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Original article

Detection and phenotypic characterization of vancomycin-resistant enterococci in pigs in Thailand

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ABSTRACT

The presence and characteristics were investigated of vancomycin-resistant enterococci (VRE) in pigs in Thailand. A total of 179 rectal swabs were collected aseptically from suckling pigs, fattening pigs and breeding sows on four commercial farms located in Central Thailand. VRE with minimum inhibitory concentrations ranging from 8 μg/mL to 16 μg/mL were detected in 43 of 179 pigs (an overall prevalence rate of 24%). VRE carriers were identified in 12 of 61 (19.7%) suckling pigs, 15 of 60 (25%) fattening pigs and 16 of 58 (27.6%) breeding sows, respectively. *Enterococcus gallinarum* was the most prevalent species for VRE in all age groups, followed by the detection of *Enterococcus casseliflavus*. All of the isolates were susceptible to teicoplanin. A large proportion of VRE isolates showed resistance to tetracycline (86.5%), erythromycin (61.5%), ampicillin (53.8%), chloramphenicol (34.6%) and ciprofloxacin (32.7%). Resistance to ampicillin was more prevalent in *E. gallinarum* isolates than in *E. casseliflavus* isolates. The results of this study indicate that VRE isolates of pigs are of the VanC phenotype and commonly exhibit multiple drug resistance. Different antimicrobial susceptibility is present between VanC species, while *E. gallinarum* is less susceptible than *E. casseliflavus*.

Introduction

Enterococci are ubiquitous, Gram-positive, catalase-negative, facultative-anaerobic bacteria and while they inhabit humans and animals and present as a part of the normal intestinal flora (Noble, 1978), they are now recognized as one of the leading causes of hospital-associated infections (Hidron et al., 2008). Many infections with enterococci are life-threatening and can be difficult to treat, due to their resistance to several antimicrobials (Huycke et al., 1998). Of particular concern has been the emergence of strains with resistance to glycopeptides. Vancomycin and teicoplanin are the glycopeptide antibiotics currently in use for the treatment of Gram-positive bacterial infections (Murray, 2000). Vancomycin-resistant enterococci (VRE) are typically multidrug-resistant and treatment options are significantly limited. Moreover, certain VRE genotypes have the potential to transfer the resistant genes to the more virulent Gram-positive pathogen, *Staphylococcus aureus* (Noble et al., 1992).

VRE were first reported in the late 1980s (Leclercq et al., 1988). Since then, VRE have been detected worldwide. In the United States, there was a 20-fold increase in VRE infection rates within a 5-year period and the percentage of VRE identified from patients in the intensive care unit with nosocomial infections was 28.5% in 2003 (National Nosocomial Infections Surveillance, 2004). In Europe, a high frequency of VRE has been reported among food animals, retail meats and non hospitalized people (Bager et al., 1997; Bonten et al., 2001). It was found that the use of avoparcin (a glycopeptide analogue) during livestock production was an important factor for the emergence of VRE (Bager et al., 1997). Due to the possible transmission of VRE and their resistant genes from farm animals to humans, a ban was enforced throughout the European Union on the use of avoparcin (Casewell et al., 2003). A study that followed the change resulting from the ban found a decrease in the prevalence of VRE in farm animals (Bager et al.,

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1999). However, this trend has not been observed in some European countries (Aarestrup, 2000). It has been hypothesized that continued use of other antibiotics in animal husbandry is one important factor for the persistence of VRE, since co-selection for resistant genes located on the same genetic element can occur (Aarestrup, 2000). In the United States, avoparcin has never been permitted for use with food animals, but VRE have been recently isolated from pigs (Donabedian et al., 2010). Therefore, avoparcin may not be an absolute factor for the development of vancomycin resistance in animals.

In Thailand, VRE have been isolated from patients, companion animals, frozen foods and environmental water (Chalermchaikit et al., 2005; Tansuphasiri et al., 2006; Thongkoom et al., 2012). In addition, in the 1990s, contamination of VRE in the poultry products exported to Japan was first detected (Ike et al., 1999) and resulted in severe economic losses. In order to implement an effective prevention and control program for VRE, all potential sources should be investigated. Although antimicrobials are commonly used in swine production, there has been no report of VRE detection from pig farms in Thailand. The goals of this study were: 1) to isolate VRE from pigs in Central Thailand; 2) to identify the species; and 3) to characterize antimicrobial susceptibility profiles.

Materials and methods

Pig farms, antimicrobial use and collection of samples

Sample collection was performed during 2011 in four commercial pig farms, located in Lopburi, Saraburi, Suphanburi and Ratchaburi provinces, Central Thailand. The farm in each province had a sow herd size of about 500, 400, 1200 and 4000 breeding sows, respectively. Data on antimicrobial use in the pig farms were also collected. In all farms, antimicrobial agents were used as feed supplements for swine disease control. The use of antimicrobials was under strict veterinary supervision and the specific types of antimicrobials used were penicillin (amoxicillin), tetracycline (chlortetracycline), macrolide (tilmicosin and valosin), polypeptides (colistin), pleuromutilin (tiamulin) and phosphonic acid (fosfomycin).

Rectal swabs were collected aseptically from randomly sampled pigs of different age groups: suckling pigs, fattening pigs and breeding sows. Fecal samples were preserved in Cary-Blair medium and transported to the laboratory on ice and processed within 18 h.

Isolation of vancomycin-resistant enterococci from fecal samples

The protocol for screening VRE in pigs has been described elsewhere (Pimarn et al., 2011). Fecal samples (approximately 0.5 g) were subjected to a 10-fold serial dilution with buffered peptone water. Diluted fecal slurries were then inoculated onto bile-esculin azide (BEA) agars (Becton Dickinson and Company; Franklin Lakes, NJ, USA) supplemented with 6 μg/mL vancomycin (Sigma–Aldrich; St. Louis, MO, USA). BEA with vancomycin (BEAV) was used as the VRE selective agar, as recommended by Clinical and Laboratory Standards Institute (2011). After incubation at 37 °C for 48 h, culture plates were examined for typical enterococcal colonies. From each sample, colonies with a brown to black halo from the BEAV plates were isolated and then tested for catalase and Gram stain. An isolate demonstrating Gram-positive cocci and negative for catalase was prepared as stock culture in Luria-Bertani broth with 20% glycerol and stored at −80 °C until subsequent identification and characterization of VRE.

Identification of vancomycin-resistant enterococci isolates

The genus identity of presumptive VRE isolates was confirmed by testing on a bile-esculin reaction, 6.5% salt-tolerance test, medium for growth at 45 °C, L-pyrrolidonyl-β-naphthylamide (PYR) and leucine-β-naphthylamide (LAP) tests (Facklam and Elliott, 1995). The PYR and LAP tests were performed, according to the manufacturer's instructions (Oxoid Ltd.; Hampshire, UK). Identification to the species level followed the procedures and biochemical key for *Enterococcus* spp., as previously described (Facklam and Elliott, 1995; Teixeira et al., 2007). Any isolates with ambiguous identification were confirmed by species-specific polymerase chain reaction (PCR) analysis (Jackson et al., 2004). *Enterococcus faecalis* ATCC 14506, *Enterococcus gallinarum* ATCC 49573 and *Enterococcus casseliflavus* ATCC 25788 were included as reference strains.

Antimicrobial susceptibility testing

Levels of vancomycin resistance were determined using minimal inhibitory concentration (MIC) testing with the agar dilution technique (Clinical and Laboratory Standards Institute, 2011). Briefly, the MIC testing was carried out on Mueller-Hinton agars (Neogen Corporation; Lansing, MI, USA) containing serial two-fold dilutions (1–128 μg/mL) of vancomycin. The agar plates were then spot inoculated with 1×10^4 colony-forming units per spot using a microplanter and incubated at 37 °C for 24 h. *E. faecalis* ATCC 29212 and 51299 were used as sensitive and resistant controls, respectively. The Clinical and Laboratory Standards Institute (2011) breakpoints for vancomycin susceptibility are 4 μg/mL or less for susceptible, 8–16 μg/mL for intermediate resistance and 32 μg/mL and above for resistant. Strains with intermediate resistance are also clinically significant, so in this paper, isolates with vancomycin MICs of 8 μg/mL or more were regarded as VRE.

VRE isolates were tested for susceptibility to a selection of antimicrobials using the Kirby–Bauer disc diffusion method (Bauer et al., 1966). The antimicrobials consisted of 13 antimicrobial agent discs (Oxoid Ltd.; Hampshire, UK) of teicoplanin (30 μg), ampicillin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), nitrofurantoin (300 μg), rifampin (5 μg), chloramphenicol (30 μg), quinupristin–dalfopristin (15 μg), linezolid (30 μg), fosfomycin (200 μg), streptomycin (300 μg), gentamicin (120 μg) and erythromycin (15 μg). *Staphylococcus aureus* ATCC 25923 was used as a control strain and inhibition zones were evaluated following the guidelines of Clinical and Laboratory Standards Institute (2011). Isolates were classified as multidrug resistant based on the occurrence of resistance to more than two antibiotics.

Statistical analysis

Fisher's exact and Student's t tests were undertaken using the Quickcals Program (GraphPad Software Inc.; La Jolla, CA, USA). The results were deemed statistically significant if $p < 0.05$.

Results

Species distribution and prevalence rates of vancomycin-resistant enterococci in pigs of different age groups

In total, 179 fecal samples were collected from 61 suckling pigs, 60 fattening pigs and 58 breeding sows. Using isolation with the VRE screening agars and identification the genus level, 71 presumptive isolates were detected. These isolates were each identified to the species level and the levels of vancomycin resistance were determined by the MIC method. With biochemical phenotyping and PCR analysis, 44 isolates were identified as *E. gallinarum*,