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Characterization of vancomycin-resistant *Enterococcus* isolates from broilers in Selangor, Malaysia

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Abstract. Vancomycin-resistant *Enterococcus* (VRE) is an emerging nosocomial pathogen in humans. The use of antibiotics in human therapy and in the production of food animals has been incriminated in the emergence of this organism. The present study describes the distribution of VRE species, the vancomycin-resistant genes detected, the vancomycin resistance pattern observed, and the genetic diversity of the isolates found in live broiler chickens in Malaysia. Overall 140 VRE were isolated with species comprising *Enterococcus faecalis* (48%), *Enterococcus faecium* (25.7%), *Enterococcus gallinarum* (12.1%), *Enterococcus casseliflavus* (1.4%) and other *Enterococcus* species (12.8%). Vancomycin resistance gene *van*A and intrinsic genes *van*C1 and *van*C2/3 were detected in the study population. *VanA* was detected in 15 (63.9%) of *E. faecium*, 23 (22.4%) of *E. faecalis* and in 3 (17.6%) *E. gallinarum* isolates. E-test was conducted on randomly selected 41 of the isolates and the minimum inhibition concentration (MIC) of vancomycin for five (11.9%) of tested isolates is more than 256µg/ml. Genotypic analysis using random amplified polymorphic DNA (RAPD) showed genetic diversity within the *Enterococcus* species.

INTRODUCTION

Vancomycin-resistant *Enterococcus* (VRE) is an emerging major nosocomial pathogen (Cetinkaya *et al.*, 2000). The emergence of VRE is associated with extensive vancomycin use in hospitals and use of avoparcin in farm animals as growth promoter (Aarestrup, 1995; Bager *et al.*, 1997; Dowling *et al.*, 2006). VRE infection in humans has been reported worldwide (Chiew, 1997; Bell *et al.*, 1998; Eom *et al.*, 2004; Jones, 2006). Although uncommon, it has been reported in Malaysia (Raja *et al.*, 2005). VRE is widespread and can be readily found in domestic animal faeces (Aarestrup, 2006) and carcasses (Pedonese *et al.*, 2005). However, since the link between VRE in animals and humans was suggested (Bates, 1997), chicken and duck farmers in Malaysia have incurred additional costs from VRE screening procedures and disposal of tested positive products. This is due to the demand for VRE-free products from Singapore, one of the main importers of local poultry (Dr. Che Zalina MZ, Department of Veterinary Service, Putrajaya). According to Zaini *et al.* (2000; 2000a), in 1999 the fear of VRE had resulted in the ban of 90% of Malaysia chickens and poultry products exported to Singapore.

Given the significant impact of the organism to public health and animal industry, additional information of VRE in animals, especially poultry is essential. This paper describes the distribution of VRE species, the vancomycin-resistant genes detected, the vancomycin resistance patterns observed, and the genetic diversity of the isolates found in live broiler chickens in Selangor, Malaysia.

MATERIALS AND METHODS

VRE Isolates

Isolates were obtained from a previous study (Hassan *et al.*, 2006). One hundred and forty isolates recovered from 540 live chickens (from six farms) that were resistant to 8µg/ml vancomycin (Sigma, USA) were included in this study.

Multiplex PCR analysis

Identification of VRE species and vancomycin-resistance genes was carried out using multiplex polymerase chain reaction (Kariyama *et al.*, 2000; Elsayed *et al.*, 2001) where seven primer sets targeting the genes *vanA*, *vanB*, *vanC1*, *vanC2/C3*, *Enterococcus faecalis*-specific, *Enterococcus faecium*-specific and *rrs* (16S rRNA) were used in one reaction tube. The *rrs* gene is used as an internal PCR control to improve reliability. The amplification of the gene indicates that an optimal condition for detection of *van* genes and the *Enterococcus* species-specific genes (Kariyama *et al.*, 2000). DNease® Blood and Tissue DNA extraction kit (Qiagen®, Germany) was used to extract the genomic DNA according to the protocol for Gram-positive bacteria as described by the manufacturer. For each analysis, negative and positive American Type Culture Collection (ATCC) control strains of *Enterococci*: *van*A strain *E. faecium* ATCC 51559 (MIC>256), vanB strain *E. faecalis* ATCC 51299 (MIC=24), *van*C1 strain *Enterococcus gallinarum* ATCC49573 (MIC=12), *van*C2 strain *Enterococcus casseliflavus* ATCC 25788 (MIC=4), and *E. faecalis* ATCC 19433 (MIC=4) were included.

E-test

Vancomycin susceptibility test was carried out on 41 randomly selected isolates using E-test kit (AB Biodisk, Sweden). The protocol was performed according to the manufacturer's guidelines.

RAPD-PCR Analysis

Random Amplified Polymorphic DNA analysis method (Martin *et al.*, 2005) with arbitrary nucleotide sequences of 5'-CTT GAG TGG A-3' and 5'-TCC TCA AGA C-3' was used to produce a distinguishable RAPD profile in *E. faecalis* and *E. faecium* isolates. Reproducibility was confirmed by using a control isolate in all of the reactions. FPQuest DNA fingerprinting software (BioRad Laboratory Inc) was used to analyse the agarose gel electrophoresis image Pearson correlation coefficient and cluster analysis by the unweighted pair group method with arithmetic average (UPGMA) were used to compare the banding patterns and strain grouping coefficients of similarity of 60% for RAPD typing was applied.

RESULTS AND DISCUSSION

Of the 140 VRE from cloacal swabs of broilers chickens, the VRE species isolated were *E. faecalis* (48%), *E. faecium* (25.7%), *E. gallinarum* (12.1%) and *E. casseliflavus* (1.4%) and the remaining isolates comprising other enterococcal species (Table 1). *Enterococcus faecalis* and *E. faecium* are two of the most often encountered *Enterococcus* species in chickens. Other species occasionally isolated from chickens are *E. casseliflavus*, *E. gallinarum* and *E. mundtii* (Simjee *et al.*, 2006). A previous study in Malaysia by Radu *et al.* (2001) showed that *E. faecalis* and *E. faecium* were present in 58.6% and 2.8%, respectively, of 70 VRE isolates from poultry meat samples. In other parts of the world, *E. faecium* is also noted to be the most common species in poultry (Butaye *et al.*, 1999).

Vancomycin resistance genes *van*A, *van*C_1 and *van*$C_{2/3}$s were detected but none of the isolate carried the *van*B gene (Table 1). Overall, *van*A gene was present in 36.4% of the poultry isolates. This is higher than those found by Poeta *et al.* (2005) in Portugal (9.2%) but much lower than those found in Costa Rica (100%) (Bustamante *et al.*, 2003). *Van*A was observed in 63.9% of *E. faecium*, 22.4% of *E. faecalis* and in 17.6% of *E. gallinarum* isolates.

*Van*A and *van*B types of resistance have been associated with outbreaks of VRE and these types of resistance are acquired and may potentially be transferred to other organisms, including *Staphylococcus aureus*

Table 1. Distribution of *van* genes based on vancomycin-resistant *Enterococcus* species isolated from broiler chickens

VRE species	No. isolates (%)	Vancomycin-resistance genes				
		*van*A	*van*B	*van*C_1	*van*$C_{2/3}$	ND
E. faecalis	67 (48%)	15	–	–	–	53
E. faecium	36 (25.7%)	23	–	–	–	13
E. gallinarum	17 (12.1%)	3	–	17	–	–
E. casseliflavus	2 (1.4%)	–	–	–	2	–
Enterococcus spp.	19 (13%)	10	–	–	–	9
Total	140 (100%)	51	0	17	2	73

ND; not detected

(Ruef, 2004). Many studies have suggested that the presence of *van*A VRE in faeces or intestines of farm animals may put humans at risk of contracting the organism or its resistant genes either by direct contact or through the ingestion of contaminated products (Bates, 1997; Donabedian *et al.*, 2003; Devriese *et al.*, 2006). However, findings from several recent studies that compared the molecular characteristics' of chickens and human VRE isolates did not support this hypothesis (van den Bogaard & Stobberingh, 2000; Willems *et al.*, 2000; Borgen *et al.*, 2002; Kuhn *et al.*, 2005; Jung *et al.*, 2006). Some authors argue that there is no sufficient evidence on exchange of enterococci or resistance genes between humans and food animals (Aarestrup *et al.*, 2002). To date, the established risk factors for VRE infection in humans are hospitalisation and treatment with antibiotics (Askarian *et al.*, 2008; Song *et al.*, 2009). Nevertheless, the possible transmissibility of VRE via non-nosocomial routes (Simjee *et al.*, 2002) indicates that great care should be taken to avoid introducing these organisms. *Enterococcus gallinarum* which carry both *van*A and intrinsic resistance genes *van*C1 as seen in the present study, were also reported by other authors (Radu *et al.*, 2001; Camargo *et al.*, 2004; Neves *et al.*, 2009). Intrinsically resistant to vancomycin enterococci such as *E. gallinarum* and *E. casseliflavus / flavescens* rarely causes human clinical infection (Schouten *et al.*, 2000).

Minimum inhibition concentration (MIC) of vancomycin for five (11.9%) of tested isolates is greater than 256ìg/ml (Table 2). This is higher than the finding of Chan *et al.* (2008) who observed only three isolates (1.3%) of MIC>256µg/ml from poultry in Pulau Pinang, Malaysia. The MIC value for eight of *E. faecalis* and three of *E. faecium* was between 32 and 128µg/ml. Thirteen of 26 isolates (50%) in the present study that possessed *van*A and *van*C were resistant to high levels of vancomycin (MIC >32µg/ml). More than 52% (73 of 140) of the VRE isolates did not posses any of the *van* genes tested (Table 1). Consequently, when 13 of the 73 isolates with non-detected *van* genes were randomly tested using E-test, 4 (30%) had MIC>32µg/ml. Resistance to glycopeptides is a complex system involving several genes (Périchon & Courvalin, 2009). Seven types of glycopeptide resistance have been described to date in enterococci (VanA, B, C, D, E, G and L) (Werner *et al.*, 2008). However, *van*A and *van*B are the most commonly reported with clinical relevance, due to conjugative transfer, which may occur via plasmids or transposons and be passed on to other pathogens (Ruoff *et al.*, 1988; Cetinkaya *et al.*, 2000; Périchon & Courvalin, 2009). In this study we report *E. gallinarum* that had acquired *van*A, but surprisingly with lower resistance level (MIC<32µg/ml)

Table 2. Distribution of *van* genes and *Enterococcus* species isolated from broiler chickens based on MIC to vancomycin

		No of isolates (%)				
		MIC ranges (µg/ml)				Total
		<8	8-24	32-128	≥256	
*van*A	*E. faecalis*	0	2 (20.0)	8 (80.0)	0	10 (100.0)
	E. faecium	0	3 (37.5)	3 (37.5)	2 (25.0)	8 (100.0)
	E. gallinarum	1 (33.3)	2 (66.7)	0	0	3 (100.0)
*van*C1	*E. gallinarum*	1 (20.0)	2 (40.0)	1 (20.0)	1 (20.0)	5 (100.0)
*van*C2/3	*E. casseliflavus*	0	2 (100.0)	0	0	2 (100.0)
Van genes not detected	*E. faecalis*	3 (60.0)	0	0	2 (40.0)	5 (100.0)
	E. faecium	2 (28.6)	3 (42.9)	2 (28.6)	0	7 (100.0)
	Enterococcus spp.	1 (100.0)	0	0	0	1 (100.0)

towards vancomycin. The absence of resistant behaviour even when *van*A and *van*B genes are present was also observed by Ribeiro *et al.* (2007). According to Ribeiro *et al.* such observation could be due to incomplete and/or unfunctional Tn1546 mobile genetic element that encodes high-level vancomycin resistance.

The RAPD-PCR analysis classified the *E. faecalis* isolates unique banding pattern into fourteen RAPD types (Fig. 1). Five profiles were discerned for *E. faecium* (Fig. 2). These findings imply that the poultry VRE are genetically and phenotypically diverse which are consistent with findings of other authors who reported considerable genetic variability in enterococci species (Son *et al.*, 1999; Braak *et al.*, 2000). However, the result from this analysis should be interpreted with caution because of the RAPD inherent limitations resulting from the lack of standard analysis methods and relatively low reproducibility as compared to, for example, PFGE (Arber, 2000).

Genetically diverse VRE isolates with *van*A gene were detected in broiler chickens in Selangor while VRE with *van*B gene were absent. Further research comparing the isolates from poultry to those of humans is required in order to validate the inference that chickens are indeed the source of VRE in humans in Malaysia.

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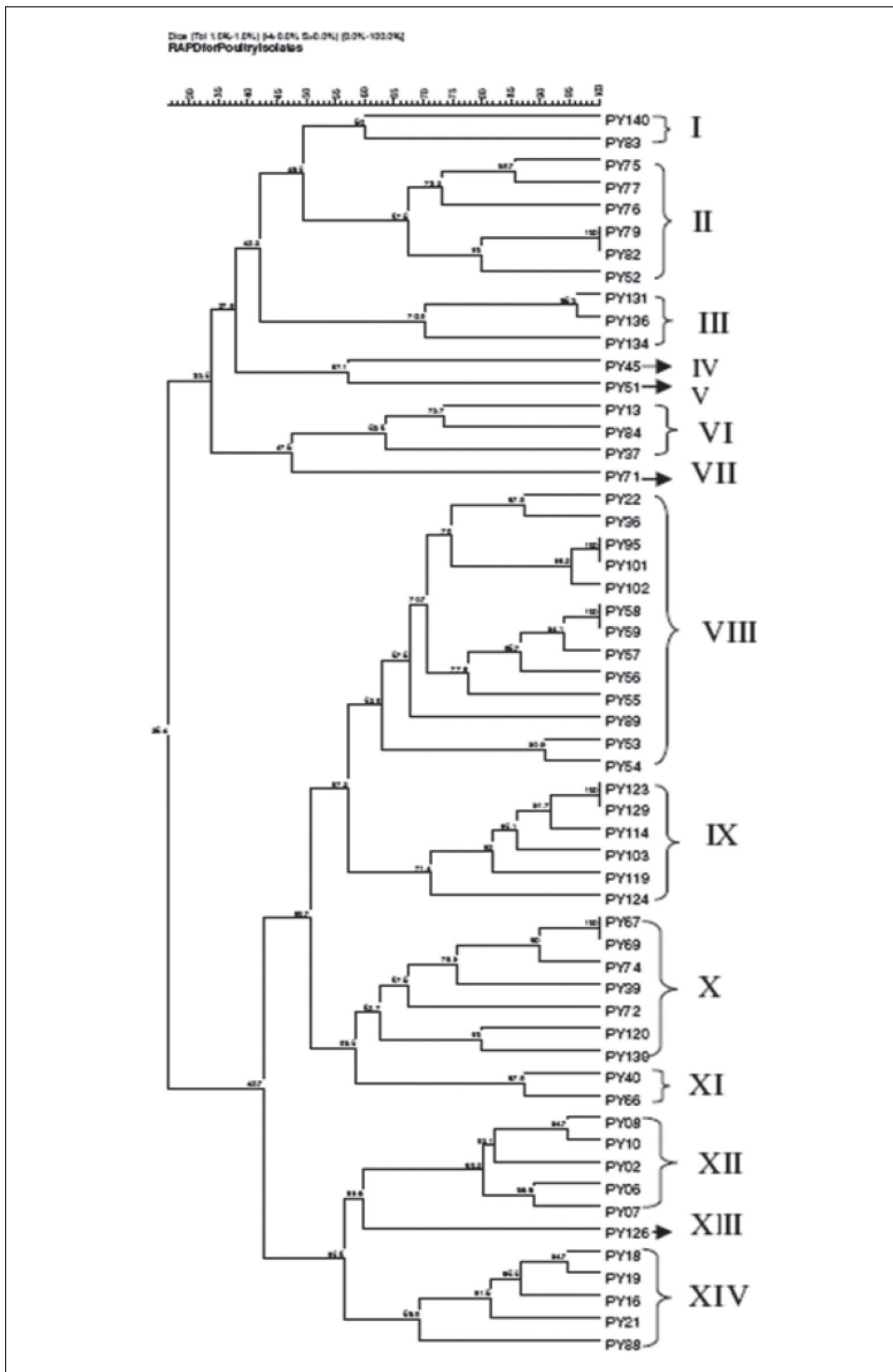

Figure 1. Dendogram from UPGMA cluster analysis of RAPD profiles of 56 *E. faecalis* isolates.

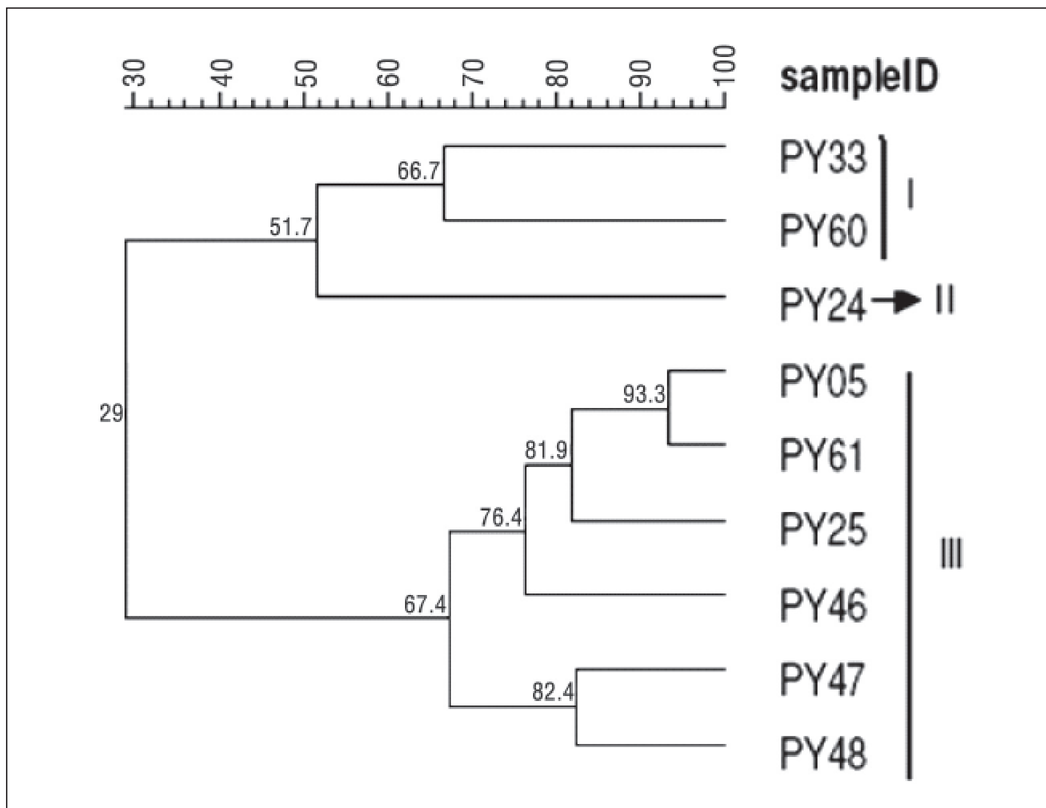

Figure 2. Dendogram from UPGMA cluster analysis of RAPD profiles of 9 *E. faecium* isolates.

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