DETECTION OF VANCOMYCIN RESISTANCE ENTEROCOCCI IN POULTRY AND CLINICAL SAMPLES AND THEIR RESISTANCE GENES BY MULTIPLEX PCR

NUR SYAFIQAH BINTI MOHAMAD NASIR

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by

NUR SYAFIQAH BINTI MOHAMAD NASIR

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LIST OF SYMBOLS

%	Percentage
/	Per
>	More than
<	Less than
≥	More than or equal to
≤	Less than or equal to
°C	Degree Celsius
=	Equal to
-	Minus
+	Addition
µg	Microgram
µl	Microliter

LIST OF ABBREVIATIONS

AST	Antibiotic Susceptibility Test
ATCC	American Type Culture Collection
BHI	Brain heart infusion
bp	Base pair
CFU	Colony Forming Unit
CHROM	Chromogenic media
CLSI	Clinical & Laboratory Standard Institute
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene-diamine-tetra-acetic acid
E-test	Epsilometer-test
EU	European Union
FDA	Food and Drug Administration
g	Gram
GN	Gram Negative
GP	Gram Positive
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric Acid
HUSM	Hospital Universiti Sains Malaysia
IC	Internal Control
ICU	Intensive Care Unit
L	Liter
LMG	Laboratory for Microbiology
LOD	Limit of Detection
M	Molarity
mg	Milligram
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
min	Minutes
ml	Milliliter
mM	Millimolar

MRSA	Methicillin-resistant *Staphylococcus aureus*
NA	Nutrient agar
NaCl	Sodium chloride
ng	Nanogram
NNIS	National Nosocomial Infection Surveillance
PC	Positive control
PCR	Polymerase Chain Reaction
pg	Picogram
pH	Potential of Hidrogen
PYR	Pyrrolidonyl Aminopeptidase
rpm	Revolution per minute
rRNA	ribosomal ribonucleic acid
spp.	species
Ta	Annealing temperature
TBE	Tris-Borate EDTA
TE	Tris-EDTA
Tm	Melting temperature
Taq	Thermus aquaticus
U	Unit
UK	United Kingdom
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Volt
van	Vancomycin
VRE	Vancomycin resistant enterococci
WHO	World Health Organization
X	Times or multiplication

LIST OF APPENDICES

PENGESANAN ENTEROCOCCI YANG RINTANG TERHADAP VANCOMYCIN DALAM SAMPEL UNGGAS DAN KLINIKAL DAN GEN RINTANGANNYA SECARA PCR MULTIPLEKS

ABSTRAK

Enterococci rintang vancomycin (VRE) telah muncul sebagai patogen nosokomial penting yang mengkhusus dalam pembentukan biofilm. Ia menyumbang kepada daya tahan yang tinggi terhadap antibiotik. Asal usul VRE tidak diketahui, tetapi kemunculannya dipercayai mempunyai kaitan dengan penggunaan kumpulan antibiotik glycopeptide avoparcin yang berlebihan yang telah digunakan sebagai sumber makanan tambahan dalam industri penternakan. Tujuan kajian ini dijalankan adalah untuk menilai kelaziman VRE di ladang ayam dan menentukan gen rintangannya dan juga menentukan gen rintang dalam VRE klinikal. Sebanyak 300 sampel swab kloaka ayam (*Gallus gallus domesticus*) telah diambil setelah ladang ayam komersial dikenalpasti dan dipersetujui oleh pemilik untuk persampelan manakala 38 arkib VRE daripada pelbagai sampel klinikal telah dikumpulkan dari Jabatan Mikrobiologi, HUSM dari tahun 2016 hingga 2019. Sampel swab kloaka ayam diambil dengan menggunakan sapu kapas yang steril dan disimpan di dalam media yang tinggi nutrien. Sampel isolat telah dikenalpasti secara morfologi dan kaedah biokimia. Ujian kepekaan antibiotik juga telah digunakan untuk menyaring *Enterococcus* yang rintang kepada vancomycin. Di samping itu, PCR multipleks dioptimumkan untuk mengenal pasti gen rintangan vancomycin (*van*A, *van*B, *van*C, dan *van*D) dalam sampel kloaka ayam dan sampel arkib VRE klinikal. Sebanyak 97 *Enterococcus* telah dipencilkan daripada 300 sampel swab kloaka ayam. Antara *Enterococcus* yang dipencilkan, 71.1% (n=69/97) adalah *Enteroccus casseliflavus* dan

28.9% (n=28/97) adalah *Enterococcus gallinarum* yang dikenal pasti dengan kaedah fenotipik dan genotipik. Di antara semua *Enterococcus* yang dipencilkan daripada ladang ayam, tiada isolat yang menunjukkan kerintangan terhadap vancomycin. Isolat menunjukkan 100% sensitif terhadap teicoplanin dan tigecycline. Walaubagaimanapun, 2/97 (2.1%) meununjukkan kerintangan terhadap gentamicin peringkat tinggi manakala 48/97 (49.5%) mempunyai kerintangan terhadap ampicillin, dan 28/97 (28.9%) lagi kerintangan terhadap penisilin. Menariknya, antara dua strain yang dipencilkan daripada ladang ayam, 18.6% (n=18/97) daripadanya rintang terhadap linezolid. Walaupun tiada VRE yang dipencilkan daripada ladang ayam, Kesemua 38 arkib VRE dari sampel klinikal menunjukkan kerintangan terhadap vancomycin dengan kehadiran gen *van*A yang menunjukkan kerintangan peringkat tinggi. Kehadiran strain baharu enterococci rintang linezolid (LRE) telah menyumbang kepada masalah yang lebih membimbangkan kepada industri penternakan haiwan dan kesihatan awam. Ini boleh mengakibatkan kemungkinan jangkitan enterococcal yang tidak dapat dirawat. Lebih membimbangkan lagi, penyebaran LRE ke kawasan geografi yang sebelum ini tidak dilaporkan berpotensi membawa kepada ancaman kesihatan global. Oleh itu, pengawasan antimikrob dalam penternakan ayam adalah sangat perlu untuk mencegah penyebaran organisma yang mempunyai kadar rintang yang tinggi terhadap perlbagai antibiotik.

MOLECULAR DETECTION OF VANCOMYCIN RESISTANCE ENTEROCOCCI IN POULTRY AND CLINICAL SAMPLES AND THEIR RESISTANCE GENES BY MULTIPLEX PCR

ABSTRACT

Vancomycin resistance enterococci (VRE) has emerged as an important nosocomial pathogen that specializes in biofilm formation which contributes to their virulence and antibiotic resistance. VRE's origin is unknown, but the emergence is believed to be associated with the overuse of antibiotic glycopeptides of avoparcin as a growth promoter in animal husbandry. This research was carried out to determine the prevalence of VRE and their resistance genes in poultry and to determine the resistance genes in archived clinical VRE. A total of 300 broiler's cloaca swab samples *(Gallus gallus domesticus)* were collected after commercial poultry farms were identified and consented by the owners for sampling, while 38 of the archived VRE isolates were retrieved from various clinical samples from the Department of Medical Microbiology between 2016 and 2019. Broiler's cloaca swab samples were taken using a sterile cotton swab and kept in an enriched broth. *Enterococcus* spp. were identified using morphological and biochemical testing, and an antibiotic susceptibility test was used to screen for vancomycin resistance. Following that, a multiplex PCR assay was optimized to simultaneously identify vancomycin resistance genes (*van*A, *van*B, *van*C, and *van*D) in poultry and clinical isolates. A total of 97 *Enterococcus* were obtained from 300 broiler's cloaca swab samples. The isolates were identified *Enterococcus casseliflavus* (n=69/97; 71.1%) and *Enterococcus gallinarum* (n=28/97; 28.9%) by phenotypic and genotypic method. Among all the *Enterococcus* isolated from broiler's cloaca swab samples, none of them were found to exhibit resistant to vancomycin.

Isolates demonstrated 100% susceptibility to teicoplanin and tigecycline. Of all, 2/97 (2.1%) were exhibit resistance to high-level gentamicin. Whereas 48/97 (49.5%) were observed to be resistant to ampicillin, and 28/97 (28.9%) were resistant to penicillin. Interestingly, among the two species isolated, 18.6% (n=18/97) of it were resistant to linezolid. Despite no VRE was found in broiler farms in this study, persistence of VRE was detected in clinical setting. All the archived clinical VRE samples were found to possess a *van*A gene, which has been linked to high-level vancomycin resistance. Preventing and controlling the spread of vancomycin resistance will require coordinated and concerted efforts from all involved hospital departments to reduce the persistence and transmission of VRE. New identified linezolid resistant *Enterococcus* (LRE) in this study causes an alarming problem to the animal husbandry as well as to the healthcare setting worldwide. This could result in possibly untreatable and severe enterococcal infections. Even more worrying, the spread of LRE to geographical regions that these resistant was previously unreported, potentially lead to global health threat. Therefore, antimicrobial surveillance in poultry husbandry is dimly necessary to prevent wide spread of multidrug-resistant organism.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Background of studies

Europe was the first continent reported of Vancomycin-resistant *Enterococcus* (VRE) species isolated from clinical specimen in 1988 (Courvalin, 2006). It was later spread unexpectedly on the East Coast of the United States and currently encountered almost in all hospitals worldwide. Meanwhile in Asia, Malaysia had reported the first case of hospital-acquired VRE back in 1996 from the University of Malaya Medical Centre (Riley *et al*., 1996). Later on, it has been reported in one of the chronic renal failure patients from Kuala Lumpur Hospital in 2006 (Zubaidah *et al*., 2006). Enterococci nosocomial infections have been a major concern to public health especially at many hospitals, it well known antibiotic-resistant opportunistic pathogens commonly associated and recovered from those who are immunocompromised and received multiple course of antibiotics (Agegne *et al*., 2018). *Enterococcus* becomes more threatening to the regimen as it could transfer their resistant gene to more deadly virulence pathogenic bacteria (Mohamed *et al*., 2015). *Enterococcus* inhabit the intestines of animals and considered normal flora of the bowel (Toosa *et al*., 2001). Even though the origin of VRE is unknown but its emergence is believed to be associated with the overuse of antibiotics as growth promoters in animal husbandry. Avoparcin is a glycopeptide antibiotic that has been used as a growth promoter for farm animals in Europe, resulting in the VRE epidemic (Acar *et al*., 2000). Livestock, mainly in chickens and pigs, are frequently reported to implicate as a reservoir of VRE for humans (Vignaroli *et al*., 2011). The use of avoparcin growth promoters was banned by the European Union in 1997 because it put selective pressure on the emergence and spread of vancomycin-resistance genes (van den Bogaard, 2000).

1.2 *Enterococcus* species

Enterococcus species inhabit the intestines of humans and animals thus considered as normal flora of the bowel (Toosa *et al.*, 2001). There are 17 species of enterococci recognised by the European Union (EU), but *E. faecalis* and *E. faecium* are the most isolated from humans. *Enterococcus avium, Enterococcus gallinarum, Enterococcus casseiliflavus, Enterococcus durans, Enterococcus raffinosus, and Enterococcus mundtii* are among the other species known (Murray, 1990; Raza *et al.*, 2018). Aside from *E. faecalis* and *E. faecium*, other species such as E. *casseliflavus, E. gallinarum and E. raffinosus* have been linked to infections (Gilmore *et al.*, 2002).

Previously, enterococci were described to be part of genus *Streptococcus* before it has been regrouping into their own genus and known to be an important human pathogen that has been reported to antimicrobial agent resistant. Previously, it is reported to cause up to 15% cases of endocarditis that best treated by antibiotics of penicillin and vancomycin (Murray, 1990). Furthermore, *Enterococcus* species have evolved to become resistant to multiple antimicrobial drugs such as penicillin, cephalosporins, clindamycin, and aminoglycosides (Mundy and Sahm, 2000). Various enterococcal species have been identified and are presumed to be less virulent and causing minimal disease in healthy people, and yet these two *E. faecium* and *E. faecalis* are known to cause human diseases such as bacteremia, endocarditis, and a variety of other nosocomial infections. (Raza *et al*., 2018).

1.2.1 Taxonomic classification

The *Enterococcus* genus is a member of the Enterococcacea family, and it was isolated from the streptococci genus by studying DNA-DNA and DNA-rRNA hybridization (Schleifer and Kilpper-Balz, 1984). Using 16S rRNA sequence analysis, this genus was later distinguished from lactococci and other gram-positive cocci, showing that it was distinct from them (Ludwig *et al.*, 1985). The details taxonomic classification is shown in the hierarchy below.

Kingdom: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Enterococcaceae

Genus: *Enterococcus*

1.2.2 Morphology

Gram-positive bacteria *Enterococcus* are facultative anaerobic species that can withstand temperatures as high as 60°C for short periods of time. Singles, pairs, or short chains of this species can be found, and the cells can be ovoid to coccobacillary in shape. In addition, the colony morphology on blood agar appears to be nonhemolytic or alpha haemolytic after 24 hours of incubation; rare strains may be beta haemolytic with diameter of 1 to 2 mm. Enterococci species were classified as group D streptococci before it has been regrouped into their genus as it can react with group D antiserum.

1.3 Epidemiology of VRE

1.3.1 VRE in human

VRE was first identified in 1986 in England and France, and it has since spread throughout the world as a major nosocomial pathogen (O'driscoll and Crank, 2015). Enterococci commonly infect immunosuppressed patients and to cause bacteraemia, endocarditis, infections of the abdomen and pelvis, infections of the urinary tract, and infections of the skin system (O'driscoll and Crank, 2015). From January to December 2003, according to data from the National Nosocomial Infection Surveillance (NNIS) (Cardo *et al*., 2004), it is reported to have more than 28% recovered vancomycin-resistant enterococci (VRE) in ICUs from more than 300 participant hospitals in United States. In Malaysia, the first case of hospital-acquired VRE was in 1996 at the University of Malaya Medical Centre (Riley *et al*., 1996). Paradoxically, it has been spread in the community setting as one of the community-acquired VRE (Raja *et al.*, 2005).

Later on, it was noted in one of the chronic renal failure patients from Kuala Lumpur Hospital in 2006 (Zubaidah *et al.*, 2006). The broaden and improper use of vancomycin was reported to become the root cause of VRE augmentation in the hospital setting. The overuse of avoparcin, which is one of the glycopeptide antibiotics is the main factor for the dispersion of VRE in the community setting. Avoparcin function as a growth promoter in the animal livestock which believes it could be postulated to human as a food source (Acar *et al*., 2000).

1.3.2 VRE in poultry

Following the incidence of VRE in hospitals and communities, Tan *et al.*, (2006) found a high prevalence of VRE isolated from chicken farms, which was found to be more than 44% in 2005, comparable to that of Getachew *et al*., (2009) who found that more than 40% of poultry samples identified VRE in 2006. While a study conducted by Ong *et al.*, (2002) and Radu *et al.*, (2001) was reported lower VRE occurrence in chicken meats from the markets of Malaysia which are 21.8% and 2% respectively. The origin of VRE is unknown, but the emergence of VRE in Malaysia was too believed to affiliate from the overuse of avoparcin and a feed supplements in livestock farms (Bach Knudsen, 2001) before it has been banned in Malaysia. Avoparcin use as a prophylactic agent in food-producing animals has also been related to rise in VRE in livestock (Bach Knudsen, 2001; Manson *et al.*, 2004; Nilsson, 2012). As a result of the latent spread of resistance across the food chain, a worldwide ban on the use of avoparcin in livestock feed was enacted (Bach Knudsen, 2001).

1.4 Pathogenicity

1.4.1 Vancomycin resistance enterococci (VRE)

VRE has emerged as an important nosocomial pathogen that specializes in biofilm formation which contributes to their virulence and antibiotic resistance (Hashem *et al.,* 2017). Increasing cases of VRE were reported by the rampant use of antibiotics, prolonged hospitalizations, and increased intensive care unit (ICU) admissions (Prematunge *et al.,* 2015). The following enterococcal virulence genes are coded for a protein that responsible for biofilm formation associated with the production of gelatinase (*gelE*), aggregation substance (*asa1*), capsule formation, and enterococcal surface protein (*esp*) (Maasjost *et al.,* 2019).Adherence, an early phase in biofilm

formation, enables bacteria to attach to a variety of medical devices such as urinary and IV catheters, which contributes to disease persistence by limiting antimicrobial penetration (Dautle *et al.,* 2003; Oli *et al.,* 2012).

The study by Tan *et al.,* 2018 reported of 99% and 57% of the *E. faecalis* and *E. faecium* consist of at least one of the virulence genes, notably *efa* was the most prevalent virulence detected in *E. faecalis* while *a`sa1* in the *E. faecium.* In the other hand, the use of certain medically important antimicrobials in animal feed is the main factor of dispersion and persistence of genetically diverse antimicrobial resistance enterococci harbouring a virulence factor. Those enterococci inhabit in broiler chickens and their environment could potentially contaminate the soil upon land application (Rehman *et al.,* 2018).

1.5 Antibiotic resistance in *Enterococcus*

Enterococci undoubtful less virulent compared to other common pathogens such as *Mycobacterium tuberculosis* and *Staphylococcus aureus*. However, the disease risk posed by these enterococci is compounded by their limited antibiotic tolerance, which is attributable to both intrinsic and acquired antibiotic resistance. Because of their natural inherent resistance, enterococci are well suited to acquiring additional resistances on mobile genetics components (Selleck *et al*., 2019). Nonetheless, this organism's resistance genes could be transferred to another virulent pathogenic bacteria, such as methicillin-resistant S*taphylococcus aureus* (MRSA) causing a series of infections in hospital around the world. Molecular techniques have been used to determine the virulence and pathogenicity of enterococci, and several genes (*agg, ace, cyILLS, esp, cpd, fsrB*) have been identified that encode for virulence factors like

gelatinase and hemolysin synthesis, adherence to caco-2 and hep-2 cells and their ability to form biofilms (Appelbaum', *et al*., 1986). Enterococci have been identified as one of the most common nosocomial pathogen in the US, accounting for more than 10% of hospital-acquired infections (Weiner *et al.,* 2016), and also discovered to be the cause of community-acquired endocarditis (Megran, 1992).

After β-lactams are no more effective towards *Enterococcus*, a combination therapy was applied and becoming the standard of care. The aminoglycosides were synergistic with β-lactams and capable of achieving a consistency treatment (Moellering and Weinberg, 1971). Just a few years later, aminoglycoside resistance in enterococci was discovered to be caused by plasmid borne resistance factor acquisition. Later, in the late 1970s and early 1980s, vancomycin was introduced and found to be effective against aminoglycoside resistant enterococci, making it the leading alternative therapy (Bisno *et al.*, 1989). However, the rapid emergence of vancomycin resistance enterococci at the turn of the twenty-first century raised concerns, as it signalled the loss of an essential bactericidal, the last line of defence. The first oxazolidinone antibiotic, linezolid, was approved by FDA in 2000 (Leach *et al*., 2011) and its use in clinical practice was soon followed by the advent of linezolid-resistant VRE strains in the United States in 2001 (Gonzales *et al*., 2001) and the United Kingdom in 2002 (Auckland *et al*., 2002).

Following this spike in antibiotic resistance, the glycylcycline tigecycline has been found to be useful in treating the VRE (Fiore *et al.*, 2019; Overbeek *et al*., 2005). It has been used to treat VRE infection effectively both alone and in combination with other antibiotics, but the resistance has arisen (Fiedler *et al.*, 2016). Nonetheless, enterococci tend to have developed resistance to all antimicrobial groups that have been used in clinical practice to date. Furthermore, enterococci's ability to survive for

long periods of time in specific environment has implications for the clonality of their infections, especially in the healthcare setting, where they are directly accessible to trigger person-to-person transmission (Chenoweth and Schaberg, 1990; Fiore *et al.*, 2019). Interaction with healthcare staff and inanimate items available in hospital setting most likely allowing the virus to spread (Porwancher *et al.*, 1997). Furthermore, the capacity to thrive in low-nutrient conditions increases the likelihood of infection and leads to nosocomial infections (Figdor *et al.*, 2003; Heim *et al.*, 2002).

1.6 Mechanism of glycopeptide resistance in enterococci

One of the most common nosocomial pathogens, Enterococci, is fitted with a variety of intrinsic antibiotic resistance genes and is intelligent enough to acquire new resistance and/or mutations (Hollenbeck and Rice, 2012). Nowadays, many kinds of research have been reported on how this genus of bacteria promptly conquer the resistance towards mostly powerful and newest antimicrobials. With the ability to acquire new genes, enterococci have a variety of mechanisms for transmitting DNA. One of the mechanisms involving pheromone-responsive plasmids is conjugation, which results in plasmid interchange across the species (Dunny *et al.*, 1995; Miller *et al.*, 2014). Vancomycin and teicoplanin are glycopeptides that prevent cell wall cross-linking by binding to the terminal "D-alanyl-D-alanine" of peptidoglycan precursors (Tian *et al.*, 2014; Xu *et al.*, 2010). As a consequence, acquired glycopeptide resistance is caused by changes in peptidoglycan precursors that are integrated into the cell wall, resulting in a lower binding affinity for vancomycin and teicoplanin, or both. Moreover, it is suggested that the changes in peptidoglycan precursor, "D-Alanyl-D-Alanine" depsipeptide was cause by the substitution to "D-alanyl-D -lactate" or to "D-

alanyl-D-serine" (Arduino and Murray, 1993; Arthur and Quintiliani R., 2001; Denyer *et al.,* 2004). Up until now, resistant of *Enterococcus* to the glycopeptide group are corresponds to eleven different *van* gene clusters based on DNA sequences and organization (Ahmed and Baptiste, 2018). The transition in the terminal amino acids of the peptidoglycan precursor from D-Alanine-D-Alanine to D-alanine-D-lactate or less often to D-alanine-D-serine causes it to be classified as high-level (MIC > 64 g/ml) or low-level (MIC: 4-32 g/ml) (Lebreton *et al.,* 2011). The level of resistance to glycopeptide is determined by the form of amino acid transition. Thus, the D-Alanine-D-Serine-ending precursor confers low-level resistance, while the transition of the terminal pentapeptide to D-Alanine-D-Lactate confers high-level resistance. Substitution into D- Alanine- D- Lactate eliminates one of the five hydrogen bonds needed for vancomycin to bind to the peptidoglycan chain, reducing its affinity by about 1000-fold, while substitution into D- Ala- D- Ser reduces the antibiotic's binding affinity by about sevenfold, leading to low level resistance (Miller *et al.,* 2014).

1.6.1 Resistance genes associated with VRE

Several genes have been linked to vancomycin resistance. It is a gene that encodes for a cell wall-forming enzyme. The *van*A, *van*B, *van*C, *van*D, *10ert*, *van*G, *van*L, *van*M, and *van*N are nine genes that have been identified in various enterococci species (Ahmed and Baptiste, 2018). These genes were distinguishable by their degree of resistance to glycopeptide, transferability, and inducibility. In addition, *van*A and *van*B genes are mainly found in *E. faecium* and *E. faecalis*, which are essential organisms. Because of their therapeutic significance, transferability capacity, high prevalence in clinical isolates, colonised organisms, and environmental materials, these species have sparked the most controversy in human health care (Ahmed and Baptiste, 2018). The two genes of *van*A and *van*B are important genes encoding to the high-level vancomycin-resistant with MIC > 64 µg/ml. Whereases, the *van*C gene was reported to low level resistant to vancomycin (MIC ≤8 µg/ml) which commonly be found in *E. casseliflavus*, *E. gallinarum* and *E. flavescens*. In the other hand, the *van*D operons are exclusively chromosomal and similar to *van*A and *van*B (Depardieu *et al.*, 2004). It represents a number of different combinations of mutations (Boyd *et al.*, 2004). However, the *van*D is described sparsely among the enterococci species and also can be carried by the *van*C *E. gallinarum* (Boyd *et al.*, 2006).

The virulence factors together with antibiotic resistance have been regarded as important determinants of bacterial colonization, establishment, and persistence of VRE in the hospital setting. Enterococcal surface protein (*esp*), hyaluronidase (*hyl*), and collagen-binding adhesin (*acm*), which are mainly carried by *E. faecium*, encode and are associated with colonisation, host invasion, persistence, biofilm development, and pathogenicity (Arshadi *et al.*, 2018). Table 1.1 shows the resistance genes of VRE and their phenotypic and genotypic characteristics.

Table 1.1 Resistance genes of VRE and their phenotypic and genotypic characteristics. Adapted from (Ahmed and Baptiste, 2018)

Van- operon	Common species associated with the genes	Level of resistance (vancomycin and teicoplanin)	Phenotypic expressions	Location and mobility
***van*A**	*E. faecium* *E. faecalis*	High for both	Inducible	Chromosome (transferable)
***van*B**	*E. faecalis* *E. faecium*	High-variable to vancomycin Susceptible to teicoplanin	Inducible	Chromosome (transferable)
***van*C**	*E. gallinarum* *E. casseliflavus* *E. flavescens*	Low to vancomycin Susceptible to teicoplanin	Constitutive Inducible	Chromosome
***van*D**	*E. faecium*	Low – high for both	Constitutive Inducible	Chromosome
***van*E**	*E. faecalis*	Low-moderate to vancomycin Susceptible to teicoplanin	Inducible	Chromosome
***van*G**	*E. faecalis*	Low to vancomycin Susceptible to teicoplanin	Inducible	Chromosome (transferable)
***van*L**	*E. faecalis*	Low to vancomycin Susceptible to teicoplanin	Inducible	Chromosome
***van*M**	*E. faecium*	High for both	Inducible	Unknown (transferable)
***van*N**	*E. faecium*	Low to vancomycin Susceptible to teicoplanin	Constitutive	Plasmid (transferable)

1.7 Isolation of *Enterococcus*

1.7.1 *Enterococcus* selective media

Enterococcus is a gram-positive, non-fastidious cocci that can be grown on a variety of media, including nutrient agar (NA), brain heart infusion broth (BHI), and *Enterococcus* agar selective media (Slanezt and Bartley Medium). A colony of *Enterococcus* appears in pink to red colour on their selective media with a 3-4 mm diameter.

1.7.2 Chromogenic culture media (CHROM Agar)

The chromogenic culture media are a form of culture medium that was developed to isolate, classify, and distinguish specific microorganisms from a diverse population. chromID VRE was the first chromogenic media to be established for the isolation of enterococci that confer acquired resistance to glycopeptides (Ledeboer *et al.*, 2007). This media had the advantage of being able to distinguish between *E. faecalis* and *E. faecium* (Perry, 2017). This is accomplished by incorporating chromogenic substrate, which the microorganisms use to generate distinct coloured colonies by detecting the presence of α-glucosidase and β-galactosidase (Orenga *et al.*, 2009). The substrate is a soluble colourless molecule which is the target of the microorganism's basic enzymatic activity and a chromophore. The chromophore is released when the microorganism's enzyme activity breaks the bond between the substrate and the chromophore. In its unconjugated state, the chromophore has a distinct colour and forms a precipitate that gives the specific colony a distinct colour. The advantages of using chromogenic culture media are less labour intensive and economic, less time consuming and easy identification.

1.8 Identification of *Enterococcus*

1.8.1 Conventional identification method

Microorganisms can be identified and characterised using a variety of techniques. Gram staining, culture, and biochemical tests are commonly used and have become the gold standard for preliminary identification of microorganism genus and species. On the other hand, genotypic methods such as polymerase chain reaction (PCR) also used together with conventional methods to increase the chances of detections. Despite the fact that conventional approaches are time-consuming and labour intensive, this routine has always been the preferred method for phenotypic detection of *Enterococcus* and other bacteria. This study involves a sample from the broiler's cloaca swab. The swabs were immersed in a bijou bottle containing 3 ml of Brain Heart Infusion broth plus 6.5% NaCl for 24 hours to grow microorganism presence from the cloaca part of the broiler.

1.8.1(a) Gram staining

Gram staining is one of the crucial and essential procedures to differentiate and classified between gram-positive and gram-negative bacteria. It is also important to determine the morphology of bacteria. The cell wall components of microorganisms were used as a principle in the gram staining procedure. Gram-positive bacteria have a thick cell wall made up of protein-sugar complexes, peptidoglycan, and little lipid. The thicker cell wall dehydrates and shrinks during the decolorization process, causing pores to close and preventing staining from entering the cell wall. Crystal violet-iodine complex bound to the thick peptidoglycan layer and resistant to ethanol decolorization. Gram-positive bacteria will appear blue or purple.

1.8.1(b) Catalase test

Catalase test plays an important role in primarily used to differentiate among gram-positive cocci. This test mainly used in this study to distinguish the genus of catalase-negative *Enterococcus* from catalase-positive staphylococcus. The catalase enzyme produces by microorganisms that grow in oxygenated environments play role in neutralize toxic forms of oxygen metabolites Hydrogen Peroxide, H_2O_2. Catalase enzyme act as a mediator in breaking down of H_2O_2 into oxygen and water, thus the oxygen bubbles produce indicates the inoculum of bacterial isolate mixed with H_2O_2 is catalase positive while no bubbles production indicating the catalase negative isolates. The test was tested to be 100% on their specificity and sensitivity by Anyanwu *et al.,* (2013).

1.8.1(c) Bile-esculin azide test

Enterococcus, formerly known as group D streptococci, was isolated and distinguished from non-group D streptococci using bile esculin with azide media. Esculin, ferric citrate to supply ferric ions, and 1% oxbile to suppress most other non-group D streptococci strains are all present in the media. Sodium azide is applied to the medium to inhibit gram-negative bacteria while esculin is broken down by group D streptococci to form dextrose and aesculetin. As this compound interacts with ferric ions in the medium, the colour changes from dark brown to black. The ability to hydrolyze esculin and resistance to bile suggest that the isolates belong to the *Enterococcus*, previously known as group D streptococci.

1.8.1(d) Pyrrolidonyl Aminopeptidase (PYR) disk test

PYR test is a method of colorimetric analysis for presumptive identification groups of bacteria which are *Enterococcus*, *Lactococcus*, and *Leuconostoc* based on the enzyme pyrolidonyl arylamidase. This test was carried out using the disk that L-pyroglutamic acid beta-naphthylamide is impregnated on it and works as the substrate for the detection of pyrolidonyl arylamidase.

1.8.1(e) Speciation of *Enterococcus* species

1.8.1(e)(i) Carbohydrate fermentation test

The carbohydrate fermentation test is used to assess bacteria's ability to ferment particular carbohydrates. The fermentation patterns of bacteria to a specific carbohydrate are useful to differentiate among its bacterial group or species. The principle is to test for acid and/or gas emitted during carbohydrate fermentation. The media used were incorporated with a 1% carbohydrate source such as mannitol, sorbitol, sorbose, inulin, arabinose, melibiose, sucrose, raffinose, trehalose, lactose, glycerol, salicin, or maltose (Facklam and Collins, 1989). Also present in the medium was a pH indicator, such as phenol red or bromocresol purple (BCP), which detects the medium's pH dropping due to acid development. The colour of the pH indicator would change if the pH dropped.

1.8.1(e)(ii) Production of pigment

After an overnight growth on tryptic soy agar, the pigmentation was observed. A cotton swab used to pick up growth from the tryptic soy agar plate turned yellowish in colour if the culture developed pigment (Facklam *et al.,* 1989). To distinguish this species from others, researchers look at the yellow pigment produced by *E. casseliflavus.*

1.8.2 Automated identification method

1.8.2(a) VITEK 2 System for identification

VITEK 2 compact (bioMerieux) is an automated device that uses a fluorogenic methodology for organism identification and a turbidity method for susceptibility testing with a 64-well card barcoded with details. The ID-GN (gram negative bacillus identification), ID-GP (gram positive cocci identification), AST-GN (gram negative susceptibility) and AST-GP (gram positive susceptibility) are some of the commercial kits available. The VITEK system began as an automated system for identification and AST in the 1970s and has advanced into the VITEK 2 system, which performs all of the steps needed for identification and AST automatically after a primary inoculum has been prepared and configured. By reading each test every 15 minutes, the VITEK 2 device allows for kinetic analysis (Ligozzi *et al.*, 2002). To monitor fluorescence, turbidity, and colorimetric signals, the optical device incorporates multichannel fluorimeter and photometer readings (Ligozzi *et al.,* 2002). Automated instruments have the advantage of improving workflow and providing quicker results than traditional methods.

1.8.3 Molecular identification method

1.8.3(a) Polymerase chain reaction (PCR)

Mullis first invented and patented the polymerase chain reaction (PCR) in 1983. This reaction is based on the use of DNA polymerase, which is used to replicate unique DNA sequences in vitro. This concept can produce tens of billions of DNA copies from an extracted DNA containing a sequence of interest, DNA of interest, or target DNA (Kadri, 2020). The technique is based on the replication of a double-stranded DNA template to obtain large quantities of unique DNA sequence from a DNA sample. The denaturing process, the hybridization phase with primers, and the elongation phase were all used to break down the double-stranded DNA (Lorenz, 2012). The PCR is performed in a reaction mixture that contains the DNA extract (template DNA), *Taq* polymerase, primers, and an excess of four deoxyribonucleoside triphosphate (dNTPs) in buffer solution (Agne *et al.,* 2009). DNA cloning for sequencing, gene cloning and manipulation, and pathogen detection in nucleic acid tests for infectious disease diagnosis are all examples of PCR applications.

1.8.3(b) Multiplex polymerase chain reaction (PCR)

Multiplex PCR is a method use in simultaneously detection of multiple targets in a single reaction tube, with a different pair of primers for each target. The multiplex PCR has been broadly applied in life science research, clinical diagnostics, and forensic laboratories. The most common uses of multiplex PCR include SNP genotyping, pathogen detection, mutation and polymorphism analysis and gene deletion analysis. As an extension to the practical use of PCR, this technique is time saving, cost effective, fewer pipetting error and increased accuracy of data normalization. For the multiplex PCR, the primers used must be selected carefully to have similar annealing

temperatures and must be not complementary to each other. In addition, the amplicon sizes should be different enough to form distinct bands when visualised by gel electrophoresis (Shen, 2019). Even though the use of multiplex PCR can reduce costs and time to simultaneously detect more than one target in a reaction, multiplex PCR is more complicated to develop and often is less sensitive than single-primer-set PCR. In the other hand, the advantage of multiplex PCR is that a set of primers can be used as internal control which can eliminate the possibility of false positives or negatives.

1.8.3(c) MALDI-TOF mass spectrometry

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a potential tool for microbial identification and diagnosis. The microbes were identified using either intact cells or cell extracts during this process. This method is based on the protein profile of microorganisms, which is a distinctive and unique molecular fingerprint of a species (Stepien-Pysniak *et al.,* 2017). This platform was not only used in clinical diagnostic as well increasingly used in the veterinary microbiological laboratory for rapid identification of microorganisms. Before MALDI-TOF MS was used to identify isolates, proteins were extracted and put in a MALDI Biotyper chamber for examination (Stepien-Pysniak *et al.,* 2017). The Ultraflextreme mass spectrometer and MALDI-Biotyper programme were used to automatically calculate the spectrum and compare it to reference spectra of bacteria. The MALDI Biotyper system's identification reliability was then expressed in points. A log(score) ≥2.0 indicate identification to the species level, and a log(score) ≥1.7 and <2.0 indicated identification to the genus level. (Stepien-Pysniak *et al.,* 2017; Stępień-Pyśniak *et al.*, 2021). MALDI-TOF MS is a useful tool for differentiating species that has a closely related without require numerous sample preparation steps but only

proteins extraction. It is less expensive and time-consuming than a gene sequencing method.

1.8.3(d) 16S rRNA gene sequencing

The 16S rRNA gene sequences have become the most common housekeeping genetic marker used for numerous studies including identification of bacterial phylogeny and taxonomy. The 16S rRNA gene (1500 bp) is large enough for informatics purposes, and it is found in almost all bacteria as part of a multigene family or operon. Furthermore, its function has not changed over time, and even spontaneous sequence changes within the gene have become a measure for evolution research (Patel, 2001; Janda and Abbott, 2007). Moreover, the 16S rRNA gene has the ability to identify genus and species for isolates that do not match known biochemical profiles and strains that are rarely associated with human infectious diseases (Winand *et al.,* 2020). Despite the growing importance of 16S rRNA gene sequence information in bacterial identification in clinical and public health settings, it may not be suitable in certain situations. To date, few studies indicate that 16S rRNA gene sequencing can identify genus in the vast majority of cases more than 90%, but not species lower than 83 percent(Drancourt *et al.,* 2000; Woo *et al.,* 2003; Mignard and Flandrois, 2006; Janda and Abbott, 2007) . However, the using 16S rRNA sequencing as a tool in species identification is still adaptable in most clinical laboratories.

1.9 Antimicrobial susceptibility test (AST)

1.9.1 Disk diffusion test

The Kirby- Bauer disc diffusion susceptibility test was created to assess the vulnerability or resistance of pathogenic aerobic and facultative anaerobic bacteria to different antimicrobial compounds in order to help clinicians choose treatment choices for patients and for researchers to study antibiotic resistance microorganisms (Hudzicki, 2016). The pathogenic organism is grown on Mueller-Hinton agar in their respective turbidity with the presence of antimicrobial disks. The capability of an antimicrobial compound to inhibit the pathogen is measured indirectly by the presence or absence of growth around the disc at 37°C for 18-24 hours (Liu *et al.,* 2012).

1.9.2 Epsilometer test (E-test)

E-test is a quantitative design that has been established to provide a direct quantification of antimicrobial susceptibility to microorganisms. It is an exponential gradient method to determine antimicrobial resistance and applied for dilution and diffusion of antibiotics into the medium. The E-test strips, which consist of a gradient concentration of antibiotics, can be used to assess the minimum inhibitory concentration (MIC) by observe at where the zone edge meets the strip.

1.9.3 Broth microdilution MIC

The semi quantitatively broth microdilution MIC is an *in-vitro* method using to measure the activity of an antimicrobial agent against a bacterial isolate. The first step in broth microdilution is to prepare a standardized number of test bacteria and inoculated in sterile plastic tray containing various concentrations of antimicrobial

agents. The MIC is determined by observing the lowest concentration of an antimicrobial agent which will inhibit visible growth of the bacterium after overnight incubation at 35°C (Garcia, 2010). MIC interpreted as susceptible, intermediate, or resistant, based on the criteria listed in the CLSI MIC standard. Normally, five to eight concentrations representing a therapeutically achievable range for each agent are tested for full-range MIC testing.

1.9.4 VITEK 2 System for antibiotic susceptibility test (AST)

VITEK 2 compact (bioMerieux) is an automated system uses a fluorogenic methodology for organism identification and turbidity method for susceptibility testing using a 64 well card that is barcoded with information on the card. The VITEK 2 AST cards provide AST results and resistance detection for clinically important Gram-positive cocci, Gram-negative bacilli and yeast. The system is proven to be rapid, reliable and accurate. It is widely used in the clinical setting for diagnostic purposes. The bacterial AST results are available in minimum of 4 hours while yeast AST results in 13 hours (VITEK® 2 AST Cards, 2018). The systems offer a number of antimicrobials and resistance tests such as ESBL, cefoxitin screen, high level aminoglycoside resistance, inducible clindamycin resistance, vancomycin resistance, etc.

1.10 Control and prevention of VRE

1.10.1 Healthcare setting

VRE known to be one of the significant nosocomial pathogens carried diverse multidrug-resistant genes. It is maintained by the selective pressure of widespread use of broad-spectrum antimicrobial drugs in the health care setting. Control and prevention are compulsorily needed in health care settings to avoid more problems arise.

To begin with, the healthcare infection control program should consider active VRE surveillance. It is because enterococci are resilient bacteria that can survive on the hands of health care workers and inanimate objects for months (5days- 4 months) (Noskin *et al*., 1995; Kramer *et al.,* 2006). Continuously disinfection of surfaces in a specific patient-care area is significant to reduce the risk of transmission of nosocomial pathogens from inanimate surfaces to susceptible patient. Moreover, it is dimly necessary to develop on-the-job training programs for the health care workers, a solid training in bacterial resistance and the prudent use of the antibiotics. In addition, developing point of care and rapid diagnostic tests, which can be used at the patient's bedside or in the doctor's office can be considered.

1.10.2 Animal husbandry

VRE can be categorized as one of the rising multidrug resistance bacteria that circles around animal husbandry cause by the overuse of avoparcin. Even though avoparcin has been banned years ago, the genes persist in a selective farm in a selective country. The World Health Organization has implied basis for the establishment of measures toward controlling antibiotic resistance (WHO, 2020). The measures should reduce the emergence and spread of antibiotic-resistance bacteria, improve the use of antimicrobials, establish effective surveillance systems, enforce legislation, and encourage the development of new drugs and vaccines.

To begin with, veterinary health care communities should establish and standardise a universal surveillance of antibiotic use, as well as continuously track the emergence and spread of new bacterial resistance strains. Secondly, it is compulsory by the policy makers and health authorities to stop the administration of antibiotics used in human medicine to the animals. It is imperative to only use and reserve the most important classes of antibiotics for humans. In addition, authorities could banish the use of antibiotics as growth promoters in animal feed in all countries. Thirdly, regulate the sale of antibiotics for use in human medicine and prohibit over the counter sales worldwide. This priority actions need to be done precisely to control and prevent dispersion of multidrug resistance bacteria in animal husbandry as proposedly by the Third World Healthcare Associated Infections Forum.

1.11 Rationale of the study

Enterococcus is indeed a normal flora of bacteria that mostly inhabit in humans and animals. However, the potential for the transfer of antimicrobial resistance from enteric bacteria in animals to humans is a global public health concern, especially in *Enterococcus* spp. It is therefore important to monitor antimicrobial resistance of zoonotic and commensal bacteria in animals. International surveillance has been conducted to monitor and track their susceptibility to medically important antibiotics such as The European Antimicrobial Susceptibility Surveillance in Animals (EASSA) (Jong *et al.*, 2018). This study is focused on the detection of VRE in poultry husbandry and their resistance genes as well as detection of resistance genes in archived VRE isolated from clinical samples. Antimicrobial surveillance in poultry husbandry is dimly necessary to prevent the widespread of multidrug-resistant organisms. Data provided in poultry samples can further be used for management and preventative measure of VRE and another antibiotic resistance organism in poultry husbandry and healthcare setting. Moreover, an early and consistency of VRE surveillance in animal husbandry are great potential to overcome the worst possible scenario in a public health as currently more reported cases of VRE and vancomycin susceptible *Enterococcus* (VSE) to get a resistant to the latest antibiotic used for *Enterococcus* infections. In the other hand, VRE also known to be one of the main causes of the hospital-acquired infection. An early detection of the genes is a great treatment strategies and control of antibiotic and antiseptic use in the health care setting. Therefore, this study has investigated the presence and distribution of the resistance genes presence in the clinical VRE isolates. Besides, it proves from this study for the efficiency of multiplex polymerase chain reaction (PCR) assay for the rapid detection of the genes simultaneously.