Presence of Vancomycin Resistance among Enterococcus Isolates from Stray Cats in Universiti Putra Malaysia and Selected Neighbourhood in Sri Serdang, Selangor, Malaysia

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Abstract

The present study was undertaken to determine the distribution of *Enterococcus* species from stray cats in Universiti Putra Malaysia and Seri Serdang and the presence of vancomycin resistance among the isolates. Fifty-five rectal swabs were collected from stray cats found in UPM and around the area of Sri Serdang. The Enterococcus species isolated were inoculated onto vancomycin resistant enterococci (VRE) agar supplemented with 8 µg/mL of vancomycin. Biochemical tests such as catalase, bile-aesculin and 6.5% NaCI were conducted to further confirm VRE isolates. Multiplex polymerase chain reaction assay (PCR) were performed for *Enterococcus* genus and species identification and vancomycin-resistant gene detection. Presence of *Enterococcus* spp. were demonstrated in every rectal sample tested. Two species were identified: *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*). Among 55 isolates of *Enterococcus* tested, none was resistant to vancomycin at 8 µg/mL.

Keywords: stray cats, vancomycin-resistant enterococci (VRE), *Enterococcus* spp., PCR

Introduction

Enterococci have been recovered from the intestinal tract of mammals, soil, water, plants, insects, and food items (Witte et al., 1999; Giraffa, 2002). They are a leading cause of nosocomial infections and are intrinsically more resistant to antimicrobial agents commonly used in hospitals than other bacteria (Martone, 1998; Cetinkaya et al., 2000).

Vancomycin-resistant enterococci (VRE) are a group of bacteria that have developed resistance to many antibiotics, especially vancomycin (Poore, 2007). VRE was discovered in 1985 (Dixson et al., 1985) and was first reported in France and England in 1986 and in the US in 1987 (Farley, 1998). It has spread rapidly and became a major problem in many institutions both in Europe and US.

Some epidemiological studies suggested that animals carrying VRE in their gastrointestinal tract could be the source of VRE infections of humans (Stobberingh et al., 2000). These VRE of animal origin can colonize humans, thus, may transfer their resistance genes to humans (Griffiths et al., 1994, Sundsfjord et al., 1998). However, there are only a limited number of studies dealing with the occurrence of VRE in

companion animals, even though direct contact with such animals is a recognized source of pathogenic bacteria for humans (Simoons-Smit et al., 2000, Wolfs et al., 2001).

The present study aimed at establishing the presence of VRE in population of stray cats in two areas in Malaysia. The specific objective of this study is to determine the distribution of *Enterococcus* species among stray cats in colleges in Universiti Putra Malaysia and selected neighbourhood in Sri Serdang, and detect the occurence of vancomycin resistance among those isolates.

Materials and Methods

Sample and data collection

Fifty five rectal swabs were collected with 26 swabs from stray cats from colleges in UPM and 29 swabs from stray cats from selected location in Sri Serdang. The pre-package swabs contained the transport Amies medium (MEUS, Piove di Sacco, Italy) and were kept in ice during sample collection. Information was collected based on the location where each cat was found, estimated age and sex (intact, spayed or neutered).

Bacteriological Analysis

After collection, samples were labeled and kept in the refrigerator at 4˚C for 24 hours before being inserted into universal bottles containing 3 mL Brain Heart Infusion (BHI; Pronadisa™ Laboratorios Conda, Spain) broth for enrichment. Each sample was then incubated for 18 hours at 37˚C aerobically. A loopful of broth culture was streaked onto membrane filter *Enterococcus* selective agar Slanetz and Bartley (SBA; Merck Inc, Germany) and incubated aerobically for 24-48 hours at 37˚C. Red/maroon/pink colonies are presumptive enterococci colonies and 3-5 colonies of different type based on shape, appearance or size were collected and put into 1.5 PCR tubes containing 1 mL of BHI broth and mixed well. A loopful of the suspension was streaked on BHI agar using the diminishing sweep technique and incubated for 18-24 hours at 37˚C aerobically for purification. From BHI agar, single colony of different types were picked and streaked on BHI agar for purification and each subculture was labeled. These plates were then incubated for 24 hours at 37˚C aerobically.

In order to confirm the genus *Enterococcus,* biochemical tests were done on all pure colonies by doing Catalase test, Gram staining, growth in bile-aesculin, growth in 6.5% NaCI broth and growth on VRE agar supplemented with 8 µg/mL of vancomycin. The species van genes determination of VRE was performed using multiplex PCR assay method described by Kariyama et al. (2000), Ke et al. (1999) and Elsayed et al. (2001).

A drop of 3% of hydrogen peroxide was dispensed on a clean glass slide. A single colony was picked from a pure culture plate using a sterile inoculating loop and placed in the drop of 3% hydrogen peroxide, mixed well and observed for formation of bubbles. A positive result was indicated by formation of bubbles.

A drop of normal saline was placed on the glass slide and mixed with a loopful of presumptive enterococci colonies on the same slide. The slide was heat-fixed and allowed to dry for a few seconds. It was then drained with crystal violet for one minute. Then it was washed and drained with iodine for one minute. Next, the slide was washed again and drained with acetone for a few seconds. Finally, it was washed and drained with diluted carbol fuschin for 30 seconds. The slide was then observed under 100x oil immersion light microscope for Gram-positive cocci, single or short chain. A loopful of single colony were picked from pure cultures and streaked onto the bile aesculin slant. The inoculated tubes were then incubated for 24 hours at 37˚C. Positive growth on bile aesculin slant was indicated by changes of the colour from yellow to black.

A few pure single colonies were inoculated into 6.5% sodium chloride (NaCI) and mixed well. The tubes were then incubated for 24 hours at 37˚C. Changes of the broth colour indicate growth.

For confirmation of VRE colonies, representative enterococcal colonies were cultured onto VRE agar supplemented with 8 µg/mL of vancomycin. It was then incubated for 48 hours at 37˚C. On VRE agar, enterococci appear as round grey/pale brown colonies about 1mm in diameter surrounded by black zones indicates positive growth.

For DNA extraction a fresh cell suspension was made (McFarland's 2) in 500 µL of deionised sterile water and boiled in water bath at 100˚C for 10 minutes. It was then centifuged at 13,000 rpm for 5 minutes. The supernatant was transferred into clean 2 µL PCR tube for PCR.

PCR wase run for genus confirmation, species identification and van gene detection. Master mix constituents are prepared for single reaction of volume 25 µL. It contains 2.5 µL PCR buffer (1X), 0.5 µL dNTPs (200 mM each deoxynucleoside triphosphate), 0.5 µL of each primer from 2X stock solution (Primers at concentration given in Table 1), 0.5 µL Taq polymerase (1U) and water to make 25 µL. Twenty microlitre of the master mix were dispensed into 0.2 mL PCR tubes and 5µL of DNA from samples were added. The PCR tubes were then spin briefly and keep on ice. Positive and negative controls were also included. PCR will consists of initial denaturation for 4 minutes at 94˚C, 30 cycles of denaturation 94˚C for 1 minutes, annealing 54˚C for 1 minutes and extension 72˚C for 1 minutes. Followed by final extension for 10 minutes at 72˚C and holding temperature at 4˚C. 2% agarose gel was prepared with 0.5X TBE buffer for analysis. Electrophoresis was run by mixing 7 µL of PCR product with 1.5 µL of 6X loading die at 80V for 1 hour.

Results

Presence of *Enterococcus* spp. was demonstrated in every rectal sample tested. Two different species were identified: *E. faecalis* and *E. faecium*. There were 20 samples with unknown strains and 7 samples with more than one species isolated from the same cat. *E. faecalis* was the species most frequently isolated (39/55, 70.9%).

None of the *Enteroccous* isolated were resistant to vancomycin.

Discussion

In this study, all cats were positive for enterococci which may be attributed to their outdoor lifestyle and an increase in environmental contact. This is not uncommon as enterococci were commonly found in the environment (Ferguson et al., 2004). The importance of enterococci is largely attributable to their resistance to many antibiotics (Ossiprandi et al., 2006). As a result of the close contact between companion animals and humans, the ease at which bacteria can be shared is magnified (Jackson et al., 2008). Humans are exposed to various zoonotic bacteria that can be transferred from companion animals and in this case, even from stray cats due to their proximity with humans

In most studies on enterococci from dogs and cats, five or fewer species of enterococci have been reported with *E. faecalis* and / or *E. faecium* isolated most frequently (Rodrigues et al., 2002; Poeta et al., 2006). *E. faecalis* and *E. faecium* are also the predominant species indicated in human infections (Murray, 1990) and *E. faecalis* was the most prevalent in poultry (Poeta et al., 2005). From this study, *E. faecalis* was the predominant species isolated (70.9%) followed by *E. faecium* (5.5%). Other species were also isolated but could not be detected due to limited number of primers used in this study. *E. faecalis* and *E. faecium* were also the two most common species, comprising 80 to 90% and 5 to 10% of clinical isolates each (Flores et al., 1996) found in humans.

VRE were not detected from all *Enterococcus* isolates from stray cats found in colleges in UPM and around Sri Serdang area. The absence of VRE in these cats possibly suggests that the cats have not been exposed to the environment where selective pressure allows for the development of resistance to antimicrobes, or/and the stray cats had been exposed to prolong antimicrobial treatments or food with antibiotic residues. According to a study, colonisation of pets may be a consequence of eating raw meat contaminated with VRE (Van Belkum et al., 1996).

From this study, absence of VRE in stray cats may be associated with the environment the cats were found and where there is limited contact with humans. The stray cats do not have much contact with farm animals that may harbour VRE. Negative results for VRE from cats that had been neutered (i.e. have been exposed to hospital environments for neutering and spaying) may be due to short duration for hospital stays therefore denies the possibility of VRE from healthcare settings.

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