturally resistant, may contribute to the emergence of resistant strains (Kak and Chow, 2002). In Malaysia, farmers can purchase antibiotics without prescription from any pharmaceutical representatives. The widely accessible antibiotics for use on farms may have led to an indiscriminate usage of antibiotics which may have contributed to the observed level of resistant enterococci. However, since there is no absolute ban on avoparcin, the negative response from the farmers about the use of avoparcin may be unreliable. In many instances, the farmers may not know the exact ingredients added into the commercially-prepared or premix

feed. In addition, the time of study coincided with the beta-agonist screening program by the Malaysian authorities. Therefore, this might have hampered farm owners from providing genuine information.

A total of 117 VRE were isolated from 41.8 % of the sampled pigs. There are no published data on the prevalence of VRE in pigs in Malaysia, to which a comparison of the study findings can be made. In addition, comparison with the findings of other studies from elsewhere is difficult as no standardized methods were used for isolation and identification of VRE. Among other, Butaye *et al.* (1999) used enrichment methods with $6\mu\text{g ml}^{-1}$ vancomycin and plating on Slanetz and Bartley media isolated VRE from 53% of sows, but they could not isolate VRE through direct streaking on selective agar. Meanwhile, Garcia-Migura *et al.* (2005) detected a low prevalence (2 to 5%) of *E. faecium* in pig samples using Slanetz and Bartley agar supplied with $6\mu\text{g ml}^{-1}$ vancomycin. Nevertheless, a high prevalence of VRE was found in urban sewage water (100%), pig slurry (34%) and pig faeces (16%) after enrichment with vancomycin at $8\mu\text{g ml}^{-1}$ and plating on m-*Enterococcus* agar with $20\mu\text{g ml}^{-1}$ vancomycin (Manero *et al.*, 2006).

The species most broadly distributed in nature are *E. faecalis* and *E. faecium*. Manero *et al.* (2006) indicated the dominance of *E. hirae* in addition to these two species in pig and its environment. In the present study, vancomycin-resistant *E. faecium*, *E. casseliflavus*, *E. gallinarum* were isolated but *E. faecalis* was not. This finding is consistent with that of Seo

et al. (2005) in Korea who isolated neither *E. faecalis* nor *E. faecium* from 70 pigs, but instead found that *E. gallinarum* and *E. casseliflavus* were prevalent. Furthermore, the findings gathered in the present study are in agreement with those of Manero *et al.* (2006) who found vancomycin-resistant *E. casseliflavus* and *E. gallinarum* from pig slurry. The significance of the isolates other than *E. faecium* and *E. faecalis* was emphasised by Willey *et al.* (1999, cited in Kirschner *et al.*, 2001), who noted that the incidence of less commonly found species such as *E. casseliflavus*, *E. gallinarum*, *E. durans*, and *E. hirae* had increased significantly in humans admitted to the hospital. In view of this finding, the role of these isolates in clinical cases due to *Enterococcus* must be investigated.

The multiplex PCR identified *E. casseliflavus* as the predominant species among those tested. However, a high percentage (63%) of VRE isolates, tested by PCR, were not identified to the species level since the multiplex PCR used was limited to the common and clinically important *Enterococcus* species. The results of this study indicate that there are other species to consider. These include *E. hirae* and/or *E. durans* as previously reported by Willey *et al.* (1999), Kirschner *et al.* (2001), and Manero *et al.* (2006) who indicated that these two species were isolated from pigs.

Six different gene clusters, mediating glycopeptide resistance, have been described in enterococci, including *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG* (Gilmore *et al.*, 2002). The gene *vanA* has been the subject of much work because it encodes resistance to all glycopeptides and is associated with the plasmid mediated transposable element Tn1546. More importantly, *vanA* signifies that the gene is not inherent but it is acquired, and is associated with high levels of vancomycin resistance. The Tn1546 element plays a major role in transferring resistance to *Staphylococcus aureus* (Dowling, 2006), which is another important hospital acquired nosocomial agent (Lowy, 1998). In addition, vancomycin-resistant *S. aureus* has also been reported (Tiwari and Sen, 2006; Smith *et al.*, 1999). *Enterococcus faecium* with *vanA* isolated

in this study is consistent with the findings of Centinkaya and colleagues (2000) who stated that *vanA* is primarily found in *E. faecium*. Furthermore, they also described *vanA* in *E. gallinarum* which was also observed in the present study. Patel *et al.* (1997) and Devriese *et al.* (1996) described similar findings in *E. gallinarum*. In addition, *E. gallinarum* with an acquired *vanA* gene has been reported to cause clinical infection in humans (Camargo *et al.*, 2004). In the present study, *vanA* was not detected in other isolates even though the bacteria were initially isolated on 32 µg ml⁻¹ vancomycin supplemented media. After isolation, the isolates were transferred into BHI vancomycin-free broth and stored at -20 °C for more than four months pending DNA extraction. The lack of stimulation due to the absence of vancomycin and long storage at low temperature could possibly explain the absence of *vanA*. Manson *et al.* (2003) also reported a rapid loss of *vanA* gene from *E. faecalis* when the isolates were transferred into vancomycin-free media. Khan *et al.* (2005) reported *E. gallinarum* isolates which did not possess any of the *van* genes, but they found resistant to high level of vancomycin instead.

Vancomycin resistance test using the E-test indicated different levels of susceptibility in the isolates. According to the National Committee for Clinical and Laboratory Standards (NCCLS, 2004) (presently known as Clinical and Laboratory Standards Institute-CLSI), enterococci with MIC₅₀ ≥ 32 are resistant and those showing MIC₅₀ ≤ 4 are susceptible. Using this categorisation, none of the *Enterococcus* isolates were determined as susceptible to vancomycin. Table 3 shows that the majority of isolates had MIC₅₀ > 8 (84%). Moreover, 40% of *E. faecium* and 23.5% of *E. casseliflavus* were completely resistant to vancomycin. In addition, a single isolate of *E. gallinarum* and *E. faecium* had a high level of vancomycin resistance.

Resistance gene *vanB*, which is also acquired and encodes for intermediate level of resistance to vancomycin, was not identified from any of the VRE. A previous study conducted in Malaysia (Radu *et al.*, 2001) could not detect *vanB* from

70 VRE isolated from poultry. Meanwhile, Ooi (2003), who studied 81 isolates from poultry, raw vegetables and clinical sources, did not isolate any *vanB* VRE. This suggests that *vanB* is possibly not present in Malaysia VRE isolates.

CONCLUSIONS

This study found VRE were present in 83% of the farms and 42% of the pigs sampled. *Enterococcus casseliflavus* (21.7%), *E. faecium* (14%), *E. gallinarum* (1.1%) and other *Enterococcus* species make up 63% of the total 92 VRE isolates examined by M-PCR. Meanwhile, Vancomycin resistance gene *vanA* was only detected in two of the VRE isolates (2.2%). The *vanA* was observed in *E. faecium* and *E. gallinarum*. Detection of *vanA*, with high level of vancomycin resistance (MIC>256), highlights potential public health threat associated with the pig industry since the resistance gene is capable of transfer within the genus *Enterococcus* and other pathogenic bacteria such as *Staphylococcus aureus*. More importantly, the multiplex PCR used in this study proved to be suitable for VRE screening. However, the incorporation of new primer sets may be required due to the high numbers of other *Enterococcus* spp. detected. In this study biochemical tests were used to identify the genus *Enterococcus*. However, using this method other bacteria with similar features (e.g. vancomycin - resistant *Pediococcus* species) may have been identified as *Enterococcus* spp. Therefore, the results should be interpreted with caution.

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