UNIVERSITI PUTRA MALAYSIA

DISTRIBUTION AND ANTIBIOTIC REGULATION OF axe-txe TOXINANTITOXIN SYSTEM IN Enterococcus faecium CLINICAL ISOLATES

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By

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Toxin-antitoxin (TA) system consists of a pair of genes which are a stable toxin and an unstable antitoxin. The unstable antitoxin is known to be able to neutralize the toxin by interferes with the lethal action of toxin. These global regulators are ubiquitously present in some bacteria and involve in many metabolic functions. Due to the diversity of TA system, this research aimed to determine the distribution of *axe-txe* system among *E. faecium*. Recent finding has postulated the association of epsilon/zeta (*ω-ε-ζ)* TA system and cell wall synthesis (Mutschler, Gebhardt, Shoeman, & Meinhart, 2011). In the meantime, penicillin is capable to disturb cell wall synthesis of bacteria. Thus, synergy action that has been postulated at the target site by these two macromolecules (antibiotic and TA systems) was determined in this study. Meanwhile, chloramphenicol action as translational inhibitor has been associated with *mazEF* TA system and this was well studied in *E. coli* (Sat et al., 2001). Thus, give an insight for potential antimicrobial target. Hence, this current research was conducted to observe the association between *axe-txe* TA system in *E. faecium* and whether the expression of these two genes (*axe-txe*) would be induced or de-activated by penicillin and chloramphenicol in *E. faecium*. Twenty *E. faecium* isolates was collected and identified the *axe-txe* genes in both plasmid and DNA. Isolates with *axe-txe* TA system were chosen for determining the MIC of penicillin and chloramphenicol by visual turbidity and colorimetric assay followed by the MBC value. The colony forming unit of treated and untreated cultures within given time intervals were calculated and compared for both penicillin-resistant and chloramphenicol-sensitive strains. In both conditions, all RNAs were extracted and cDNAs were synthesized. The expression level of *axe* and *txe* genes were evaluated by real-time quantitative PCR (RT-qPCR) and the C_T value obtained was calculated by using comparative C_T method. All tests were done in triplicate. Twenty *E. faecium* clinical isolates possess the *axe-txe* TA system on both plasmid and chromosome. The MIC and MBC value were determined and will be used for next objective. The MIC and

MBC values of chloramphenicol-sensitive *E. faecium* were 4 µg/ml and 32 µg/ml, respectively. Whereas, the *E. faecium* strain exhibited a resistance pattern towards penicillin with 256 µg/ml of MIC and 1024 µg/ml MBC. The expression level of *axe* and *txe* genes demonstrated the neutralizing effect to one another by forming a toxin-antitoxin complex. Under the stress conditions, the expression of *axe* gene within a given time interval was inhibited leaving the Txe toxin alone to react with intracellular target thus programmed cell death (PCD) was induced. The expression of Txe toxin in chloramphenicol-sensitive strain was gradually increased compared to penicillin-resistant strains. *E. faecium* showed a complex regulation of *axe-txe* TA system under stress conditions between penicillin-resistant and chloramphenicol-sensitive strains. The *axe-txe* TA system is functional and transcribed in both chromosome and plasmid in *E. faecium*. The regulatory mechanism of this TA system could be explored further for potential antimicrobial targets in this pathogenic bacterium in future.

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

TABURAN DAN REGULASI ANTIBIOTIK SISTEM *axe-txe* TOKSIN-ANTITOKSIN DI DALAM PENCILAN *Enterococcus faecium* KLINIKAL

Oleh

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Pengerusi : Profesor Madya Rukman Awang Hamat, PhD
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Sistem toksin-antitoksin (TA) terdiri daripada sepasang gen toksin yang stabil dan antitoksin yang tidak stabil. Antitoksin yang tidak stabil dapat meneutralkan toksin dengan mengganggu tindakan maut toksin. Regulator global ini boleh didapati dalam beberapa bakteria dan terlibat dalam banyak fungsi metabolik. Oleh kerana heterogenitas sistem TA, kajian ini bertujuan untuk menentukan taburan sistem *axe-txe* di *E. faecium*. Temuan terkini telah mengandaikan terdapat hubungan di antara sistem epsilon / zeta (ω-ε-ζ) TA dan sintesis dinding sel (Mutschler, Gebhardt, Shoeman, & Meinhart, 2011). Sementara itu, penisilin mampu mengganggu sintesis dinding sel bakteria. Oleh itu, tindakan sinergi yang telah diandaikan di tapak sasaran oleh kedua-dua makromolekul ini (sistem antibiotik dan TA) telah ditentukan dalam kajian ini. Sementara itu, tindakan chloramphenicol sebagai perencat translasi telah dikaitkan dengan sistem TA *mazEF* dan ini dikaji dengan baik dalam *E. coli* (Sat et al, 2001). Oleh itu, kajian ini memberikan gambaran untuk sasaran antimikrob yang berpotensi. Kajian ini juga bertujuan untuk menyiasat pola ekspresi gen sistem *axe-txe* TA di dalam *E. faecium* dalam keadaan normal dan tertekan (cabaran antibiotik). Dua puluh pencilan *E. faecium* dikumpul dan dikenal pasti gen *axe* dan *txe* di dalam plasmid dan DNA. Pencilan yang mempunyai sistem *axe-txe* TA dipilih untuk menentukan MIC di antara penisilin dan kloramfenikol oleh kekeruhan visual dan analisis kolorimetri diikuti dengan penentuan nilai MBC. Unit pembentuk koloni di antara kultur yang dirawat dan tidak dirawat dalam selang masa yang diberikan telah dikira dan dibandingkan di antara strain rintang penisilin dan strain sensitive kloramfenikol. Di dalam kedua-dua keadaan, semua RNA diekstrak dan cDNA disintesis. Tahap ekspresi gen *axe* dan *txe* telah dinilai dengan kaedah Real-Time Quantitative PCR (RT-qPCR) dan nilai C_T yang diperolehi dikira menggunakan kaedah perbandingan C_T. Tahap ekspresi sistem *axe-txe* TA dibandingkan di antara strain rintang penisilin dan strain sensitif kloramfenikol. Semua ujian telah dijalankan sebanyak tiga kali. Dua puluh pencilan *E. faecium* klinikal mempunyai sistem *axe-txe* TA pada

kedua-dua plasmid dan kromosom. Nilai MIC dan MBC dikaji dan akan digunakan untuk objektif seterusnya. Nilai-nilai MIC dan MBC bagi *E. faecium* yang sensitif terhadap kloramfenikol adalah 4 µg / ml dan 32 µg / ml masing-masing. Manakala, strain *E. faecium* menunjukkan pola ketahanan terhadap penisilin dengan 256 µg / ml MIC dan 1024 µg / ml MBC. Tahap ekspresi gen *axe* dan *txe* menunjukkan tindakan meneutralkan di antara satu sama lain dengan membentuk kompleks toksin-antitoksin. Di bawah keadaan tekanan, penghasilan ekspresi gen *axe* dalam selang masa yang ditetapkan telah dihalang dan meninggalkan toksin Txe sahaja untuk bertindak balas dengan target intraselular lalu mengaktifkan kematian sel terprogram (PCD). Ekspresi toksin Txe dalam strain sensitif chloramphenicol meningkat secara bertahap berbanding dengan strain rintang penisilin. *E. faecium* mempamerkan regulasi sistem *axe-txe* TA yang kompleks di dalam keadaan tekanan di antara strain rintang penisilin dan strain sensitif kloramfenikol. Sistem *axe-txe* TA mempunyai fungi dan hadir di dalam kedua-dua kromosom dan plasmid di dalam *E. faecium*. Mekanisme pengawalaturan sistem TA ini boleh diterokai dengan lebih lanjut untuk mengenal pasti sasaran antimikrobia yang berpotensi di dalam bakteria patogen ini pada masa akan datang.

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This thesis was submitted to the Senate of the 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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This is to confirm that:

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LIST OF ABBREVIATIONS

TA	Toxin-antitoxin
PCD	Programmed cell death
MIC	Minimal inhibitory concentration
MBC	Minimal bactericidal concentration
PBPs	Penicillin-binding proteins
CC17	Clonal complex-17
NNIS	National Nosocomial Infection Surveillance
SCOPE	Surveillance and Control of Pathogens of Epidemiological
CANWARD	Canadian Ward Surveillance Study
PCR	Polymerase chain reaction
CFU	Colony-forming unit
RT-qPCR	Real-time quantitative PCR
noRT	No reverse transcriptase
NTC	No template control

CHAPTER 1

INTRODUCTION

1.1 Research Background

Enterococcus spp. are gram-positive cocci, facultative anaerobic, catalase-negative and able to hydrolyze esculin in the presence of bile. Enterococci can be easily differentiated from other streptococci by the appearance of black color on bile esculin agar. Enterococci can survive under a harsh condition such as 6.5% of NaCl. This alone can distinguish enterococci from nonenterococci. Enterococci are considered as part of normal intestinal flora of human and animal but can behave as opportunistic pathogens in certain conditions such as in patients with indwelling catheters and immunocompromised statues (Higuita & Huycke, 2014). The most predominant enterococcal infections are caused by *Enterococcus faecalis* and *Enterococcus faecium*. However, recent evidence has shown that enterococci are responsible for nosocomial infections with the emergence of multidrug-resistant isolates especially in *E. faecium* (Cattoir & Leclercq, 2013). The emergences of multidrug-resistance strains are responsible for approximately 11.7% of nosocomial blood stream infections in the United States (Jones et al., 1997). In addition, *E. faecium* and *E. faecalis* are responsible for most of enterococcal infections such as intra-abdominal infections, endocarditis, urinary tract infections and bacteraemia. In 1995-1996, 59.6 % of nosocomial blood stream infections in the United States were caused by *E. faecalis*, followed by *E. faecium* with 19.4 % (Jones et al., 1997). Nosocomial enterococcal infections usually occur in serious ill patients who have been exposed to wide-spectrum antibiotics. In United States enterococci are the fourth most common cause of nosocomial infection (Salata, Donskey, & Fraser, 2016).

Mortality rates associated with enterococcal infections may exceed 50% in critically ill patients such as patient with solid tumors and transplant patients (DiazGranados, Zimmer, Mitchel, & Jernigan, 2005). The mortality rate for enterococcal bacteraemia at 30 days was 26% as reported in Denmark (Suppli et al., 2011). The mortality rate for patients with *E. faecium* bacteraemia was 36%, compared to 18% for patients with *E. faecalis* bacteraemia. Another case study in Canada found that the fatality rate was 22.8% and was higher for *E.faecium* infections (Billington et al., 2014).

Toxin-antitoxin (TA) systems are regulatory systems discovered in the bacteria that consist of a pair of genes coding for a toxin and its antitoxin. The toxin and its antitoxin are neutralizing one another by creating a toxin-antitoxin complex. The degradation of antitoxin would enable the toxin to trigger bacterial cell death or stasis. Moreover, TA systems have recently been associated in numerous cellular pathways including stress response, programmed cell death, starvation-

induced cell stasis, gene regulation, gene stabilization, virulence, persistence, biofilm formation and antiphage protection (Engelberg-Kulka, Amitai, Kolodkin-Gal, & Hazan, 2006).

TA systems are ubiquitous on mobile genetic elements. TA system have been detected in numerous pathogenic bacteria and associated with antibiotic resistance and virulence which contributes to its pathogenicity (Amato, Orman, & Brynildsen, 2013). One of the major roles of TA systems is to stabilize and maintain the plasmid population. This has been proved in the study on a *Shigella flexneri* virulence plasmid Pmysh6000, the *mvpA-mvpt* (*vapBC*) TA system stabilize and maintain the plasmid population (Sayeed, Reaves, Radnedge, & Austin, 2000). Another study also found the same function of *higBA* TA system in *Proteus vulgaris* (Hurley & Woychik, 2009). In addition, omission of *vapBC* homologues in nontypeable *Haemophilus influenzae* leads to great reduction of virulence in tissue and animal models for otitis media (Ren, Walker, & Daines, 2012).

The *axe-txe* system was first observed on the multidrug resistant pRUM plasmid found in a clinical isolate of *E. faecium* (Grady & Hayes, 2003). Initial analysis of *axe–txe* system has established its capabilities as a function TA system in which the expression of Txe would become toxic to cells, and Axe would alleviate Txe-induced toxicity, and *axe–txe* would increase plasmid maintenance (Grady & Hayes, 2003). It has also been presented that Txe is an endoribonuclease which able to cleave mRNA and thereby inhibits protein synthesis (Halvorsen, Williams, Bhimani, Billings, & Hergenrother, 2011). Three-component TA system (*ω-ε-ζ* system) and *axe-txe* system were discovered on several plasmids from a collection of 93 geographically and epidemiologically diverse strains of *E. faecium*. These strains revealed that 42 (45%) and 18 (19%) harboured genes for *axe-txe* and *ω-ε-ζ* respectively (Rosvoll et al., 2010). Due to the prevalence of the *axe–txe* genes on plasmids in enterococcal isolates, artificial activation of Txe (a toxin) could present an attractive antimicrobial strategy. In addition, molecular functions of few TA systems have similar target sites with antibiotics. For instance, *ω-ε-ζ* system targets the cell wall synthesis which is similar to the cellular target of penicillins (Wen, Behiels, & Devreese, 2014).

Programmed cell death (PCD) is defined as an active process of “cellular suicide” which is an essential mechanism in multicellular organisms. Traditionally, PCD is associated with eukaryotic multicellular organisms. However, recent data has interestingly documented that PCD systems in bacteria have evolved for the microorganisms to survive under an unfavorable condition (Sat et al., 2001). *mazEF* TA system was the first TA system in bacteria that has been associated with PCD. This TA system was located on chromosome of *Escherichia coli* (Aizenman, Engelberg-Kulka, & Glaser, 1996).

Thus, one of the aims of this current research was to determine the distribution of *axe-txe* TA system among *E. faecium* clinical isolates which has been reported to be commonly detected in this bacterium (Rosvoll et al., 2010). *E. faecium* exhibits high resistant patterns toward a wide spectrum of antibiotics. Thus, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of penicillin (intrinsic resistance) and chloramphenicol (sensitive) in *E. faecium* were investigated. Observation of these two antibiotics and *axe-txe* system at different time intervals among *E. faecium* strains was conducted by gene expression study.

Later, the expression of *axe-txe* system and antibiotics inducement will be associated briefly. The regulation of *axe-txe* TA system in *E. faecium* will be observed.

1.2 Research Problem

E. faecium has been recognized as pathogenic bacterium that is responsible for a wide spectrum of clinical diseases. TA systems are heterogeneous in nature in terms of its distribution and functionality. TA system has been distributed in both plasmid and chromosome of bacteria (Williams & Hergenrother, 2012). Due to this heterogeneity in terms of its distribution, this research aimed to determine the distribution of *axe-txe* system among *E. faecium*. Penicillin is capable in disrupting the cell wall synthesis of bacteria by binding to penicillin-binding proteins (PBPs) (Zapun, Contreras-martel, & Vernet, 2008). Recent finding has postulated the association of epsilon/zeta (*ω-ε-ζ)* TA system and cell wall synthesis. Synergy action has been postulated at the target site by these two macromolecules (antibiotic and TA systems). A recent study has demonstrated the action of zeta toxin PezT of *Streptococcus pneumoniae* which hindered cell wall synthesis and triggered autolysis in *E. coli* (Mutschler et al., 2011). Meanwhile, chloramphenicol action as translational inhibitor has been associated with *mazEF* TA system and this was well studied in *E. coli* (Sat et al., 2001). Hence, this current research was conducted to observe the association between *axe-txe* TA system in *E. faecium* and whether the expression of these two genes (*axe-txe*) would be induced or de-activated by penicillin and chloramphenicol in *E. faecium*. It is known that TA system also contributed to programmed cell death (PCD) in bacteria. In addition, the degradation of antitoxin would result in activation of bacterial cell death via the action of toxin to enable the bacterium to survive under harsh stressful conditions or to achieve effective cell death. Recent study showed that, *mazEF* system can mediated killing by antibiotics in *E. coli* (Sat et al., 2001). Thus, the current research could give an insight on the mechanism of regulation of *axe-txe* TA system in *E. faecium*.

1.3 Research Objectives

1.3.1 General Objective

To determine the distribution and regulation of *axe-txe* TA system in *E. faecium* clinical isolates.

1.3.2 Specific Objectives

- To determine the distribution *of axe-txe* TA system of *E. faecium* clinical isolates.
- To determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of penicillin and chloramphenicol in *E. faecium* clinical isolates.
- To investigate the pattern of gene expression of *axe-txe* TA system of *E. faecium* in normal and stress conditions (antibiotic challenge).

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