

Infrequent occurrence of vancomycin-resistant enterococci in poultry from Malaysian wet markets

CHS ONG, M ASAAD, KC LIM BSc, and YF NGEOW FRCPATH, MD

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract

Fifty samples of chicken, duck and geese faeces were obtained from 13 wet markets in Kuala Lumpur to study the prevalence of vancomycin-resistant enterococci (VRE) among local market poultry. Biotyping of colonies grown on azide agar incubated at 45°C yielded *E. pseudoaerium*, *E. faecalis*, *E. faecium* and *E. gallinarum* from chicken faeces and *E. malodoratus*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae* and *E. durans* from goose and duck faeces. On agar containing 6 mg/l of vancomycin, one strain of *E. flavescens* was identified, giving a VRE detection rate of 2.0%. This isolate had a vancomycin M.I.C. of 8 mg/l as determined by the Etest, and the *van C-3* gene that was identified by PCR followed by sequence analysis. The prevalence of VRE among poultry sold in local markets appears to be low, and may reflect the infrequent use of antimicrobials in our poultry farms. Nevertheless, the possibility of human acquisition of microbes via the food chain cautions against the use of antimicrobials in animal husbandry that may encourage the emergence and spread of multi-drug resistant organisms like the VRE among animal microbial flora.

Key words: enterococci, vancomycin resistance

INTRODUCTION

Enterococci are part of the intestinal flora of humans and animals but have been associated with serious infections such as endocarditis and bacteraemia. These bacteria are inherently resistant to many commonly used antibiotics such as cotrimoxazole and cephalosporins. Infections by them are usually treated with ampicillin alone or, as in the case of endocarditis, with a combination of ampicillin and an aminoglycoside to obtain synergistic bactericidal activity. Vancomycin has been used as an alternative drug in patients who are allergic to penicillins or who are infected by strains showing high-level resistance to ampicillin and aminoglycosides. The emergence of vancomycin resistance among the enterococci, therefore, severely restricted therapeutic options for the effective treatment of multidrug-resistant enterococcal infections.

Since the first reports of vancomycin-resistant *E. faecalis* and *E. faecium* from Europe and vancomycin-resistant *E. gallinarum* from the USA,¹ vancomycin-resistant enterococci (VRE) became increasingly recognized as causes of sepsis among patients in major medical centers.^{2,3} Initially, these strains were thought to be selected

out by the extensive use of vancomycin and extended spectrum cephalosporins in the clinical setting.⁴ Recently, however, there is evidence that VRE may be brought into the hospital from the community. Asymptomatic faecal carriage of VRE in the community has been found to range from 0-28%.^{4,5} Since enterococci colonize farm animals and are found in meat and dairy products,⁶ and it is theoretically possible for animal strains to pass on plasmids carrying vancomycin-resistance genes to human strains, the food chain has been implicated as a likely source of VRE in humans. In Malaysia, there is little data on the occurrence of VRE in food animals. In this study, the prevalence of VRE and the distribution of various enterococcal biotypes are studied among poultry sold in local markets.

MATERIALS AND METHODS

Sample collection and processing

From March to April 2001, faecal samples from poultry were collected from 13 wet markets in the suburbs of Kuala Lumpur. All samples were processed on the day of collection or kept at 4°C overnight before being processed. Each sample was expressed from a piece of intestine about 2

inches long into a sterile glass bottle. To each sample, one ml of physiological saline was added, after which the sample was vortexed and allowed to stand at room temperature until large particles have settled. The supernatant consisting of a suspension of fine faecal particles was then separated into two aliquots, one for the isolation and biotyping of enterococci, and the other for VRE screening.

Isolation and biotyping of enterococci

0.1 ml of each sample was spread out on Azide agar (Oxoid, U.K.) and incubated at 45°C for 24-48 hours. From each culture plate, eight colonies were randomly selected and tested for biological and biochemical properties. Enterococci were identified as catalase-negative, Gram-positive, chain-forming cocci, growing on MacConkey agar at 45°C, bile tolerant, hydrolyzing aesculin and producing pyrrolidonyl arylamidase (PYR). For biotyping, carbohydrate fermentation tests were performed as described by Facklam & Casey⁷ and Facklam,⁸ with 1% mannitol, raffinose, sorbitol and arabinose solutions. In addition, enterococci were tested for their ability to grow on 0.04% tellurite blood agar and production of pigment on nutrient agar incubated at room temperature. The differentiation of *E. casseliflavus* from *E. flavescens* was based on hemolytic activity on sheep blood agar.⁹

VRE screening

0.1 ml of each sample was spread out on Azide agar containing 6 mg/l of vancomycin (Sigma, U.S.A.) and incubated overnight at 45°C. Colonies growing on the selective plates were Gram-stained. Gram-positive cocci in chain formation were tested for susceptibility to ampicillin and vancomycin, using ampicillin 25 mg and vancomycin 30 mg disks on Mueller-Hinton agar (Difco, U.S.A.), and inhibition zone diameters were interpreted according to N.C.C.L.S. (National Committee for Clinical Laboratory Standards, U.S.A.) guidelines. The vancomycin minimum inhibitory concentration (M.I.C.) was obtained for all these strains by the Etest (ABioDisk, Sweden). Vancomycin-resistant strains (M.I.C. \geq 4 mg/l) were biotyped as described above.

PCR

A multiplex PCR for *van A*, *van B*, *van C-1*, *van C-2*, *van C-3*, *ddl*_{*E. faecalis*} and *ddl*_{*E. faecium*} genes was carried out as described by Dutka-Malen *et al.*¹⁰ Primers were synthesized by BioSynTech,

Malaysia. Total DNA was prepared by cell lysis with proteinase-K. PCR was performed in a DNA thermal cycler (MJ PTC-200, U.S.A.) and using a final volume of 100 μ l containing 5 μ l of prepared template DNA, 1x PCR buffer, 50 pmole of each primer (Van A1, A2, B1, B2, C1, C2, D1, D2, E1, E2, F1 and F2),¹⁰ 2 units of Platinum Taq DNA polymerase (Gibco, U.S.A.), 7 mM MgCl₂, 0.5 mM (each) of the dNTPs and 10 mM β -mercaptoethanol. In the thermal cycling, an initial denaturation at 94°C for 2 minutes was followed by 30 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute, finishing with a final extension at 72°C for 10 minutes. PCR products were visualized on a 1.5% agarose gel electrophoresed at 90V for 1 hour and stained with 0.5 μ g/ml of ethidium bromide.

RESULTS

A total of 50 faecal samples were collected, of which 46 were from chicken, 3 from geese and only one from a duck. Of these 50 samples, 41 yielded Gram-positive cocci that were subsequently identified to be enterococci. Biotyping of randomly chosen enterococcal colonies identified *E. pseudoavium*, *E. faecalis*, *E. faecium* and *E. gallinarum* in chicken faeces, *E. malodoratus*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hiraeldispar*, and *E. durans* from goose faeces and *E. durans* from duck faeces.

When the 50 faecal samples were plated out on vancomycin azide agar, 12 samples (from 7 chicken and one goose) produced colonies that were found to be Gram-positive cocci. All 12 presumed VREs were sensitive to ampicillin by the disk diffusion test. Against vancomycin, 3 strains had inhibition zone diameters indicating resistance, 4 intermediate resistance and 5 susceptibility. The vancomycin MICs of the 3 strains showing resistant inhibition zone diameters were 2256 mg/l, 2256 mg/l and 8 mg/l while the other 9 isolates were all found to be susceptible to vancomycin with MICs of <4 mg/l. On biotyping, the 2 strains with vancomycin MICs of 2256 mg/l were identified to be *Leuconostoc* spp. while the strain with a vancomycin MIC of 8 mg/l was found to be *E. flavescens*. This strain was isolated from chicken faeces.

The *van* gene PCR assay was performed on the *E. flavescens* isolate. A PCR product of 439 bp (Fig. 1) was obtained indicating the presence of the *van C-2* and/or *van C-3* genes. This product was purified and sent to AMCAL DNA

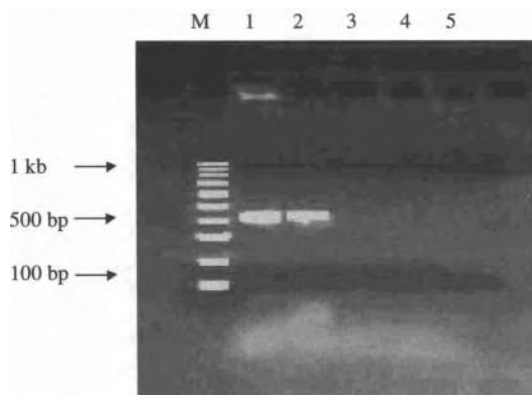


FIG. 1: Multiplex PCR using 6 sets of primers for detection of van genes in vancomycin resistant enterococci.

Lane M = 100 bp DNA ladder

Lane 1 & 2 = vancomycin resistant *E. flavescence* containing *van C-2* / *van C-3* gene

Lane 3 & 4 = vancomycin resistant *Leuconostoc species*

Lane 5 = reagent control

Laboratory, University of Malaya for DNA sequencing by the dideoxyribonucleotide chain terminating method of Sanger's et al.¹¹ Blast results showed that the PCR product was 98% identical to the *van C-2* gene of *E. casseliflavus* and *van C-3* gene of *E. flavescens*.

DISCUSSION

Glycopeptide resistance in the enterococci is expressed as different phenotypes. Currently 5 phenotypes are recognised: the *van A*, *B*, *C*, *D* and *E* phenotypes resulting from the replacement of D-Ala-D-Ala terminus on the peptidoglycan with either D-Ala-D-lactate (*van A*, *B*, *D*) or D-Ala-D-Ser (*van C* and *E*). The resistance can be constitutive or inducible and may be expressed at a high or low level." The *van A* type is characterized by inducible resistance to both vancomycin and teicoplanin while the *van B* type shows resistance to vancomycin but susceptibility to teicoplanin. The *van C* phenotype demonstrates low-level, constitutive resistance to vancomycin and is seen in *E. gallinarum*, *E. casseliflavus*, *E. flavescens* and the motile enterococci.^{13,14,15} The *van C-1* gene synthesizes the D-Ala-D-Ser in *E. gallinarum* while *van C-2* and *van C-3* genes are present in *E. casseliflavus*, and *E. flavescens* respectively. In this study, the only enterococcus isolated on vancomycin azide agar was a *E. flavescens*, and as expected, it showed low-level (8 mg/l) resistance and the presence of the *van C-2/C-3* gene in its genome.

The VRE isolation rate of 2.0% in this study gives the impression that VRE are not widespread among poultry sold in local markets. This low rate could be due to the small number of samples tested. Alternatively, it could reflect a low usage of antimicrobials in local poultry farms. It is not known what predisposes to VRE carriage in animals. As in the case of human patients treated with vancomycin for *Clostridium difficile* diarrhoea, intestinal colonization is likely to be the result of exposure to high levels of vancomycin in the gut after oral administration of the drug. In animal husbandry, antibiotics are often added to animal feeds as growth promoters. In many parts of Western Europe, the use of avoparcin, a glycopeptide antimicrobial, in animal feeds, has been considered a prime factor leading to the emergence of VRE in animals.¹⁶ In contrast, VRE has not been found in food animals in the U.S.A. where avoparcin has never been licensed as a drug for animals.¹⁷ Van den Bogaard *et al.*¹⁸ compared antimicrobial resistance rates among enterococci in swine faecal samples from abattoirs in Sweden where avoparcin has been banned since 1986, and in The Netherlands where avoparcin is extensively used until 1995, and found no VRE in Swedish pigs but an average isolation rate of 39% in Dutch samples. In addition to glycopeptides, the use of other antimicrobials can also predispose to the establishment of VRE colonization. In *E. faecium*, for instance, a transposon has been identified that carries the genes for both vancomycin and ampicillin resistance.¹⁹ This

implies the selection of vancomycin resistance by the use of ampicillin which is a commonly used antibiotic in veterinary medicine.

Antibiotics are more likely to be used in large intensive farming units both as growth promoters and as veterinary medicine. Under the selective antibiotic pressure, resistant organisms emerge and are easily transmitted among animals via faecal contact. During slaughtering, carcasses will be contaminated by faecal flora and become a vehicle for the transfer of drug-resistant organisms to humans. In the human gut, there are opportunities for animal strains to pass on transmissible drug resistance genes to human intestinal flora that are potential causes of endogenous infection. Hence, it is important to ensure that food animals do not carry drug-resistant organisms that can pose a health hazard to humans. Activities towards this end should include implementing a policy for rational use of antimicrobials in animal husbandry and regular surveillance for resistant organisms among farm animals. The prevalence of VRE in animal faeces can be used as an indication of the extent of antibiotic use and a basis for making recommendations on antimicrobial use in veterinary practice.

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