UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY OF ENTEROCOCCAL SPECIES ISOLATED FROM CATS EXPOSED TO DIFFERENT ANTIBIOTICS

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IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY OF ENTEROCOCCAL SPECIES ISOLATED FROM CATS EXPOSED TO DIFFERENT ANTIBIOTICS

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It is hereby certified that I have read this project paper entitled "Identification and Antimicrobial Susceptibility of Enterococcal Species Isolated from Cats Exposed to Different Antibiotics", by Nor Azimah binti Mohd Amin and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course

VPD 4999 – Final Year Project.

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This project is specially dedicated to

MY PARENTS

Mohd Amin bin Mohd Akhir

Nor Laili binti Ahmad

MY SIBLINGS

Mohd Nizam and Sally Shahreena

Mohd Nazim and Zahidah

MY NIECE

Nur Na’ila Sara

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µm	Micrometer
ºC	Degree Celcius
AST	Antimicrobial Susceptibility Test
E. avium	*Enterococcus avium*
E. Coli	*Escherichia coli*
E. durans	*Enterococcus durans*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
E. gallinarum	*Enterococcus gallinarum*
E. hirae	*Enterococcus hirae*
FLUTD	Feline Lower Urinary Tract Disease
ICU	Intensive Care Unit
ILD	Inflammatory Liver Disease
MDR	multidrug resistant
PDR	pandrug resistant
UTIs	urinary tract infections
VRE	vancomycin resistant enterococci
XDR	extensively drug resistant
mm	Millimeter

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY OF ENTEROCOCCAL SPECIES ISOLATED FROM CATS EXPOSED TO DIFFERENT ANTIBIOTICS

By

Nor Azimah binti Mohd Amin

2016

Supervisor: Assoc. Prof. Dr. Siti Khairani Bejo

Enterococci are part of normal microbial flora in the gastrointestinal tract of human and animals. They have emerged to pose a significant public health concern through its emergence of being opportunistic in causing nosocomial infections as well as acquiring high level of resistance to many antimicrobial agents. Vancomycin-resistant enterococci (VRE) appearance has caused serious problems in human and veterinary medicine. The objectives of this study were to isolate and identify enterococci in cats exposed to different antibiotics and to determine its antimicrobial susceptibility pattern. Thirty (30) rectal swabs of cats were collected from a veterinary clinic and processed for isolation and identification of enterococci by inoculating the samples onto blood agar and followed by biochemical tests. The isolates were then subjected to antimicrobial susceptibility test (AST) to six antibiotics namely amoxicillin, amoxicillin and clavulanic acid, enrofloxacin, marbofloxacin, doxycycline, and vancomycin. Six rectal swab samples were positive for enterococci. The enterococci isolates were consists of *E. faecalis* (3 isolates), *E. durans* (3 isolates) and *Enterococcus* sp.(1 isolate). None of the isolates were resistant to all six antibiotics tested. *Enterococcus* sp. isolate was resistant to five antibiotics (amoxicillin, amoxicillin and clavulanic acid, enrofloxacin, marbofloxacin, and doxycycline). One isolates of *E. faecalis* was resistant to four antibiotics (amoxicillin and clavulanic acid, enrofloxacin, marbofloxacin, and doxycycline). One *E. faecalis* and one *E. durans* were resistant to three antibiotics (amoxicillin and clavulanic acid, enrofloxacin, and marbofloxacin). Two *E. durans* and one *E. faecalis* were resistant to two antibiotics (enrofloxacin and marbofloxacin). All enterococci isolates were resistant to enrofloxacin and marbofloxacin whilst vancomycin was the antibiotic that all the isolates were sensitive to. Two multidrug-resistant (MDR) enterococci and two extensively drug resistant (XDR) enterococci were detected in this present study.

Keywords: cat, rectal swab, enterococci, AST

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir.

IDENTIFIKASI DAN KERENTANAN ANTIMIKROB OLEH SPESIS ENTEROCOCCUS DARI KUCING YANG TERDEDAH KEPADA ANTIBIOTIK YANG BERBEZA

Oleh

Nor Azimah binti Mohd Amin

2016

Penyelia: Prof. Madya Dr. Siti Khairani Bejo

Enterokoki adalah sebahagian daripada flora normal di dalam saluran gastrousus manusia dan haiwan. Ianya telah muncul untuk menimbulkan kebimbangan terhadap kesihatan awam melalui kemunculannya sebagai organisma oportunis dalam menyebabkan jangkitan nosokomial serta memperoleh kerentanan pada tahap yang tinggi terhadap banyak agen antimikrob. Kemunculan enterokoki yang rentan terhadap vancomycin telah menyebabkan masalah yang serius dalam perubatan manusia dan perubatan veterinar. Objektif kajian ini adalah untuk mengasing dan mengidentifikasi enterokoki daripada kucing yang pernah terdedah kepada antibiotik yang berbeza serta menentukan corak kerentanannya terhadap antimikrob. Sebanyak 30 swab rektum kucing telah diperoleh daripada sebuah klinik veterinar dan diproses untuk pengasingan enterokoki dengan cara menginokulasikannya ke atas agar-agar darah serta diikuti oleh ujian biokimia. Ujian kerentanan antimikrob dengan enam antibiotik iaitu amoxicillin, amoxicillin dan clavulanic acid, enrofloxacin, marbofloxacin, doxycycline dan vancomycin kemudiannya dilakukan terhadap enterokoki yang terasing. Enam swab rektum didapati positif untuk enterokoki dan terdiri daripada *E. faecalis* (3), *E. durans* (3) dan *Enterococcus* sp. (1). Tiada enterokoki terasing yang rentan terhadap keenam-enam antibiotik yang diuji. *Enterococcus* sp. rentan terhadap lima antibiotik (amoxicillin, amoxicillin dan clavulanic acid, enrofloxacin, marbofloxacin dan doxycycline). Satu *E. faecalis* rentan terhadap empat antibiotik (amoxicillin dan clavulanic acid, enrofloxacin, marbofloxacin, dan doxycycline). Satu *E. faecalis* dan satu *E. durans* rentan terhadap tiga antibiotik (amoxicillin dan clavulanic acid, enrofloxacin dan marbofloxacin). Dua *E. durans* dan satu *E. faecalis* rentan terhadap dua antibiotik (enrofloxacin dan marbofloxacin). Kesemua enterokoki terasing rentan terhadap enrofloxacin dan marbofloxacin manakala vancomycin adalah antibiotik yang sentitif oleh kesemua enterokoki terasing. Dua enterokoki yang rentan terhadap antimikrob pelbagai dan dua enterokoki yang rentan terhadap antimikrob meluas telah dikenalpasti di dalam ujian ini.

Kata kunci: kucing, swab rektum, enterokoki, kerentanan antibiotik

1.0 INTRODUCTION

The enterococci are part of normal microbial flora in the gastrointestinal tract of human and animals. They are also common to be found in the environments contaminated from fecal materials of human and animals as well as food products derived from animals. *Enterococcus faecalis, Enterococcus faecium* and *Enterococcus durans* are the major enterococcal species (Akhter *et al.*, 2011). Generally, they are non-pathogenic bacteria and do not cause any illness in healthy human and animals. However, they have emerged to pose a significant public health concern through its emergence of being opportunistic pathogens in causing nosocomial infections as well as acquiring high level of resistance to many antimicrobial agents (Kataoka *et al.*, 2013). Recently, vancomycin-resistant enterococci (VRE) appearance has cause serious problems in human and veterinary medicine.

Enterococci is known to cause infections in human include urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteraemia and neonatal sepsis (Poh *et al.*, 2006). Besides, it has been reported that enterococci is the second most cause of wound and urinary tract infection and the third most common cause of bacteraemia (De Fátima Silva Lopes *et al*., 2005). Based on clinical information from hospital Kuala Lumpur (HKL) Malaysia, 244 cases of enterococci infected patients were identified (Ibrahim *et al.*, 2010).

In animals, particularly cats, enterococci were commonly isolated from cats with bacterial urinary tract infections (UTIs) (Dorsch *et al.*, 2015), from Norwegian cats with feline lower urinary tract disease (FLUTD) that have higher prevalence of bacterial cystitis (Lund *et al.*, 2015), from livers of cats with inflammatory liver disease (ILD) (Twedt *et al.*, 2014) and from the ileum mucosa of terminally ill kittens with clinical signs of diarrhea (Ghosh *et al*., 2013).

The increasing amount of resistant bacteria causing infections in pets indicates increase amount of antimicrobials used in pets, including agents used in human medicine (Lloyd, 2007). Human particularly pet owners and veterinary staffs have more opportunities for physical contact with pets. Thus, there is possible transmission of such bacteria from pets to human in which later may have impact on the use of antimicrobials in human medicine.

The transmission of pathogenic and antimicrobial-resistant bacteria from pets to their owners has been described in numerous reports (Buma *et al.*, 2006). There are 3.8 million pet populations in Malaysia in 2012 (Lee and Kok, 2015). However, few studies have been conducted and reported on the presence of enterococci in pets particularly in Malaysia. Therefore, as the ownership of pets has risen, monitoring enterococci in pets is important for public health and veterinary medicine.

1.1 Objectives

The objectives of this study were:

1. To isolate and identify enterococci bacteria in cats exposed to different antibiotic.
2. To determine antibiotic susceptibility pattern of enterococci bacteria isolated from cats exposed to different antibiotic.

1.2 Hypothesis

The hypothesis for this study was that cats exposed to antibiotics have enterococci resistant to antimicrobials.

2.0 LITERATURE REVIEW

2.1 Enterococci

The enterococci cells are spherical or ovoid in shape of approximately 0.6-2.0 x 0.6-2.5 µm in size which occur in pair or short chains in liquid media. They are gram positive organisms and do not form endospores. They are sometimes motile by scanty flagella but lack of obvious capsules. They are facultative anaerobes, chemoorganotrophs with fermentative metabolism whereby they ferment a wide range of carbohydrates with the production of mainly L lactic acid without production of gas and a final pH of 4.2-4.6. Their nutritional requirements are complex. The optimum temperature for growth is 37^{o}C, with pH value of 9.6 as well as with the presence of 6.5% NaCl and 40% bile. They seldom reduce nitrate yet they usually ferment lactose. They belong to Lancefield serological Group D (Holt *et al.*,1994). They also belong to group of organisms known as lactic acid bacteria (LAB) that produces bacteriocins (Fischer K. & Phillips C., 2009). The representative species are *E. faecalis, E. faecium, E. avium, E. durans* and *E. gallinarum* (Wiley *et al.*, 2011).

Macroscopically, this organism appears as small to medium gray colonies on sheep blood agar. Under anaerobic condition, their growth is more robust as compared to their growth in aerobic condition. Their hemolytic appearance on blood agar varies as they may be alpha, beta, or non-hemolytic. Unlike the streptococci, they are catalase negative or weakly positive whereby its reaction may be a little bubbles after a short delay of time (Holt *et al.*, 1994).

2.2 Enterococci in nature

According to Gilmore *et al.* (2014), in the 1960's and 1970's, enterococci were explored in the gastrointestinal tracts and feces of mammals, reptiles and birds with a percentage of 71.3%, 85.7% and 31.8% respectively. The presence of enterococci was discovered in insects with the percentage of 53%. However, they claimed that the actual rate of colonization may be near 100% as the rates of positive culture may underestimate the presence of microbes.

According to Hardie and Whiley (1997), enterococci are found in intestinal tract and feces of man and other animals. They also stated that some species have been isolated from soil, food, water and plants. The organisms can grow and survive a wide range of environmental conditions include extreme temperatures and salt concentrations which would suggest for the ubiquitous distribution of the genus.

2.3 Enterococcal infections in human

There were serial surveillance since the 1980s and based on the data, the epidemiologic impact from enterococcal infection has dramatically increased particularly in the nosocomial setting and due to the principally acquired resistant, it has become a significant public health concern (Linden, 2008). Based on a nationwide surveillance study between the year of 1995 and 2002, enterococci were the third most common cause of nosocomial infection and high level of vancomycin resistance was present in 60% of *E. faecium* strains but only 2% of *E. faecalis* (Centers for Disease Control and Prevention, 2003). The same study at the same time showed that vancomycin-resistant enterococci (VRE) accounted for 27.5% of intensive care unit nosocomial bacteraemic and nonbacteraemic infections (Center for Disease Control and Prevention, 2003). Based on clinical information from Hospital Kuala Lumpur (HKL) Malaysia, 244 cases of enterococci infected patients were identified (Ibrahim *et al.*, 2010).

Enterococcal infections in human include urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteraemia and neonatal sepsis (Poh *et al.*, 2006). Besides, it has been reported that enterococci is the second most cause of wound and urinary tract infection and the third most common cause of bacteraemia (De Fátima Silva Lopes *et al.*, 2005). In United Kingdom, there were 7066 enterococcus bacteraemia cases reported in 2005 whereby 63% of the cases due to *E. faecalis* and 28% due to *E. faecium* in which both of them have increasing antibiotic resistance (Fischer and Phillips, 2009). The most common species among the enterococci isolated from human illness is *E. faecalis* as indicated from the epidemiological data meanwhile *E. faecium* poses higher antibiotic resistance threat (Giraffa, 2002).

2.4 Enterococcal infections in animals

Animals can also be infected by enterococci (Gilmore *et al.*, 2014). While enterococci associated infections in small animals is quite uncommon, the resistant enterococci reported is important as they are able to horizontally transfer resistance traits to other bacteria including *Staphylococcus aureus* (Weigel *et al.*, 2003).

This genus of bacteria is non-pathogenic but it has been associated with feline diseases. A study on feline urinary tract pathogens showed that enterococci were the fourth most frequent bacteria to be isolated from cats with bacterial urinary tract infections (UTIs) and the enterococci isolates were resistant to a significantly higher number to antimicrobial agents than *E.coli* and *Staphylococcus* sp. isolates (Dorsch, von Vopelius-Feldt *et al.*, 2015). Norwegian cats with feline lower urinary tract disease (FLUTD) have shown higher prevalence of bacterial cystitis and enterococci were among the common bacterial species to be detected (Lund *et al.*, 2015).

Investigation on the presence and distribution of bacteria within the livers of cats with inflammatory liver disease (ILD) showed that *E.coli* and enterococci were the most predominant bacteria to be isolated (Twedt *et al.*, 2014). Twedt also stated that the type of intrahepatic bacteria, their spatial distribution within the liver, and the high prevalence of concurrent disease which reduce intestinal barrier function suggests that potential source of infection would be from enteric translocation or haematogenous seeding. Enterococcus was one of the isolates in cats with acute neutrophilic cholangitis or cholecystitis from previous reported case which suggest ascending infection with enteric bacteria (Brain *et al.*, 2006). Similar study also found that enterococci were one of the frequent isolates in cats and dogs with hepatic inflammation (Wagner *et al.*, 2007).

In healthy kittens, *E. hirae* was the most common species to be isolated from the small intestines, meanwhile, *E. faecalis* was more commonly isolated from the ileum mucosa of terminally ill kittens which shows clinical signs of diarrhea and post-mortem evidence of enteritis (Ghosh *et al.*, 2013).

2.5 Pets (dogs and cats) as source of transmission to human

Dogs and cats are known as sources of enterococci. They may also harbor and disseminate the bacteria to human through the close physical contact that occur between human and their pets (Guardabassi *et al.*, 2004). The likelihood of enterococci to be resistant may be increased through the widespread use of antimicrobials in these animals (Leener *et al.*, 2005).

Healthy dogs and cats are a source of antimicrobial resistant enterococci and may act as reservoir that can be transferred from pets to human, human to pets, and to the environment (Jackson *et al.*, 2009). In their study, enterococci were predominantly isolated from sites such as rectal, hindquarters, and belly of both dogs and cats as these sites would likely to be contaminated during or after the animal defecated and compared to any other sites, it is in continual close contact with the environment. Other sites such as teeth and nasal areas were tested positive but in a lower number of isolates. This shows that any other sites may also be contaminated at any time and this is important considering the close physical contact made between human and their pets as the risk of transmission is higher.

2.6 Multidrug-resistance (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) of Enterococci

Definition of MDR is acquired non-susceptibility of bacteria to at least one agent in three or more antimicrobial categories, definition of XDR is non-susceptibility of bacteria to at least one agent in all but two or fewer antimicrobial categories and definition of PDR is non-susceptibility of bacteria to all agents in all antimicrobial categories (Magiorakos *et al.*, 2011). *Enterococcus faecium* has emerged as multidrug-resistant enterococci in the 1980s as the leading cause of hospital-acquired infection (Lebreton *et al.*, 2013). Currently, majority of *E. faecium* are resistance towards three of the most useful antimicrobials against enterococci which are ampicillin, vancomycin and aminoglycosides whereby it exhibits high-level of resistance to aminoglycosides (Arias *et al.*, 2010).

2.7 Vancomycin Resistant Enterococcus (VRE)

In 1986, *E. faecium* strains with high-level of vancomycin resistance was first appeared in France and England (Linden, 2008). Its appearance was a major landmark in the evolution of antimicrobial resistant enterococci. There are six gycopeptide resistance phenotypes which are VanA, VanB, VanC, VanD, VanE and VanG. VanA and VanB enterococci have high-level of vancomycin resistance and have the highest prevalence and clinical importance (Linden, 2008). Linden also stated that there are three sequential processes that lead to detectable VRE colonization and potential subsequent infection with multiple modifiers; (i) Enterococci containing vancomycin-resistant genome exposure via contact with animate or inanimate source (vanA gene does not arise from a spontaneous or antibiotic-induced mutation), (ii) VRE inoculum amplification in gastrointestinal reservoir usually due to antimicrobial selective pressures, (iii) natural or iatrogenic anatomic or immune defects that lead to bloodstream or tissue invasion.

3.0 MATERIALS AND METHODS

3.1 Animal selection

A total number of 30 cats were selected from a private veterinary clinic in which all of the selected cats have history of being treated with antibiotics; amoxicillin (18/30), amoxicillin and clavulanic acid (5/30), marbofloxacin (5/30), enrofloxacin (4/30), and metronidazole (1/30). They are selected regardless of their sex, breed and age.

3.2 Collection of samples

A number of 30 cats had exposure to antibiotics were selected and they were restrained properly. Rectal swab was taken by inserting the cotton part into the lumen of the rectum. The swab was moved from side to side and swabbed the fecal materials in the lumen of the rectum. Feces should be evident on the swab. The swab was placed in Cary Blair transport media before it was transported to Bacteriology Laboratory in UPM, Serdang, Selangor for isolation and identification of enterococci bacteria.

3.3 Isolation and identification of enterococci

The swab samples collected and kept in transport media were immediately transported to the Bacteriology Laboratory. The swab sample was streaked onto blood agar (Oxoid, England) as primary culture and incubated aerobically for 24 hours at 37^{o}C. Plates with absence of bacterial growth after 24 hours of incubation were continued to be incubated for another 24 hours at 37^{o}C. Enterococci exhibited shiny gray to white, flat, circular surface colonies with entire or slightly irregular edges and variable hemolysis on blood agar.

Gram staining was done on all bacterial colonies to confirm enterococci species. Microscopically, enterococci appeared as gram positive cocci. The presumptive enterococci grown on primary culture was selected, subcultured on blood agar (Oxoid, England) to get pure culture and incubated for 24-48 hours at 37^{o}C.

Gram staining of bacterial colony on subculture was done again prior to biochemical tests for species identification. The presumptive enterococci were selected and Catalase test was done by dispensing a drop of 3% hydrogen peroxide on a clean glass slide and a single colony was picked from subculture plate using a sterile inoculating loop and placed and mixed well with the dropped of 3% hydrogen peroxide. Formation of bubbles indicates positive result. Those that were negative on Catalase test were suspected to be enterococci and species identification was done by conducting biochemical test.

3.4 Biochemical test for enterococci

Biochemical tests for species identification of *Enterococcus* spp. is as listed in Appendix III. Results from biochemical tests were able to identify *Enterococcus faecalis, Enterococcus faecium, Enterococcus* group of bacteria and other species of *Streptococcus* bacteria. Additional tests need to be done for those that were consistent with *Enterococcus* group of bacteria to differentiate among certain animal enterococci.

It can only be done only if the result of 6.5% NaCl and Bile esculin tests were positive. The additional tests are as listed in Appendix IV.

3.5 Antimicrobial susceptibility test (AST)

Testing for antimicrobial susceptibility of enterococci isolated was done by using Kirby-Bauer method. 2ml of sterile saline was dispensed into sterile test tube by using sterile pipette. Several isolated colonies from the subculture were collected by using inoculating loop and transferred into the test tube of sterile saline. The bacteria were diluted to obtain a turbidity equivalent to the 0.5 MacFarland test standard. A black-lined MarFarland reference card was held against the test tubes to accurately rate the turbidity. A sterile swab was dipped into the inoculum and streaked the entire surface of the blood agar (Oxoid, England) 3 times with the swab by turning the plate 60 degrees between streaking to obtain an even inoculation. Blood agar (Oxoid, England) was used instead of Mueller-Hinton agar in this case because appearances of enterococci on blood agar were able to be seen clearly as it gave better contrast. Six (6) antibiotics were selected based on the common antibiotics prescribed from the private clinic, from University Veterinary Hospital (UVH) and antibiotic from literature review. The antibiotics selected were Amoxicillin (10µg), Amoxicillin and Clavulanic Acid (30µg), Enrofloxacin (5µg), Marbofloxacin (5µg), Doxycycline (30µg) and Vancomycin (30µg). The antibiotic discs were placed onto the agar by using a dispenser and it was then lightly pressed down with a sterile forceps to make contact with the agar surface. The plate was then incubated at 37^{o}C for 24 hours. Zones showing complete inhibition by gross inspection were measured in millimeters (mm) by using calipers and the values obtained were compared to acceptable limits in Clinical and Laboratory Standards Institute (2010) to determine the susceptibility level of the antibiotics used.

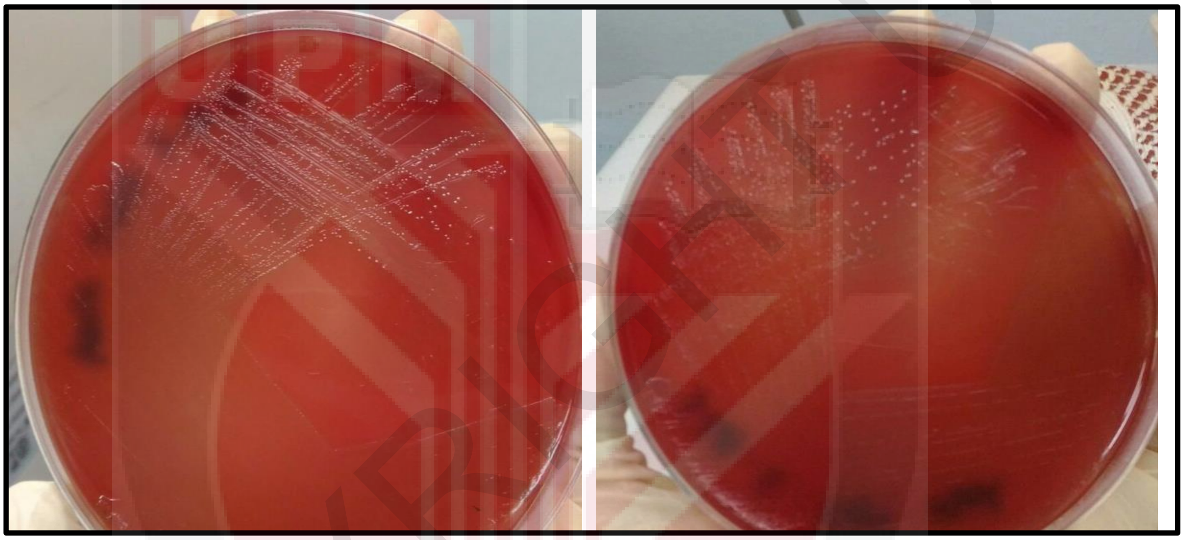


Plate 1: Pure culture of enterococci grown on blood agar. *E. faecalis* (left) and *E. durans* (right).

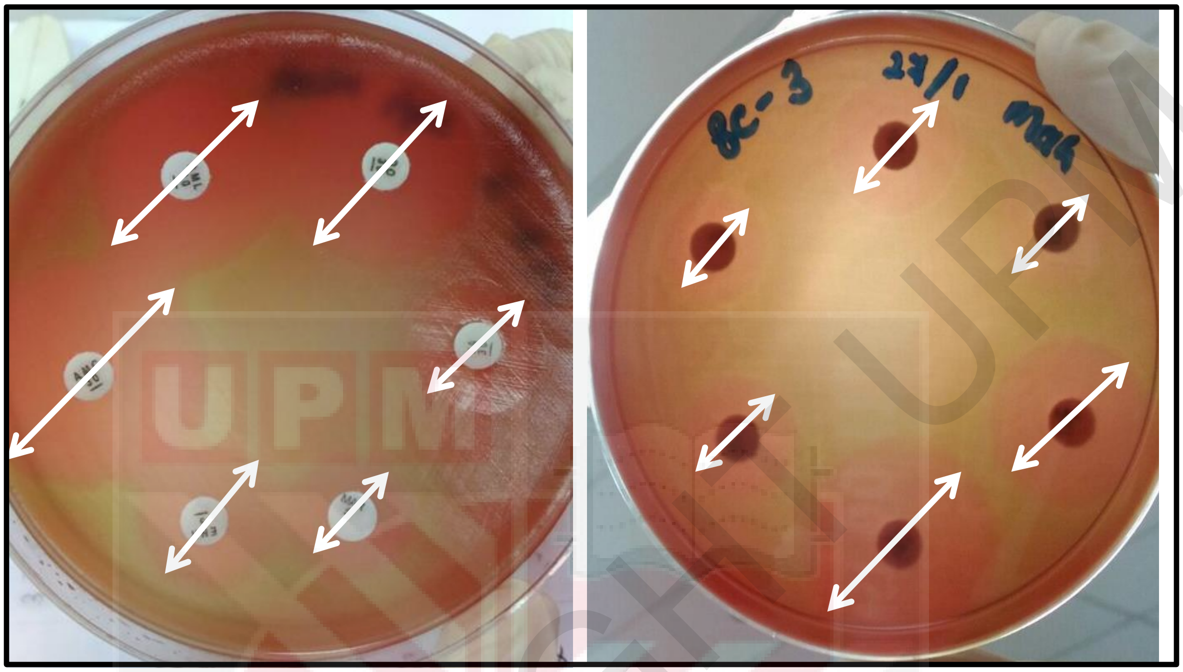


Plate 2: Antimicrobial susceptibility test on blood agar. The white arrows show the diameter of zone of inhibition. *E. faecalis* (left) and *E. durans* (right).

4.0 RESULTS

4.1 Isolation and identification of enterococci

Out of 30 samples, six (6 or 20%) samples were positive for enterococci as shown in Figure 1. Within the six positive samples, one of the samples was positive for two species of enterococci. Figure 2 shows enterococci isolates which consists of *E. faecalis* (3 isolates), *E. durans* (3 isolates) and *Enterococcus* sp.(1 isolate).

Figure 1: The isolation and identification of enterococci in 30 samples of rectal swabs from cats exposed to different antibiotics

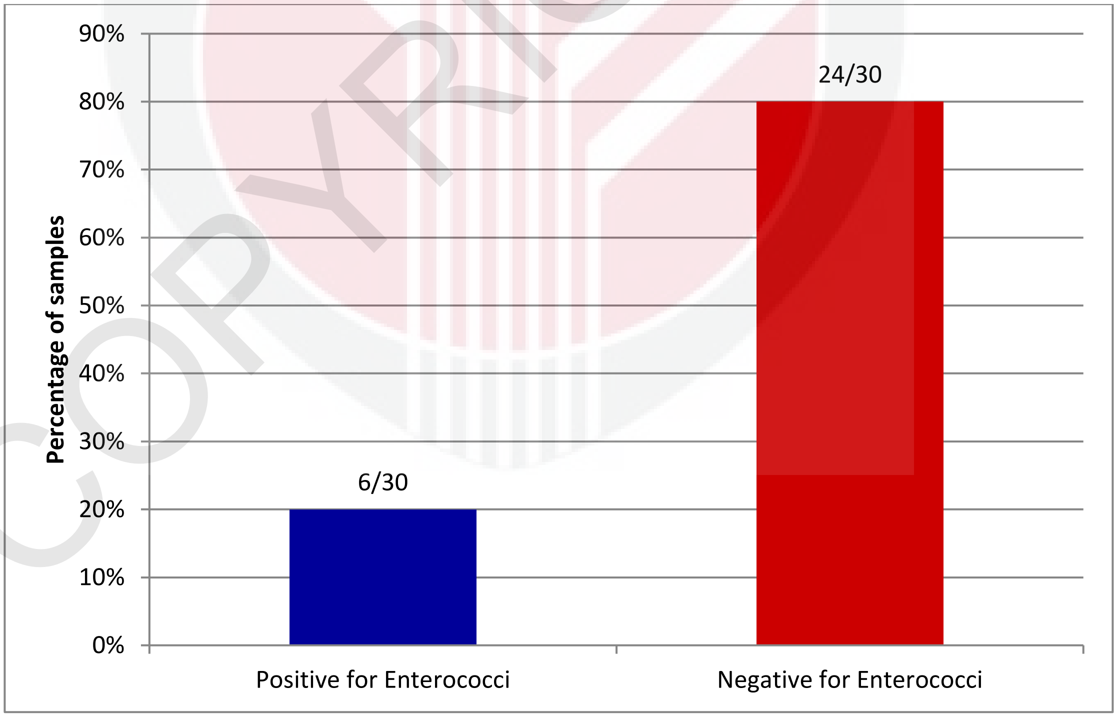
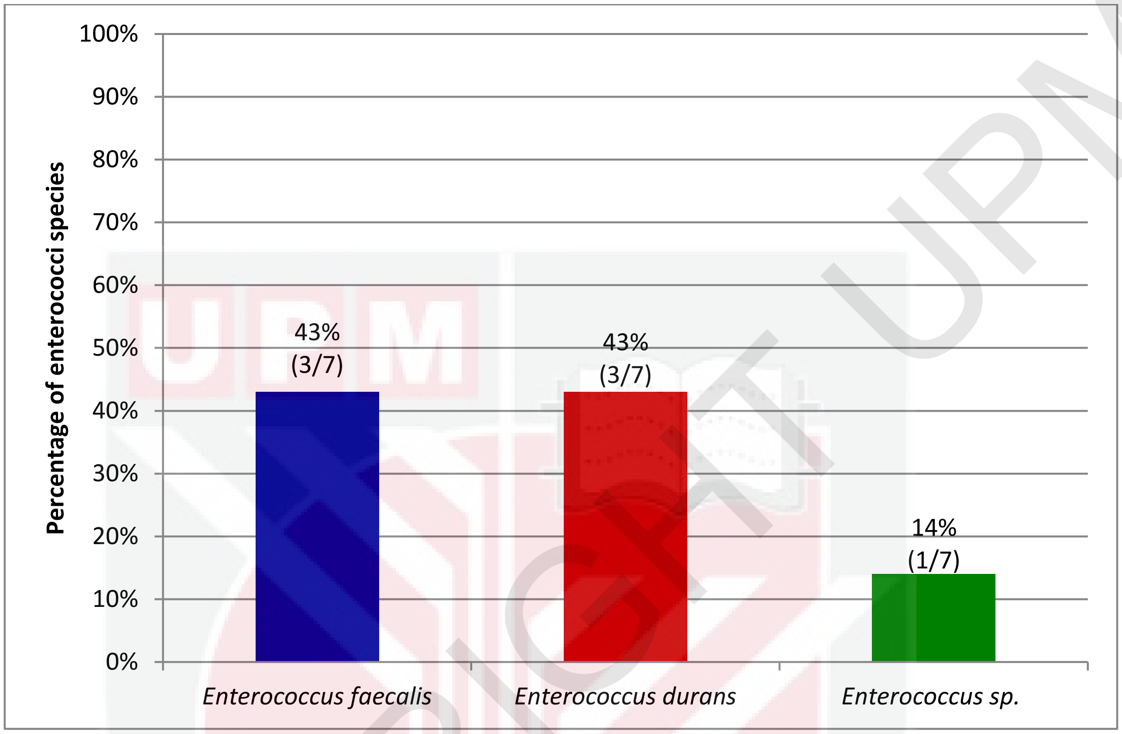


Figure 2: The species of enterococci isolated and identified



4.2 Antimicrobial Susceptibility Test

All seven species of enterococci isolated and identified were subjected to six types of antibiotics and the results are as tabulated in Table 3. None of the isolates were resistant to all six antibiotics tested. *Enterococcus* sp. isolate was resistant to five antibiotics (amoxicillin, amoxicillin and clavulanic acid, enrofloxacin, marbofloxacin, and doxycycline). One isolates of *E. faecalis* was resistant to four antibiotics (amoxicillin and clavulanic acid, enrofloxacin, marbofloxacin, and doxycycline). One *E. faecalis* and one *E. durans* were resistant to three antibiotics (amoxicillin and clavulanic acid, enrofloxacin, and marbofloxacin). Two *E. durans* and one *E. faecalis* were resistant to two antibiotics (enrofloxacin and marbofloxacin). Two multidrug-resistant

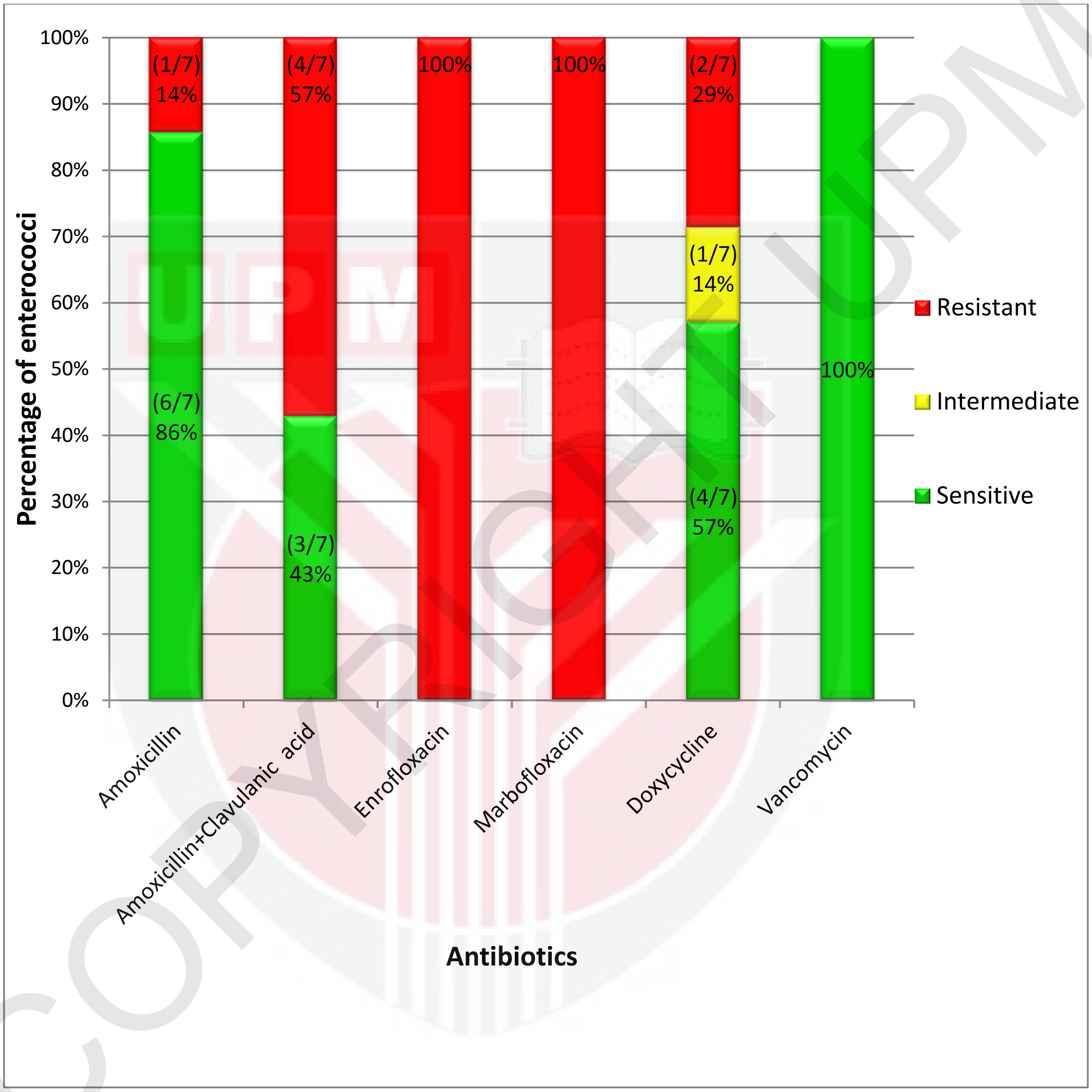
(MDR) enterococci and two extensively drug resistant (XDR) enterococci were detected. Figure 3 shows all enterococci isolates were resistant to enrofloxacin and marbofloxacin whilst vancomycin was the antibiotic that all the isolates were sensitive to.

Table 1: Antimicrobial susceptibility test result for each *Enterococcus* spp. isolated

		Antimicrobial category	Beta-lactams		Fluoroquinolones		Tetracycline	Glycopeptides	
Cat	Antibiotics exposed	Antimicrobial agent / Isolates	Amoxicillin	Amoxicillin & Clavulanic acid	Enrofloxacin	Marbofloxacin	Doxycycline	Vancomycin	
1.	Metronidazole	*E. durans*	🟩	🟥	🟥	🟥	🟨	🟩	XDR
2.	Amoxicillin	*E. durans*	🟩	🟩	🟥	🟥	🟩	🟩	
3.	Marbofloxacin	*E. faecalis*	🟩	🟥	🟥	🟥	🟥	🟩	MDR
4.	Amoxicillin	*E. faecalis*	🟩	🟩	🟥	🟥	🟩	🟩	
5.	Enrofloxacin & Marbofloxacin	*E. durans*	🟩	🟩	🟥	🟥	🟩	🟩	
6.	Amoxicillin & Clavulanic acid	*E. faecalis*	🟩	🟥	🟥	🟥	🟩	🟩	XDR
	Amoxicillin & Clavulanic acid	*Enterococcus* sp.	🟥	🟥	🟥	🟥	🟥	🟩	MDR

🟩	Sensitive	🟨	Intermediate	🟥	Resistant

Figure 3: The antimicrobial susceptibility pattern of enterococci isolated



5.0 DISCUSSION

Few studies on isolation and identification of enterococci and its susceptibility pattern to antimicrobials in pets or companion animals have been conducted worldwide to monitor its prevalence and distribution of enterococci mainly those that are resistant to many antimicrobials. In Malaysia, there was only one published study conducted on stray cats from an area in Selangor in which none of the samples were positive for vancomycin resistant enterococci (VRE) (Nurul Husna *et al.*,2010).

In this study, only six samples out of 30 samples were positive for enterococci with a percentage of 20%. Among the six positive samples, one of the samples was positive for two strains of enterococci. Based on previous study, seven out of 17 cats (43.8%) exposed to antibiotics were positive to enterococci (Kataoka *et al.*, 2014). Same goes to another study done on cats in veterinary clinics which 53 out of 121 cats (43.8%) were positive for enterococci (Jackson *et al.*, 2009). Both of these studies had higher percentage as compared to present study and this could be due to the culture medium used. In both of the studies, they used enterococcosel agar which is a selective medium and has the standard formulations for isolation and identification of enterococci. The samples that were negative for enterococci could be due to the effect of antimicrobials in which the cat was treated with at the time of sampling. Besides, culture methods are inadequate as the microorganism can be cultivated only after its physiological niche is perceived and duplicated experimentally (Ward *et al.*, 1990). Thus, it suggests that the actual rate of colonization may be higher.

The three strains of enterococci isolated in this study were *E. faecalis* (43%), *E. durans* (43%) and *Enterococcus* sp. (14%). Based on previous study, *E. faecalis* was the most frequent *enterococcus* species to be found in the gut and tonsils of cats and dogs (Devriese *et al.*, 1992). In another study, *E. faecalis* was the most isolated from rectal samples of cats with percentage of 45% (Jackson *et al.*, 2009). From human perspective, the most common species among the enterococci isolated from human illness is *E. faecalis* as indicated from the epidemiological data (Giraffa, 2002). In United Kingdom, 63% of bacteraemia cases in human were due to *E. faecalis* (Fischer and Phillips, 2009). *E. durans* is among the major strains of enterococci (Akhter *et al.*, 2011). There are reports stated that enterococci endocarditis in human can be caused by *E. durans* even though it is very rare (Stepanović *et al.*, 2004; Vijayakrishnan and Rapose, 2012; Kenzaka *et al.*, 2013).

From this study, there was only one isolate (*E. durans* from cat no. 5) which resistant to the same antibiotics it had exposed to from the history. Meanwhile, the other isolates were resistant to more antibiotics than they have been exposed. However, there was one isolate (*E. faecalis* from cat no. 4) that was resistant to antibiotics it had never been exposed to. Of all the enterococci isolated, two multidrug-resistant (MDR) enterococci (*E. faecalis* and *Enterococcus* sp.) and two extensively drug resistant (XDR) enterococci (*E. durans* and *E. faecalis*) were detected. In United States of America, MDR *E. faecalis* and *E. faecium* (three to eight antimicrobials) were detected in 116 healthy cats visiting veterinary clinics (Jackson *et al.*, 2009). There was a study conducted and reported that environment in small animal veterinary hospitals were contaminated by MDR enterococci (KuKanich *et al.*, 2012). MDR enterococci can persist for months to years once it is established in the digestive tract (Sørum *et al.*, 2006). A hypothesis was made that antibiotics used in small animal veterinary hospitals leads to the selection of specific MDR clonal lineages in the patients that may contaminate the hospital environment and becomes the source of MDR enterococci for resident cats which then become carriers or reservoirs and may re-contaminate the hospital environment (Ghosh *et al.*, 2012). This suggests that the cats from this study may acquire antimicrobial resistant enterococci from the clinic environment.

In this study, 100% of the isolated enterococci were resistant to enrofloxacin and marbofloxacin. Compared with previous studies, enrofloxacin resistant enterococci in cats with antibiotic pressures were 13% and 75% (Kataoka *et al.*, 2014; Kataoka *et al.*, 2013) which is lower compared to this current study. In Belgium, screening of cats from diverse populations demonstrated that enterococci in cats from hospitals and catteries had relatively higher prevalence of resistance to several antimicrobial agents as compared to healthy domestic cats (Leener *et al.*, 2005).

The resistant enterococci reported in this study are important especially to tetracyclines, fluoroquinolones, and beta-lactams category of antibiotics because they are commonly used in small animal veterinary medicine to treat various bacterial infections (Prescott *et al.*, 2002). Even though enterococci are not commonly causing infections in small animals, they are able to horizontally transfer their resistant traits to other bacteria including *Staphylococcus aureus* (Weigel *et al.*, 2003). The likelihood of transfer of bacteria from pets to human is high due to the close physical contact made between them as demonstrated for different bacteria including enterococci and staphylococci (Guardabassi *et al.*, 2004).

None of the enterococci isolated in this study were resistant to vancomycin. This is parallel with the findings from a study in detection of VRE in stray cats in Universiti Putra Malaysia and selected neighbourhood in Sri Serdang, Selangor (Nurul Husna *et al.*, 2010). Consumption of VRE contaminated raw meat in pets may cause the bacteria to colonize in them (Manson *et al.*, 2003). This suggests that these cats have not been exposed to vancomycin such as from VRE contaminated meat to allow resistance traits to develop. Besides, the cats have never been exposed to vancomycin from the history.

6.0 CONCLUSION AND RECOMMENDATIONS

In this study, six out of 30 samples were positive for enterococci and seven isolates were identified. The strains of enterococci isolated were *E. faecalis, E. durans* and *Enterococcus* sp. of unidentified strain. Cats exposed to antibiotics had enterococci resistant to antimicrobials.

As recommendations for this study, sites for potential contamination of enterococci such as nasal, teeth, belly and hindquarters of the animal can be sampled. It is also recommended to identify enterococci in other species of animals or pets such as dogs.

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Appendix 1: The flow chart for isolation and identification of enterococci and antimicrobial susceptibility test

DAY 1	Sample collection
	↓
	Gram staining
	↓
	Inoculate sample onto blood agar
	↓
	Incubate aerobically at 37^oC for 24 hours
	↓
DAY 2	Describe colony morphology on blood agar
	↓
	Gram staining
	↓
	Inoculate onto blood agar for pure culture
	↓
	Incubate aerobically at 37^oC for 24 hours
	↓
DAY 3	Describe colony morphology on blood agar
	↓
	Gram staining
	↓
	Biochemical tests
	↓
DAY 4	Species identification of enterococci
	↓
	Antimicrobial susceptibility test (AST)
	↓
	Incubate aerobically at 37^oC for 24 hours
	↓
DAY 5	Measure zone of inhibition

Appendix 2: Gram staining technique

Place a drop of normal saline onto a clean glass slide

↓

Mix a loopful of isolated bacteria colonies onto the glass slide

↓

Heat-fix the slide and allow to dry for few seconds

↓

Drain with crystal violet for one minute and then wash with distilled water

↓

Drain with iodine for one minute and wash with distilled water

↓

Drain with acetone for few seconds and wash with distilled water

↓

Drain with diluted carbol fuschin for one minute and wash with distilled water

Appendix 3: Biochemical tests for *Enterococcus* spp. identification

No.	Biochemical test	Method
1.	Soluble haemolysin	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
2	6.5% NaCl	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
3	Bile esculin	A loopful of isolated colonies from pure culture was collected and streaked onto the slant. It was then incubated at 37^{o}C for 24 hours.
4	Lactose	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
5	Sorbitol	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
6	Trehalose	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.

Appendix 4: Additional tests to differentiate among certain animal enterococci

No.	Biochemical test	Method
1	Voges-Proskauer (VP)	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours. 6 drops of α-naphtol and 2 drops of potassium hydroxide solution were added and it was well shaken for 1 minute. It is allowed to stand for 10-20 minutes if there is no change.
2	Motility	A needle loop was used to collect isolated colonies from pure culture and stabbed into agar butt. It was then incubated at 37^{o}C for 24 hours.
3	Mannitol	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
4	Sorbitol	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
5	Sorbose	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
6	Arabinose	A loopful of isolated colonies from pure culture was

		collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
7	Raffinose	A loopful of isolated colonies from pure culture was collected and mixed into the broth. It was then incubated at 37^{o}C for 24 hours.
8	Arginine dihydrolase (ADH)	A loopful of isolated colonies from pure culture was collected and mixed into the broth. Mineral oil was added to cover the broth and incubated at 37^{o}C for 24 hours.

Appendix 5: The colony morphology, gram staining and biochemical test results for each enterococci isolated

Sample no	4C	6B	21B	24B	27B	29B	29C
Colony morphology	Round, entire, pinpoint, grey, α-haemolysis	Round, entire, pinpoint, white, smooth, α-haemolytic	Round, entire, pinpoint, creamy white, non-haemolytic	Round, entire, convex, 1mm, bright white, smooth, α-haemolytic	Round, entire, raised, 1 mm, pale white to grey, non-haemolytic	Round, entire, raised, 1 mm, bright white, non-haemolytic	Round, entire, pinpoint, pale white, α-haemolytic
Gram stain	Gram positive cocci	Gram positive cocci	Gram positive cocci	Gram positive cocci	Gram positive cocci	Gram positive cocci	Gram positive
Subculture							
Reincubate							
Biochemical tests							
Catalase	-	-	-	-	-	-	-
Lancefield group							
Soluble haemolysin	-	-	-	-	-	-	-
6.5% NaCl	+	+	+	+	+	+	+
Bile esculin	+	+	+	+	+	+	+
Lactose	-	-	+	+	-	+	+
Sorbitol	-	-	+	+	-	+	+
Trehalose	-	-	+	+	-	-	-
	Enterococcus group	Enterococcus group	*E. faecalis*	*E. faecalis*	Enterococcus group	Enterococcus group	Enterococcus group
Additional tests							
VP	+	+			+	+	+
Motility	-	-			-	-	-
Mannitol	-	-			-	+	+
Sorbitol	-	-			-	+	+
Sorbose	-	-			-	-	-
Arabinose	-	-			-	-	-
Raffinose	-	-			-	-	+
(ADH) Arginine	+	+			+	+	+
	E. durans	*E. durans*	*E. faecalis*	*E. faecalis*	*E. durans*	*E. faecalis*	*Enterococcus* sp.