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

Getachew, Y.M.1, Hassan, L.1*, Zakaria, Z.1, Saleha, A.A.1, Kamaruddin, M.I.2 and Che Zalina, M.Z.2
1 Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, 43400 Selangor, Malaysia
2 Department of Veterinary Services, Wisma Tani, Podium Block, Lot 4G1, Precinct 462630 Putrajaya, Malaysia
Corresponding email: latiffah@vet.upm.edu.my
Received 21 August 2009, received in form 24 September 2009; accepted 2 October 2009

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 vanA and intrinsic genes vanC1 and vanC2/3 were detected in the study population. VanA was detected in 15 (63.9%) of E. faecium, 23 (22.4%) of E. faecalis and in 5 (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: vanA strain E. faecium ATCC 51559 (MIC>256), vanB strain E. faecalis ATCC 51299 (MIC=24), vanC1 strain Enterococcus gallinarum ATCC49573 (MIC=12), vanC2 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 vanA, vanC1 and vanC23S were detected but none of the isolate carried the vanB gene (Table 1). Overall, vanA 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). VanA was observed in 63.9% of E. faecium, 22.4% of E. faecalis and in 17.6% of E. gallinarum isolates.

VanA and vanB 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

<table>
<thead>
<tr>
<th>VRE species</th>
<th>No. isolates (%)</th>
<th>Vancomycin-resistance genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>vanA</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>67 (48%)</td>
<td>15</td>
</tr>
<tr>
<td>E. faecium</td>
<td>36 (25.7%)</td>
<td>23</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>17 (12.1%)</td>
<td>3</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td>2 (1.4%)</td>
<td>–</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>19 (13%)</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>140 (100%)</td>
<td>51</td>
</tr>
</tbody>
</table>

ND; not detected

(Ruef, 2004). Many studies have suggested that the presence of vanA 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; Donabeedian 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 (Sinjee et al., 2002) indicates that great care should be taken to avoid introducing these organisms. Enterococcus gallinarum which carry both vanA and intrinsic resistance genes vanC1 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 256µg/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 vanA and vanC 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, vanA and vanB 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 vanA, 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

<table>
<thead>
<tr>
<th>Van genes</th>
<th>No of isolates (%)</th>
<th>MIC ranges (µg/ml)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;8</td>
<td>8-24</td>
</tr>
<tr>
<td>vanA</td>
<td>E. faecalis</td>
<td>0</td>
<td>2 (20.0)</td>
</tr>
<tr>
<td></td>
<td>E. faecium</td>
<td>0</td>
<td>3 (37.5)</td>
</tr>
<tr>
<td></td>
<td>E. gallinarum</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>vanC1</td>
<td>E. gallinarum</td>
<td>1 (20.0)</td>
<td>2 (40.0)</td>
</tr>
<tr>
<td>vanC2/3</td>
<td>E. casseliflavus</td>
<td>0</td>
<td>2 (100.0)</td>
</tr>
<tr>
<td>Van genes not detected</td>
<td>E. faecalis</td>
<td>3 (60.0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E. faecium</td>
<td>2 (28.6)</td>
<td>3 (42.9)</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>1 (100.0)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

towards vancomycin. The absence of resistant behaviour even when vanA and vanB 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 vanA gene were detected in broiler chickens in Selangor while VRE with vanB 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.

Acknowledgements. We thank the Department of Veterinary Services (DVS) Malaysia, farm veterinarians and farmers for their cooperation. This project was a jointly funded by grants number 04/01/07/0078RU of UPM and DVS Malaysia internal fund.
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.

REFERENCES


