UNIVERSITI PUTRA MALAYSIA

ENVIRONMENTAL PERSISTENCE AND THE EFFICACY OF COMMONLY USED DISINFECTANTS ON VANCOMYCIN-RESISTANT ENTEROCOCCUS (VRE) ISOLATED FROM CHICKENS AND HUMANS

ADAMU ABUBAKAR YERIMA

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By

ADAMU ABUBAKAR YERIMA

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Masters of Veterinary Science

2012

DEDICATION

For my wife Aisha and daughters Sumayyah and Maryam;

I love you all.

Abstract of the thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment for the award of Masters of Veterinary Science.

ENVIRONMENTAL PERSISTENCE AND THE EFFICACY OF COMMONLY USED DISINFECTANTS ON VANCOMYCIN-RESISTANT *ENTEROCOCCUS* (VRE) ISOLATED FROM CHICKENS AND HUMANS

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ADAMU ABUBAKAR YERIMA

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Chairman : Latiffah Hassan, PhD

Faculty : Faculty of Veterinary Medicine

Vancomycin-resistant enterococci (VRE) are well-known ascendant nosocomial pathogens and farm animals have been implicated as the source and reservoir for VRE in humans. In Malaysia, VRE had been a great concern to poultry farmers that export their products to Singapore due to the restriction on export of only VRE-free poultry products. The present study was conducted to provide more understanding on the duration of survival of VRE in the Malaysian local weather and the factors that may affect its survival. The specific objectives of the study are: to ascertain the viability and sustainability of VRE isolates in the local Malaysian environment, to determine the factors that contributes to the survival of the isolates in the environment and to determine the efficacy of commonly used disinfectants available to farmers on the isolates.

Survival and surface experiment were performed using 10 isolates of VRE; *Enterococcus faecalis* (two each from human and chicken, one reference strain) and *Enterococcus faecium* (two each from human and chicken, one reference strain) carrying vancomycin resistance gene. In the first study, the abilities of the isolates to survive when dried on wooden surfaces in a typical Malaysian climate of high temperature and high relative humidity (RH) of $30^{o}C \pm 2^{o}C$ and 80% ± 10% RH and examined the influence of organic soiling on the survival of the isolates. The results revealed that all the isolates survived for at least 4 weeks when devoid of protective influences of substances such as protein (clean condition) and for a minimum of 8 weeks in the presence of organic soiling (soiled condition). After 4 weeks, the isolates survived with a colony count of $6.80 \times 10^{2} – 5.06 \times 10^{3}$ CFU/ml in clean condition and in a relatively higher colony counts of $1.00 \times 10^{3} – 2.02 \times 10^{4}$ CFU/ml in soiled condition. Four of the isolates survived till the end of the study (16 weeks). The isolates had an overall decay rate of -0.13 irrespective of source (human or chicken), condition (clean or soiled) and species (*E. faecalis* or *E. faecium*). The decay rates did not vary significantly by the source of organism ($p = 0.48$) but varies significantly between species ($p = 0.001$). *E. faecium* have a higher decay rate of -0.16 than -0.13 for *E. faecalis* in clean condition and the values of -0.12 and -0.11 in soiled condition. The decay rates also varies by condition ($p=0.001$) with the isolates showing decay rates of -0.144 in clean condition and -0.118 in soiled condition.This signifies that the presence of soiling as obtainable on farms significantly increases the survival time and number of surviving VRE.

In the second study, the effect of organic soiling on the disinfection procedure and the efficacy of in-use concentration of a few commonly used disinfectants (Lindores*-30®, Omnicide® and EcosTimsen®), was evaluated using the European surface test. All

disinfectants tested have an intermediate activities against the organism (ME consistently between 2 and 5) except for Omnicide® which exhibited low activity on stainless steel surface in soiled condition (MEs consistently between 0.5 and 3).In clean condition, Ecos Timsen® had the highest ME value of 5.29±1.44 while Omnicide® has the least ME value of 2.38±0.58for *E. faecalis*. On wooden surfaces, Omnicide® showed the highest ME value of 3.53±0.49 for *E. faecalis* with Lindores-*30® showing the least ME value of 2.84±0.24 for *E. faecium* in clean condition. In soiled condition, all tested disinfectants exhibited a significantly reduced activity with lower ME values. Omnicide® showed the highest value of 2.43±0.57 for *E. faecalis* and Ecos Timsen® have the least ME value of1.53±1.01 for *E. faecium* on stainless steel surface. On wooden surface, the highest ME value was observed when using Ecos Timsen® (2.27±0.58) for *E. faecium*. Lindores-*30® have the least value 1.11±0.49 for *E. faecium*. Therefore, this study signifies that the commonly used disinfectants do not altogether eliminate VRE from the farm environment especially in the presence organic soiling and that the rate of effectiveness of the disinfectant varies with type of surfaces where it is being applied.

This study has improved on the knowledge on the survival of VRE in the Malaysian climate of high relative humidity and temperatures. Furthermore the in-use concentration of some commonly used disinfectant does not altogether eliminate VRE from the farm environment especially in the presence of organic soiling which markedly reduced the activity of the disinfectants. The study has provided an insight that can be further enhanced in devising means by which VRE persistence in poultry farms can be mitigated.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Veterinar

KEMANDIRIAN ENTEROKOKUS RINTANG VANKOMISIN YANG DIPENCILKAN DARI AYAM DAN MANUSIA DALAM ALAM SEKITAR DAN KEBERKESANAN PEMBASMI KUMAN YANG BIASA DIGUNAKAN TERHADAPNYA

Oleh

ADAMU ABUBAKAR YERIMA

November 2011

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Enterokokus rintang vankomisin (VRE) sangat dikenali sebagai pathogen nosokomial yang hebat dan haiwan ternakan telah dikenalpasti menjadi punca dan takungan kepada VRE dalam manusia. Di Malaysia, VRE telah menjadi kebimbangan besar kepada penternak yang mengeksport produk mereka ke Singapura disebabkan sekatan ke atas eksport produk ayam yang tidak bebas VRE. Kajian ini telah dijalankan untuk memberi kefahaman yang lebih lanjut tentang tempoh kemandirian VRE dalam cuaca tempatan Malaysia dan juga faktor-faktor yang mempengaruhi daya ketahanannya. Objektif khusus kajian adalah seperti berikut: untuk menyiasat kemandirian dan kemampanan pencilan - pencilan VRE dalam persekitaran Malaysia, untuk menentukan faktor-faktor yang menyumbang kepada keupayaan untuk hidup pencilan – pencilan di

persekitaran dan untuk menentukan keberkesanan pembasmi kuman yang biasa digunakan oleh peladang – peladang untuk membasmi kuman ini.

Eksperimen keupayaan hidup dan permukaan dilakukan dengan menggunakan 10 pencilan VRE; *Enterococcus faecalis* (dua; setiap satunya diperoleh daripada manusia dan ayam serta satu strain rujukan) dan *Enterococcus faecium* (dua; setiap satunya diperoleh daripada manusia dan ayam serta satu strain rujukan) yang membawa gen rintang vankomisin. Dalam kajian yang pertama, kami membezakan keupayaan pencilan – pencilan untuk terus hidup apabila dikeringkan pada permukaan kayu dalam iklim Malaysia yang biasa yang bersuhu tinggi dengan kadar ketinggian kelembapan relatif (RH) $30^{o}C \pm 2^{o}C$ dan 80% ± 10% RH dan mengkaji pengaruh bahan organik terhadap keupayaan hidup pencilan - pencilan. Keputusan kajian ini mendedahkan bahawa semua pencilan terus hidup sekurang-kurangnya 4 minggu tanpa pengaruh perlindungan bahan-bahan seperti protein (keadaan bersih) dan sekurang-kurangnya 8 minggu dengan kehadiran bahan organik (keadaan kotor). Selepas 4 minggu, semua pencilan hidup dengan pengiraan koloni $6.80 \times 10^{2} – 5.06 \times 10^{3}$ CFU/ml dalam keadaan bersih dan secara relatifnya pengiraaan koloni agak tinggi iaitu pada $1.00 \times 10^{3} – 2.02 \times 10^{4}$ CFU/ml dalam keadaan kotor. Empat daripada pencilan mampu hidup hingga ke akhir kajian ini (16 minggu). Semua pencilan mempunyai kadar pereputan keseluruhan pada -0.13 tanpa mengira sumber (manusia atau ayam), keadaan (bersih atau kotor) dan spesies (*E. faecalis* atau *E. faecium*). Kadar pereputan tidak berbeza dengan ketara oleh sumber organisma ($p = 0.48$) tetapi berbeza dengan ketaranya antara spesies ($p = 0.001$). *E. faecium* mempunyai kadar pereputan -0.16 berbanding -0.13 untuk *E. faecalis* dalam keadaan bersih dan nilai-nilai -0.12 dan -0.11 dalam keadaan kotor. Kadar pereputan juga berbeza-beza mengikut keadaan ($p = 0.001$) dimana pencilan menunjukkan kadar pereputan -0.144 dalam keadaan bersih dan -0.118 dalam keadaan

kotor. Ini menunjukkan bahawa kehadiran bahan organik seperti yang ditemui di ladang dengan ketaranya meningkatkan masa keupayaan hidup dan bilangan pencilan VRE.

Dalam kajian kedua, yang kesan bahan organik terhadap prosedur pembasmian kuman dan keberkesanan penggunaan kepekatan tertentu sesetengah pembasmi kuman yang biasa digunakan (Lindores *- 30®, Omnicide®danEcosTimsen®), dinilai menggunakan ujian permukaan Eropah .Semua pembasmi kuman yang diuji mempunyai aktiviti – aktiviti yang sederhana terhadap organisma (ME konsisten antara 2 dan 5) kecuali Omnicide® yang mempamerkan aktiviti yang rendah pada permukaan keluli tahan karat dalam keadaan kotor (MES konsisten antara 0.5 dan 3). Dalam keadaan bersih, EcosTimsen®mempunyai nilai ME yang tertinggi iaitu 5.29 ± 1.44 manakala Omnicide® mempunyai nilai ME yang paling rendah iaitu 2.38 ± 0.58 untuk *E. faecalis*.Pada permukaan kayu, Omnicide® memberikan nilai ME yang tertinggi pada 3.53 ± 0.49 untuk *E. faecalis* dengan Lindores-*30® memberikan nilai ME yang paling rendah pada 2.84 ± 0.24 untuk *E. faecium* dalam keadaan bersih.Dalam keadaan yang kotor, semua pembasmi kuman yang diuji mempamerkan aktiviti yang lemah dengan ketara dengan nilai ME yang begitu rendah. Omnicide® menunjukkan nilai tertinggi 2.43 ± 0,57 untuk *E. faecalis* dan Ecos Timsen® memberikan nilai ME terendah pada 1.53 ± 1.01 untuk *E. faecium* pada permukaan keluli tahan karat. Di atas permukaan kayu, nilai ME tertinggi dicatatkan dengan penggunaan Ecos Timsen® (2.27 ± 0.58) untuk *E. faecium*.Lindores-*30® memberikan nilai yang paling rendah iaitu 1.11 ± 0.49 untuk *E. faecium*. Oleh yang demikian, kajian ini menunjukkan bahawa pembasmi - pembasmi kuman yang biasa digunakan tidak mampu menghapuskan VRE dengan sempurna dari persekitaran ladang terutamanya dengan kehadiran bahan organik dan kadar keberkesanan pembasmi kuman adalah berbeza bergantung pada jenis permukaan di mana ia digunakan.

Kajian ini telah meningkatkan pengetahuan terhadap keupayaan VRE untuk hidup dalam iklim Malaysia yang secara relatifnya mempunyai kelembapan dan suhu yang tinggi.Tambahan pula, penggunaan kepekatan sesetengah pembasmi kuman yang biasa digunakan tidak dapat menghapuskan VRE secara mutlak dari persekitaran ladang terutamanya dengan kehadiran bahan organik yang dengan jelasnya melemahkan aktiviti pembasmi kuman.Kajian ini telah memberikan kefahaman yang dapat meningkatkan pemikiran untuk mengurangkan beban VRE tegar dalam ladang ayam.

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I certify that a Thesis Examination Committee has met on 7th March, 2012 to conduct the final examination of Adamu Abubakar Yerima on his Masters of Veterinary Science thesis entitled "Environmental Persistence and Efficacy of Commonly used Disinfectants on Vancomycin-Resistant *Enterococcus* (VRE) Isolated from Chickens and Humans" in accordance with the Universities and University Colleges Act 1971 and the constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommended that the student be awarded Master of Veterinary Science.

Members of the Thesis Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of **Masters of Veterinary Science.** The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotation and citations which have been duly acknowledged. I also declare that it had not been previously, and is not currently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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ADAMU ABUBAKAR YERIMA

Date: 7th March, 2012

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AFNOR	Association Francaise de Normalisation
AGP	Antimicrobial Growth Promoter
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ASC	Active surveillance cultures
ATCC	American Type Culture Collection
BHI	Brain heart infusion
BSA	Bovine serum albumin
CC	Clonal Complex
CDC	Center for Disease Control and Prevention
CEN	Comité Européen de Normalisation (European Committee for Standardization)
CFU	Colony forming unit
CI	Confidence Interval
CV	Coefficient of variation
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Program
D&E	Dey and Engley
DF	Degree of freedom
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Service
ECDC	European Center for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic Acid
EST	European surface test
GLM	General linear model

ICU	Intensive care unit
ME	Microbicidal effect
MFT	Membrane filtration technique
MIC	Minimum Inhibition Concentration
mL	Millilitre
M-PCR	Multiplex-Polymerase chain reaction
NaCl	Sodium Chloride
NH_4Cl_3	Ammonia chloride
PBP	Penicillin binding proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PHMB	Polyhexamethylene Biguanide
PYR	Pyrrolidonyl -aryl-amidase
RAPD	Random Amplified Polymorphic DNA
RCR	Rolling circle
RH	Relative humidity
S & B	Slanetz and Bartley
SD	Standard Deviation
SE	Standard Error
TNTC	Too numerous to count
USA	United States of America
UV	Ultraviolet
VRC	Vancomycin-Resistant Cocci
VRE	Vancomycin-Resistant Enterococci
VRSA	Vancomycin-Resistant *Staphylococcus aureus*
VSE	Vancomycin-susceptible Enterococci
WHO	World Health Organization

µg	microgram
σ	Standard deviation
σ^2	Variance
η^2	Effect size

CHAPTER 1

INTRODUCTION

Enterococcus species are commensal bacteria which forms part of the normal microbiota of humans and animals. They are lactic acid bacteria which are very important in environmental, food and clinical microbiology (Goldstein *et al.,* 1983). In the late 1970s, enterococci began to appear as a common cause of nosocomial infection; this coincides with and is most probably due to increased usage of third generation cephalosporins to which enterococci are resistant to. Enterococci especially the vancomycin-resistant type are currently ascendant nosocomial pathogens (Cetinkaya*et al.,* 2000; Patel, 2003). Dozens of enterococcal species have been identified, but only two; *Enterococcusfaecalis* and *Enterococcus faecium* are recognized as the most important species because they account for the majority of clinical isolates and are capable of acquiring and disseminating resistance genes*vanA*and *vanB*(Cetinkaya*et al.,* 2000; Werner, *et al.,* 2008). The acquired resistance to vancomycin is associated with transmissible element transposons (Tn1546, Tn1547 and Tn1549/Tn5382) which encode for the vancomycin resistance. Inter-species transfer of resistance within the genus enterococci and to other bacteria is by direct acquisition of these elements or via plasmids (Weaver *et al.,* 2002.; Guardabassi and Dalsgaard, 2004; Launay *et al.,* 2005; Novais *et al.,* 2008; Werner, *et al.,* 2008).

Although the rest of the species; *E. gallinarum, E. durans, E. hirae, E. cassiflavus, E. avium, E. raffinosus* and *E. mundtii* also possess the ability to acquire the

*vanA*resistance, only *vanB* has been found in *E. gallinarum* and *E. durans*, These species account for only about 5% of all clinical isolates. *E. faecalis* account for the majority of clinical isolates (80-90%) and *E. faecium* account for the remaining 10-15% due to the fact that the majority of *vanA* and *VanB* genes are found predominantly in these species (Cetinkaya*et al.*, 2000; Willems, *et al.*, 2005; Lalitagauri, 2007; Willems and Bonten, 2007; Werner, *et al.*, 2008).

Vancomycin-resistant enterococci (VRE) is a name given to all species of bacteria of the genus *Enterococcus* that exhibits resistance to multiple antibiotics especially vancomycin. They were first isolated by Uttley *et al* in England in 1986, shortly thereafter in France and other parts of United Kingdom (Friden, 1993; Leclercq*et al.*, 1988; Uttley *et al.*, 1988). Since then VRE has spread worldwide and has been isolated from a wide range of sources in humans, animals and the environment. The importance of VRE as a hospital acquired pathogen is growing, currently *E. faecalis* and *E. faecium* are considered as 3rd to 4th most prevalent nosocomial pathogen (Werner, *et al.*, 2008).

The main mode of VRE transmission in nosocomial infections is the transient contamination of the hands of health care workers (Tornieporth*et al.*, 1996; Uttley*et al.*, 1988). Enterococci has the ability to remain viable in the environment for several days to weeks and has been recovered from the environment in about 7-30% of environmental cultures obtained from several outbreaks (Zervos*et al.*, 1987; Karanfil*et al.*, 1992;Boyce*et al.*, 1994; Yamaguchi*et al.*, 1994; Morris, *et al.*,1995; Tornieporth*et al.*, 1996). This makes the contaminated surfaces of the environment

as a plausible source through which VRE can be transmitted from infected or colonized individuals to non-infected/colonized persons or animals.

Currently in Malaysia, the presence of clinically important VRE isolates carrying the high resistance *vanA* and *vanB* genes in human, pigs and poultry has been confirmed (Getachew, *et al.,* 2008; Getachew, *et al.,* 2009; Getachew, *et al.,* 2010). These and other findings signify the potential role of the environment in dissemination, persistence, and transmission of vancomycin resistance both in human and animal populations as well as in other bacteria. Thus environmental contamination in general and environmental isolates of VRE in particular constitutes a possible important means of VRE persistence, transmission and endemicity in the community.

The emergence of VRE in animal husbandry is associated with the use of antibiotics as feed additives in animals (Bates 1997; Jensen*et al.,* 2008). The antimicrobials used in animals' husbandry have analogues in human health, thus making them capable of selecting for antibiotics resistance found in humans (Boerlin*et al.,* 2006). It is in this light that, animals and their environments serve as the human reservoirs of antimicrobial-resistant bacteria or resistance genes (Aarestrup*et al.,* 1996; Dowling*et al.,* 2006). The use of avoparcin as growth promoter in animals has been implicated as the main factor for the emergence of VRE outside the hospital environment in Europe (Bates, 1997).

In Malaysia, VRE occurrence in animals has been reported by several authors (Son*et al.,* 1999; Dahlia*et al.,* 2000; Radu*et al.,* 2001; Ong*et al.,* 2002; Ooi 2003; Shah-Majid*et al.,* 2004; Hassan*et al.,* 2006; Chan*et al.,* 2008). Specifically in poultry, varying degree of occurrence have been reported; Hassan *et al.,* (2006) reported a prevalence of 43% in broilers while both Ong *et al.,* (2002) and Radu *et al.,* (2001) recorded a low rate of different VRE species and the occurrence of the *vanA* and *vanC2/C3* genes in *Enterococcus species* isolated from poultry sources in wet markets. Ever since the link between VRE and poultry has been established, Malaysian farmers were under undue pressure by Singapore, the major importer of Malaysian poultry to produce VRE-free chickens and poultry products (Kamaruddin Mat Isa, Department of Veterinary Services (DVS), Putrajaya. pers. comm. July 06, 2007).

Enterococci are frequently found in the environment as they are constantly excreted by human and animals via faecal matter. The bacteria is resistant to physical and chemical stress and are a potent environmental contaminant because of their ability to survive and persist in the environment outside the intestine of their host for a long time (Kühn *et al.,* 2000). Several studies have indicated that vancomycin-resistant *Enterococcus faecium* have persisted in broiler farms in Europe despite the lack of selective pressure following the ban on avoparcin (Borgen *et al.,* 2000; Garcia-Migura *et al.,* 2005; Sorum *et al.,* 2006). Garcia-Migura *et al.,* (2007) found that the farm environment appear to act as the major reservoir for VRE persistence in poultry.

The main concerns associated with VRE are;

1. Infection with VRE is increasing worldwide and livestock has been reported as a one of the major source of the organism. Transmission of the organism to humans is believed to be due to consumption of contaminated animal products. Therefore, among Malaysian farmers, this organism is of concern since it may result in their products to be banned from being exported.

2. Environmental contamination with VRE is one of the most important means in which VRE colonized and persists in human and animal populations. The farm environment has been recognized as the main reservoir of VRE persistence in poultry.

3. In Malaysia, the presence of VRE was confirmed by previous studies which focused mainly on;
   a. detection of VRE [Son *et al.* (1999), Radu *et al.*, (2001), Ong *et al* (2002), Latiffah (2006), Dahila *et al*, (2005)]
   b. epidemiological investigation [Getachew (2010)]

4. Farmers are concerned on how they can eliminate VRE in their farm environments. Thus, there is a need to ascertain the survival of the isolates in the environment and ways in which they will be efficiently eliminated from the environment.

The specific objectives of the study were;

1. to ascertain the viability and sustainability of VRE isolates in the local Malaysian environment.
2. to determine the factors that contributes to the survival of the isolates in the environment.
3. to determine the efficacy of commonly used disinfectants available to farmers on the isolates.

In this study it was hypothesized that;

1. due to high ambient temperatures and relative humidity, the Malaysian VRE isolates will survive in higher numbers and for longer time than that reported in literatures.
2. commonly available disinfectants in in-use concentration will eliminate the organisms from the environment.

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