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CHARACTERIZATION OF Enterococcus faecium AND Enterococcus faecalis CLINICAL ISOLATES IN A MALAYSIAN HOSPITAL.

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CHARACTERIZATION OF *Enterococcus faecium* AND *Enterococcus faecalis* CLINICAL ISOLATES IN A MALAYSIAN HOSPITAL.

By

WENG POH LENG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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CHARACTERIZATION OF *Enterococcus faecium* AND *Enterococcus faecalis* CLINICAL ISOLATES IN A MALAYSIAN HOSPITAL.

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Chair: Assoc. Prof. Malina Osman, PhD

Faculty: Medicine and Health Sciences

Enterococcus faecium and *Enterococcus faecalis* have been well documented as ubiquitous, Gram-positive cocci, opportunistic nosocomial pathogens. In recent decades, they have been recognized as the primary agent for nosocomial infections in many countries worldwide. The aims of the study were to determine the molecular characterization of *E. faecium* and *E. faecalis* isolated from patients in a Malaysian hospital focused on enterococcal surface protein (*esp*) gene, biofilm formation, association of *esp* gene with antibiotic resistance and genetic relatedness amongst the isolates collected. Samples were collected from a local tertiary hospital from May 2009 to March 2010 and subjected to characterization via biochemical identification, antibiotic susceptibitilty tests and DNA extraction. PCR was performed for genotypic identification and *esp* gene detection and MLST of isolates. Association of biofilm with the presence of *esp* gene was also examined with crystal violet assay. Enzymatic digestion with *Sma*I and PFGE typing were used to determine genetic relatedness of some strains. In this study, *E. faecalis* (n=52) was found to be most

commonly isolated amongst 80 isolates followed by *E. faecium* (n=28). The higher resistance rates were exhibited by *E. faecium* in decreasing order: tazobactam-piperacillin (96.4%), ampicillin (92.9%), high-level gentamicin (89.3%) and penicillin (82.1%). Whereas *E. faecalis* demonstrated slightly lower resistance rates: high-level gentamicin (25.0%), penicillin (7.7%), ampicillin (1.9%) and tazobactam-piperacillin (1.9%) respectively. No vancomycin and teicoplanin resistant enterococci was found. The prevalence of *esp* gene was found higher in *E. faecium* (78.5%) compared to *E. faecalis* (46.2%). However, the prevalence of this gene was more predominantly found in isolates that were resistant to ampicillin (74.1%) and tazobactam-piperacillin (65.8%). Ampicillin–resistant strains and *esp* gene were strongly associated with the genetic clustering in clonal complex-17. A significant strength of relationship between *esp* gene and biofilm formation was strongly observed in *E. faecium* than in *E. faecalis*. Some isolates without the *esp* gene were also found to form biofilms and these findings suggest *esp* might play a significant role although multiple factors might also be involved apart from environmental conditions. PFGE typing revealed high genetic diversity of enterococcal isolates and no indication of outbreaks. Clonally related ST types (ST6, ST16, ST28, *E. faecalis*; ST17, ST 78, ST203, *E. faecium*), which are circulating globally and two new ST types (ST399, *E. faecalis*; ST601, *E. faecium*) were obtained via MLST. ST6, ST16, ST28 and ST179 of *E. faecalis* were documented and of particular concern ST6 is associated with clonal-complex 2, a representative of hospital adapted complexes and the most often reported amongst hospital isolates ST type. ST type of *E. faecium*: ST17, ST78 and ST203, have been widely linked to CC17, which is associated worldwide spread and hospital outbreaks. The conclusion drawn from this study is that *E. faecium* exhibited high resistance rate, which is expected and observed in other countries. Strong

association of *esp* gene with biofilm formation in *E. faecium* would suggest *esp* gene as a potential marker for line-associated infections. In addition, detection of several ST types in both species should prompt proper surveillance system to be carried out in the near future.

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

PERINCIAN *Enterococcus faecium* DAN *Enterococcus faecalis* ISOLAT KLINIKAL DARIPADA SATU HOSPITAL DI MALAYSIA.

Oleh

WENG POH LENG

Disember 2013

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Enterococcus faecium dan *Enterococcus faecalis* telah didokumentasikan sebagai bakteria Gram positif cocci yang lasak dan sentiasa ada di mana jua. Bakteria ini merupakan patogen nosokomial opportunis yang biasa diperolehi dan dalam beberapa dekad kebelakangan ini telah menjadi penyebab utama jangkitan nosokomial di pelbagai negara seluruh dunia. Objektif kajian ini adalah untuk menyiasat ciri-ciri molekular *E. faecium* dan *E. faecalis* yang diperoleh daripada pesakit di sebuah hospital di Malaysia yang fokuskan kepada gen enterococcal surface protein (*esp*), pembentukan biofilem, perhubungan antara gen *esp* dengan rintangan antibiotic dan perkaitan genetic antara sampel. Sampel dikutip daripada sebuah hospital tertier tempatan di Malaysia dari Mei 2009 sehingga Mac 2010 dan sampel diperiksa melalui pengesahan biokimia, ujian rintangan terhadap antibiotic dan pengesktrakan DNA. PCR juga dilakukan pada isolat untuk pengesahan genotipik dan gen *esp* serta MLST. Kaitan antara biofilem dan kehadiran gen *esp*

dikaji melalui cerakin kristal ungu (crystal violet assay). Pencernaan enzim dengan *Sma*I dan analisa PFGE digunakan untuk mengkaji perkaitan antara strain. Didapati *E. faecalis* (n=52) merupakan isolat yang paling sering ditemui daripada 80 isolat yang dikaji dan diikuti oleh *E. faecium* (n=28). Kadar rintangan antibiotik mengikut susunan menurun yang dipamerkan oleh *E. faecium* adalah: tazobactam-piperacillin (96.4%), ampicillin (92.9%), gentamicin aras tinggi (89.3%) and penicillin (82.1%). *E. faecalis* pula menunjukkan kadar rintangan yang lebih rendah iaitu: gentamicin aras tinggi (25.0%), penicillin (7.7%), ampicillin (1.9%) and tazobactam-piperacillin (1.9%) masing-masing. Tiada rintangan terhadap antibiotik vancomycin dan teicoplanin dijumpai di dalam enterococci. Prevalens gen *esp* didapati lebih tinggi pada *E. faecium* (78.5%) daripada *E. faecalis* (46.2%). Tetapi, prevalens gen ini didapati lebih banyak tertumpu di isolat yang rintang kepada ampicillin (74.1%) dan tazobactam-piperacillin (65.8%). Bakteria yang jenis rintang kepada ampicillin dan gen *esp* dikait rapat dengan penggugusan genetik pada klonal kompleks-17. Didapati bahawa terdapat hubungan yang ketara dan kuat antara gen *esp* dan pembentukan biofilem pada *E. faecium* berbanding dengan *E. faecalis*. Sesetengah isolat tanpa gen *esp* didapati boleh membenruk biofilem dan penemuan ini mencadangkan gen *esp* memainkan peranan yang penting, namun pelbagai faktor terlibat dalam proses pembentukan biofilem selain faktor persekitaran. Jenis ST (ST6, ST16, ST28, *E. faecalis*; ST17, ST 78, ST203, *E. faecium*) yang berhubung-kait secara klonal yang tersebar secara global, serta menemui dua jenis ST yang baru (ST399, *E. faecalis*; ST601, *E. faecium*) melalui MLST. ST6, ST16, ST28 dan ST179 daripada *E. faecalis* telah didokumentasikan di dalam pangkalan data MLST di mana ST6 dihubungkaitkan dengan klonal kompleks-2, iaitu wakil gugusan kompleks yang telah beradaptasi dengan hospital, dan merupakan jenis ST yang

paling kerap dilaporkan di kalangan isolat hospital. Dalam analisis MLST *E. faecium* pula, ST17, ST78 dan ST203 terkenal sebagai kumpulan utama klonal kompleks-17 yang dihubungkaitkan dengan penularan jangkitan dan peletusan di hospital. Kesimpulannya, daripada kajian ini, *E. faecium* menunjukkan kadar rintangan yang tinggi seperti mana dijangka dan diperhatikan di negara-negara lain. Hubungkait yang ketara antara gen *esp* dan pembentukkan biofilem mencadangkan gen *esp* mempunyai potensi sebagai penanda untuk jangkitan berkaitan saluran parenteral intravena. Tambahan pula, pengesanan beberapa jenis ST di dalam kedua-dua spesis sepatutnya mengingatkan supaya sistem pengawasan yang sepatutnya dilaksanakan pada masa akan datang.

I certify that a Thesis Examination Committee has met on **2nd December 2013** to conduct the final examination of **Weng Poh Leng** on her thesis entitled "**Characterization of *Enterococcus faecium* and *Enterococcus faecalis* clinical isolates in a Malaysian Hospital**" 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 recommends that the student be awarded the Master of Science.

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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 Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

Declaration by graduate student

I hereby confirmed that:

- this thesis is my original work
- quotations, illustrations and citations have been duly referenced
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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in Rule 41 in Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

%	percentage
bp	base pair
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
h	hour
kb	kilobase
kg	kilogram
min	minute
MLST	multi locus sequence typing
mm	milimetre
mM	milimolar
mg	miligram
ml	militre
ng	nanogram
nm	nanometer
°C	degree celcius
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RNA	ribonucleic acid
s	seconds
ST	sequence type
TE	Tris-EDTA
TBE	tris-borate EDTA

Taq	*Thermus aquaticus*
U	unit
µl	microlitre
µg	microgram
UV	ultra violet
V	volt
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

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Enterococci are mainly isolated from patients with bacteremia, urinary tract infections or soft skin tissue infections and many more. In Malaysia, resistant strains of enterococci specifically vancomycin-resistant enterococci (VRE) have been isolated from animal and food sources (Radu *et al.*, 2001). The first case of hospital-acquired VRE was reported in 1996 by Riley, Parasakthi and Teh (Riley *et al*., 1996).

Two main species have been identified related to the enterococcal infections in humans accounting for 75% and 25% prevalence such as *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) respectively (Shankar *et al*.,1999). Nosocomial infections caused by enterococci have been reported to demonstrate an ascending trend of high morbidity and mortality, with the incidence of bacteremia has increased as much as 77% in 1980s (Lautenbach, *et al.*, 1999). Enterococcal surface protein (*esp*) which was firstly discovered by Shankar *et al.* (1999) revealed this novel surface protein was a significant enrichment in infection-derived isolates. In a study conducted by Valdezate *et al*. (2009) the *esp* gene was likely shown to be present in *E. faecium* and *E. faecalis* related infections. These enriched *esp E. faecium* isolates were likely to be in the Clonal Complex-17 (CC17) group, a clonal complex group of which highly associated with hospital outbreaks and it is part of the worldwide vancomycin-resistant enterococci (VRE) epidemic clone (Khan *et al*., 2008).

Strain typing of bacterial pathogens with increased virulence and/or transmissibility and antibiotic resistance (example of *E. faecium*, Clonal Complex-17 group) not only raised concerns but also highlighted the necessity to have effective identification methods of these strains and track their spread (Enright and Spratt, 1999). The current investigations encompassing the interface of taxonomy, genetics, evolutionary and epidemiology with the use of molecular typing such as multi-locus sequence typing (MLST) was to illustrate the importance of genetic variability in a single microbial species. And the examination of either gene polymorphism or genome has implications for the identification of microbial genetic types (Belkum *et al.*, 2001).

There are still insufficient data to elucidate the current *E. faecium* and *E. faecalis* infections in Malaysian hospitals in comparison to other countries (AbadÍa-Patiño, 2010). Therefore the aims of this study were to determine the molecular characterisation of *E. faecium* and *E. faecalis* isolated from patients in a Malaysian hospital with special focus on enterococcal surface protein (*esp*) gene, biofilm formation, association of *esp* gene with antibiotic resistance and genetic relatedness amongst the isolates collected in the study.

1.2 General objectives

i) To determine the molecular characteristics of *Enterococcus faecium* and *Enterococcus faecalis* isolated from patients by Pulsed-Field Gel Electrophoresis and Multi Locus Sequence Typing in a Malaysian hospital.

1.3 Specific objectives

i) To investigate the demographic of isolates, prevalence of enterococcal related infections and part of the microbiological characteristics (antibiotic profiles) of enterococci isolated from patients with enterococci related infections.

ii) To determine the prevalence of *esp* gene (associated with antibiotic profiles) among the isolates.

iii) To examine the association between *esp* gene and biofilm formation.

iv) To study the genetic relatedness among the isolates.

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