UNIVERSITI PUTRA MALAYSIA

RELATIONSHIP BETWEEN VIRULANCE FACTOR AND TOXIN ANTITOXIN SYSTEM IN ENTEROCOCCUS

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By

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RELATIONSHIP BETWEEN VIRULANCE FACTOR AND TOXIN ANTITOXIN SYSTEM IN ENTEROCOCCUS

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September 2014

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Toxin-antitoxin system is a regulatory system where two sets of genes encodes the toxin and its corresponding antitoxin. There is no documented study that showed the association between virulence genes and TA system in *Enterococcus faecium* and*Enterococcus faecalis*.

For this purpose,the prevalence of TA systems in independently isolated clinical isolates of *E. faecium* and*E. faecalis*were determined, then the dominant TA systems were identified, different virulence genes in *E. faecium* and*E. faecalis*are surveyed and subsequently the level of expression of virulence and TA genes in normal and stress conditions were determined and finally their association with TA genes are defined.Remarkably, sequences for one particular TA pair, *mazEF*and *higBA*TA systems, were present on plasmid and chromosome of all *E. faecium* and*E. faecalis*strains, respectively. Additionally, toxin transcript expression levels were increased when bacteria were grown under stressful conditions. The *pilB, fms8, efaAfm* and *sgrA* are the most prevalent virulence genes in *E. faecium* and*E. faecalis.* These virulence genes were reported for first time in*E. Faecalis*, so this study demonstrated the importance of horizontal transformation in *E. faecium* and*E. Faecalis.* Peptide nucleic acid assay demonstrated with silencing of *mazF* by *anti-mazF PNA* a decrease of expression level of virulence genes were observed. The findings showed association between TA systems and virulence factor. The *mazEF* TA locion plasmidand *higBA* TA genes on chromosome of all *E. faecium* and*E. faecalis* were dominant. Also, there was decreasing of expression of virulence genes in presence of *anti mazF-PNA*. Therefore, it is recommended that *mazEF*
TA systems are potent and sensitive targets in of all E. faecium and E.

Faecalis.
Hence, deactivation of the toxin proteins in the *mazEF* TA system should be investigated further as an effective antibacterial strategy against this bacterium.

Keywords: Toxin antitoxin system, *mazEF*, *Enterococcus,* Peptide Nucleotide Acid.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluanuntuk Master Sains

HUBUNGAN ANTARA FAKTOR VIRULENCE DAN SISTEM ANTITOXIN TOKSIN DALAM BAKTERIA ENTEROCOCCUS

Oleh

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Sistem toksin-antitoksin merupakan system aturan dimana dua set gen mengkodkan kepada toksin dan antitoksinnya tersendiri. Tiada penyelidikan secara dokumentasi telah ditunjukkan tentang hubungan gen virulen dengan system TA di dalam *Enterococcus faecium* dan*Enterococcus faecalis*.
Oleh itu, bagi merealisasikan tujuan ini, kadar sistem TA telah ditentukan melalui klinikal sampel daripada *E. faecium* dan*E. faecalis*. Di samping itu, beberapa parameter dikaji dan ditentukan; iaitu dominan bagi system TA dikenalpasti, kepelbagaian virulen gen dalam *E. faecium* dan*E. faecalis* diukur seterusnya paras ekspresi gen virulen dan sistem TA dalam keadaan normal dan stress. Akhir sekali, perhubungan antara gen-gen TA ditentukan.Keputusan kajian amatlah memberangsangkan di mana jujukan bagi satu pasangan TA, *mazEF*dan *higBA*sistem TA, telah dijumpai dalam plasmid dan kromosom daripada kesemua strain *E. faecium* dan*E. faecalis***.** Tambahan pula, paras ekspresi toksin yang ditranskrip menunjukkan peningkatan apabila bacteria ditumbuhkan dalam keadaan stress. Beberapa virulen gen paling kerap dijumpai dalam*E. faecium* dan*E. faecalis*adalah *pilB, fms8, efa* dan *sgr*.Gene-gene virulen tersebut telah dilaporkan untuk pertama kalinya dalam *E. Faecalis*, oleh itu kajian ini menunjukkan kepentingan proses transformasi mendatar di dalam *E. faecium* dan*E. Faecalis*. Ujian peptid asid nukleik membuktikan dengan penyenyapan pada *mazF* oleh *anti-mazF PN*, paras ekspresi bagi virulen gen telah menurun.Penemuan ini menunjukkan hubungan antara sistem TA dengan faktor virulen.*mazEF* TA lokus pada plasmid dan gene TA *higBA* pada kromosom
daripada semua *E. faecium* dan*E. faecalis* adalah dominan. Berikutan dengan itu juga, terdapat penurunan ekspresi dalam gen virulen dengan kehadiran

anti mazF-PNA. Oleh itu, dicadangkan bahawa sistem TA adalah dan kuat dan sensitive terhadap target dalam semua *E. faecium* dan*E. Faecalis*. Dengan demikian, keberkesanan strategi terhadap bakteria melalui proses penyahaktif toksin protein dalam sistem TA mazEF perlu dikaji dengan lebih mendalam pada masa hadapan.

Keywords: Sistem toksin-antitoksin ,*mazEF*, *Enterococcus,* Peptide Nucleotide Acid.

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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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LIST OF ABBRIVATION

TA system	Toxin antitoxin system
DNA	Deoxyribonucleic acid
VRE	Vancomycin Resistant Enterococcus
RNA	Ribonucleic acid
PNA	Peptide Nucleotide Acid
PMO	Phosphorodiamidate Morpholino Oligomer
LNA	Locked Nucleic Acid
PSK	Post Segregation Killing
PCD	program cell's death
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase PCR
RT-qPCR	Real-Time quantitative PCR

CHAPTER 1

INTRODUCTION

1.1 Introduction

Toxin-antitoxin (TA) systems, first described in the mid 1980s, are regulatory loci that encode a toxin and its corresponding antitoxin. The toxin and antitoxin system could be a RNA or protein, but in all TA systems reported to date, the antitoxin is found to be unstable, and the toxin is stable (Van Melderen *et al.,* 2009).Toxin-antitoxin loci are often transferred by horizontal transformation and are more associated with pathogenic bacteria and most of them were found on plasmids containing antibiotic resistance(Mine *et al.,* 2009) and the virulence genes might harbor TA plasmids. The use of toxin has been proposed as a new approach for antimicrobial therapy in pathogenic bacteria (Mutschler & Meinhart, 2011). Antisense therapy, which is sequence dependent, silences a specific gene. The antisense components are analogue of mRNA; therefore, this technology is involved in the inhibition of gene expression. Many techniques are available for antisense therapy that use different RNA analogue, such as phosphorodiamidate morpholino oligomers (PMOs), locked nucleic acids (LNAs) and peptide nucleic acids (PNA). Among these, the properties of PNA make it particularly appropriate for antisense therapy in bacteria. This technique is applied for molecular bioengineering, therapeutic methods and antibiotics (Lee & Roth, 2003; Janson & Duringm, 2006; Rasmussen *et al.,* 2007).The *Enterococcus* is Gram positive, facultative anaerobic organisms. The most important diseases are caused by *Streptococcus pneumoniae, Staphylococcus aureus,* and *Enterococcus faecalis* in Gram-positive bacteria. This is due to firstly, the high rate of morbidity and mortality, secondly, their ability to resist antibiotics (Alekshun & Levy, 2007), and thirdly, because of the horizontal spread of virulence genes among them (Anderson & Seifert, 2011). *Enterococcus faecalis* and *E. faecium* are known to be the third and fourth most prevalent nosocomial pathogens worldwide (Bereket *et al.,* 2012).

1.2Statement of problem

The majority of clinical enterococcal infections are caused by *E. faecalis* and *E. faecium*. *Enterococcus faecalis* is considered to be more virulent however, *E.*

*faecium*is more likely to be resistant to antibiotics. Twenty years ago only 10% of the nosocomial enterococcal infections were caused by *E. faecium* (Willems & Van Schaik, 2009). Now about 40% of the enterococcal nosocomial infections worldwide are caused by *E. faecium* (Van Schaik & Willems, 2010). This ratio changed in favor of *E. faecium* in the US during the late 1990s and in Europe around the year 2000 (Treitman *et al.,* 2005). In the last two decades the emergence of enterococci as an important nosocomial pathogen has been increasingly documented. The relative proportions of *E. faecalis* and *E. faecium* in Norwegian blood culture isolates in 2008 were 4% and 1.4%, respectively. On the one hand, the pathogenesis of enterococcal infections is only partly understood. However, several adhesins, hemolysin, hyaluronidase, aggregation substances, gelatinase, and genes encoding pili are now considered possible virulence factors (Sava *et al.,* 2010). So far, at least 22 different genes, collectively called *fms* (*E. faecium* surface protein-encoding genes) are considered putative virulence factors in *Enterococcus*. Virulence factors encoded by *acmfm* (*fms8*), *hyl*, *espfm, sgrA* and *ecbA*, are most strongly associated with clinical lineages in *E.faecium* (Sillanpaa *et al.,* 2009). Despite this, virulence genes are more detected in *E.faecium* but based on blast sequence analysis, these virulence genes have a high rate of similarity and consequently prevalent in *E. faecalis.* There has been no study to survey the distribution of the current virulence genes in *E. faecalis.* However, TA system could be a potent target for antibiotic therapy. In theory, the activation of a toxin or inhibition of an antitoxin is an attractive strategy for antimicrobial therapy (DeNap & Hergenrother, 2005; Engelberg-Kulka *et al.,* 2004). Amitai and colleagues demonstrated that 5% of bacterial cells were viable and 95% were killed after toxin activation because the increased toxin could not be neutralized by the antitoxin. However, when co-expressing *mazE (*antitoxin) and neutralizing *mazF* (toxin), 85% of the cells were viable because the toxin was neutralized and inhibited by the antitoxin (Amitai *et al.,* 2004).However, the most important step for potency of TA system, as a target, is to identify a TA system that is prevalent in all pathogenic clinical strains and determine its functionality. While the analysis of TA system can be instructive, up to now, there is no information available on the prevalence and identity of TA systems in pathogenic *E.faecium* and *E. faecalis*. Therefore, it is necessary to study a TA system that is prevalent and transcribed in all clinical pathogenic *E.faecium* and *E. faecalis* and evaluate the TA system as a potent target in *E.faecium* and *E. faecalis.*

1.3 Statement of objectives

1.3.1 General objective

To study TA system as a target in pathogenic *E.faecium* and *E. faecalis.*

1.3.2 Specific Objectives:

1. To determine the prevalence of different TA systems, their location on plasmid or chromosome and the dominant TA system in clinical isolates of *E.faecium* and *E. faecalis.*

2. To identify the prevalence of different virulence genes in *E.faecium* and *E. faecalis.*

3. To evaluate the functionality of virulence factors and TA genes and identification of dominant TA system as a potent antimicrobial target in *E.faecium* and *E. faecalis.*

1.4 Statement of hypothesis

Based upon these data, it hypothesized that the identification of a dominant and functional TA system in *E.faecium* and *E. faecalis*would provide an association between pathogenicity of *E.faecium, E. faecalis* and TA system.

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