ESTABLISHMENT OF A REAL TIME RT-PCR ASSAY TO DETECT EXPRESSION OF SELECTED GENES FROM MULTIPLE-DRUG RESISTANT *ACINETOBACTER BAUMANNII*

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ABSTRACT

Acinetobacter baumannii has an important role in nosocomial infections. However, in addition to the respiratory infections caused by this bacterium it also causes other infections, such as, bacteremia, urinary tract infections, meningitis, eye and skin infections. Recently the antibiotic resistance of *A. baumannii* has increased in respect of almost all of the current antibiotics. The present study focuses on the expression of genes ABAYE0161 (hypothetical protein) and ABAYE3501 (general secretion pathway protein C) in the presence and absence of the antibiotic imipenem. These two genes were chosen from the previous studies concerning the effect of microgravity on the expression of *A. baumannii* genes. The multiple antibiotic resistant strain of *A. baumannii* was collected from a patient at University of Malaya Medical Centre (UMMC) in 1996. The identity of the tested strain was confirmed by sequencing and analysing of the 16S rDNA gene. The quality and quantity of the extracted RNA were checked out and the related cDNA was synthesized. Real time RT-PCR was done in the last step to evaluate the expression of the interested gene. In comparing our findings and the results from the microgravity study, we found that imipenem reduces the expression of the ABAYE0161 and ABAYE3501 genes, while microgravity has an inhibitory effect on ABAYE0161 and enhances ABAYE3501 gene expression.

ABSTRAK

Acinetobacter baumannii mempunyai peranan yang berkesan dalam jangkitan nosokomial. Walau bagaimanapun, tambahan kepada jangkitan pernafasan yang disebabkan oleh bakteria ini juga akan menyebabkan jangkitan lain , seperti, bakteremia , jangkitan saluran kencing , meningitis , mata dan jangkitan kulit. Baru-baru ini rintangan antibiotik *A. baumannii* telah meningkat kepada hampir semua antibiotik semasa. Kajian ini memberi tumpuan kepada gen ABAYE0161 (*hypothetical protein*) dan ABAYE3501 (rembesan laluan protein C) dengan kehadiran dan ketiadaan antibiotic imipenem . Kedua-dua gen telah dipilih daripada kajian sebelum ini mengenai kesan mikrograviti pada ungkapan gen *A.baumannii*. Pelbagai rintangan antibiotik *A. baumannii* telah dikumpul daripada pesakit Pusat Perubatan Universiti Malaya (PPUM) pada tahun 1996. Identiti yang diuji telah disahkan oleh penjujukan dan analisis daripada gen 16S rDNA . Kualiti dan kuantiti daripada RNA yang telah diekstrak diperiksa dan cDNA berkaitan telah disintesis. Masa nyata PCR telah dilakukan dalam langkah terakhir untuk menilai gen yang berminat. Perbandingan penemuan kami dan hasil kajian mikrograviti , kami telah mendapati bahawa imipenem mengurangkan kemunculan gen ABAYE0161 dan ABAYE3501, manakala mikrograviti mempunyai kesan yg melarang pada ABAYE0161 selain meningkatkan kesan ABAYE3501

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1 CHAPTER I: INTRODUCTION

1.1 Introduction

The genus *Acinetobacter* is a Gram-negative coccobacilli bacteria and classified in the *Moraxellaceae* family. Rather than *Acinetobacter baumannii, Acinetobacter haemolyticus, Acinetobacter lwoffi* and *Acinetobacte*r bereziniae are the other species of this family (Rossau *et al.*, 1991). *Acinetobacter* spp. not necessarily related to infections, but responsible for 1.4% of nosocomial infections in USA during the years 1971-1981. The species *Acinetobacter baumannii* is associated with the outbreak of nosocomial infection, especially in immunocompromised patients. This bacterium can cause pneumonia, bacteremia, urinary tract infections, meningitis, eye and skin infections. *A. baumannii* is capable of rapidly adapting to the hospital environment (García-Garmendia *et al.*, 2001). In addition, *A. baumanniii* has shown resistance to several antibiotics, including aminopenicillins, first- and second- generation cephalosporins and choloramphenicol, which are used to treat infections caused by Gram-negative bacteria (Dijkshoorn *et al.*, 2007; Karah *et al.*, 2011).

Infections caused by *A. baumanniii* occur through several virulence factors, such as outer membrane protein (OMP) (Choi *et al.*, 2005), Lipopoly saccharide (LPS) and the presence of extracellular compounds (Gordon & Wareham, 2010). However, the important roles of biofilm (Smith *et al.*, 2007) and pili (Lee *et al.*, 2006) in the heightening of infections by this bacterium have been proven.

Although the ABAYE3501 gene is coding GspC protein, which is part of the secretion pathway II and is responsible for the transduction of energy to GspD in the outer membrane, the exact function of the ABAYE0161 gene is unknown (Kanehisa & Goto, 2000; Kanehisa

et al., 2014). Recent study had been done on bacterial profile gene expression in the presence and absence of microgravity. This study concluded that ABAYE3501 and ABAYE0161 genes were up-regulated and down-regulated, respectively, in microgravity conditions. To continue this study, the expression of the selected genes have been investigated in the presence and absence of antibiotic.

1.2 Objective

1. To evaluate the expression level of ABAYE3501 and ABAYE0161 genes of *Acinetobacter baumanii* via real time RT-PCR..
2. To analyse the effect of imipenem on gene expression of ABAYE3501 and ABAYE0161 genes.

2 Chapter II: REVIEW OF RELATED LITERATURE

2.1 Morphological characteristics of *Acinetobacter*

The genus *Acinetobacter* comprises Gram-negative, aerobic and non-motile coccobacilli. The G+C content of DNA is 39 to 47 mol% and the optimal bacterial growth temperature is 20-30^{o}C. *Acinetobacter* strains can grow on a simple mineral medium and a growth factor is not required.

According to Bergey's Manual of Systematic Bacteriology, the genus *Acinetobacter* is classified in the *Neisseriaceae* family (Holt *et al.*, 1984). However, more recent taxonomic development has classified members of the genus in the *Moraxellaceae* family including *Moraxella, Acinetobacter, Psychrobacter* and related organisms (Rossau *et al.*, 1991).

Acinetobacter spp. are short, plump, rod shaped and often coccoid in the stationary phase. Colonies of *Acinetobacter* spp. are smooth, sometimes mucoid and pale yellow to whitish-grey in colour on agar media.

Acinetobacter baumannii is known to be the main species associated with nosocomial infections (Bouvet *et al.*, 1987). In one study, it was determined that out of 584 *Acinetobacter* strains that were isolated from 420 patients from 12 different hospitals during one year, 426 (72.9%) strains were *A. baumannii* (Seifert *et al.*, 1993).

2.2 Epidemiology

The frequency of nosocomial infection caused by *Acinetobacter* spp. is not exactly estimated since the isolation of this bacterium from the patient does not necessarily reflect infection (Struelens *et al.*, 1993). During the 10 years from 1971 to 1981, *Acinetobacter*

species caused 1.4% of the nosocomial infections in a university hospital in the USA (Larson, 1984). *A. baumannii* can be the cause of different types of infection, such as pneumonia, bacteremia, urinary tract infections, meningitis, and eye and skin infections as well as surgical wound infections.

Acinetobacter can count as micro flora of the skin and some studies have proposed that 25% of normal people carry *Acinetobacter* spp. on their skin as normal flora (Somerville *et al.*, 1970; Taplin *et al.*, 1963). The oral cavity and respiratory tract of healthy adults are among the other places that *Acinetobacters* can be found (Glew *et al.*, 1977; Rosenthal & Tager, 1975). The carriage rate of hospitalized patients is usually higher than non-hospitalised patients (S. Rosenthal, 1974). Studies have been dedicated to the presence of *Acinetobacter* spp. in the hospital environment, such as hospital floors and sink traps (S. L. Rosenthal, 1974).

2.3 Virulence factors

Acinetobacter baumannii is the cause of severe infections, especially in immunocompromised patients (Bergogne-Berezin & Towner, 1996). However, the exact mechanism of the pathogenicity of this bacterium is unknown. It is believed that certain features in this microorganism can promote the virulent effect. The outer membrane protein is an instance for these pathogenicity enhancers. Frequently, OmpA, which is present in the cell membrane of the bacteria, can interact with the host cell membrane causing cytoskeletal disarrangement through which *A. baumannii* will be enclosed by the host cell (Choi *et al.*, 2008).

Another outer membrane protein that may be involved in the pathogenesis of *A. baumannii* is Omp38. Based on a study, Omp38 releases some compounds that promote apoptosis of cells by disarrangement of the mitochondria (Choi *et al.*, 2005).

Lipopoly saccharide (LPS) is another virulence factor that plays an important role in the pathogenicity of *A. baumannii* (Luke *et al.*, 2010). LPS contains lipid A, inner core and O antigen. The most toxic portion of LPS is lipid A (Raetz & Whitfield, 2002).

The presence of extracellular compounds, such as the capsule (Jennifer A. Gaddy *et al.*, 2009) and pilus, form the biofilm (Tomaras *et al.*, 2003), which is a community of bacteria that forms on surfaces and is acknowledged to be a source of human infection (Loehfelm *et al.*, 2008). For forming biofilm, chemical cell signalling between cells is required. These signals allow bacteria to recognise neighbouring bacteria and also respond to environmental variations (Musk *et al.*, 2006).

Biofilm can increase the tolerance of bacteria against antibiotics and disinfected reagents; thus, antimicrobial resistance will be increased (Høiby *et al.*, 2010). In other words, antimicrobial agents penetrate the bacteria slowly and incompletely (Stewart & William Costerton, 2001). The steps involved in forming biofilm include the formation of a conditioning layer, bacterial adhesion, bacterial growth and biofilm expansion (Kokare *et al.*, 2009). The importance of certain factors in forming biofilm has been proven. Biofilm-associated proteins (bap) can be an instance of these genes. Research has indicated that bap increase the bacterial cell surface hydrophobicity, which can increase the adherence of bacteria to eukaryotic cells. Thus, bap are important for the colonization and hosting of infections (Brossard & Campagnari, 2011).

The usher-chaperone assembly system encoded by the *csu* gene is responsible for pili synthesis. This gene is considered as another requirement for the formation of biofilm (Jennifer A Gaddy & Actis, 2009). Recently, the association between the development of biofilm and a phenomenon called quorum sensing has been proven (Popat *et al.*, 2012). Quorum sensing is defined as the communication of bacteria with each other through the use of cell-signalling known as autoinducers. Autoinducers are certain compounds, such as acyl-

homoserine lactone (AHL), which interact with repressors and activators in DNA. Therefore, the production of certain genes including biofilm production genes would be under the control of these autoinducers.

2.4 Antibiotic resistance

A. baumannii could be resistant to numerous antibiotics, including penicillin, cephalosporin, carbapenem and cholamphenicol (Urban *et al.*, 2001). The resistance to antibiotics of *A. baumannii* can occur through a broad spectrum of β-lactamase enzymes that prevent the antibiotic action through the changes that occur in the penicillin-binding protein (PBP) or via a reduction in the gene expression of the outer membrane protein or the expression of multidrug efflux pumps. In the resistance of *A. baumannii,* some genetic elements, such as bacterial plasmids and transposons, are involved (Roca *et al.*, 2012).

2.4.1 β- lactamases

The four-atom ring of B-lactam antibiotics is the main target for β-lactamase enzymes, such as penicillins, cephalosporins and carbapenem. Inactivation of β-lactam antibiotics occurs through hydrolysis of the β-lactam ring via β-lactamase enzymes. Extended spectrum beta-lactamases (ESBLs) or class A beta-lactamase contain *veb-1, tem-92, tem-116, shv-12, per-1* and *per-2*, which involve genes in *A. baumannii* that are resistant against antibiotics (Huang *et al.*, 2004 ; Naiemi *et al.*, 2005; Yong *et al.*, 2003). Metallo-beta-lactamases (MBLs) constitute another class of β-lactamases used against *A. baumannii,* especially carbapenem. In the structure of this enzyme, a metal ion, which is usually zinc, exists and participates in the catalysis. A cluster of genes is involved, including *imp, vim* and *sim* (T. R. Walsh, 2005; Timothy R. Walsh *et al.*, 2005)

Class D OXA beta-lactamases constitute another type of enzyme that is involved in the antibiotic resistance of *A. baumannii*. The importance of this class concerns OXA beta-

lactamases that inactivate carbapenem. The genes involved that contribute to this class of enzyme are *oxa-23, oxa-24* and *oxa-58* (Brown & Amyes, 2006; Coelho *et al.*, 2006; Jeon *et al.*, 2005; Marque *et al.*, 2005; Walther-Rasmussen & Høiby, 2006), which are encoded by plasmid. On the other hand, *oxa-51/69*-like beta-lactamase are "naturally occurring" in *A. baumannii* with a chromosomal source. The multidrug efflux pump and changes in the outer member protein (OMP) are the non-enzymatic mechanisms for the resistance of *A. baumannii*. Resistance through the OMP occurs by decreasing the permeability for antibiotics; normally, antibiotics pass through the pores present in the cell membrane. When the number of pores decreases, the entrance of antibiotics will also decrease. Studies have shown that proteins sized 22, 33, 37, 44 and 47 KDa cause expression reduction in antibiotic resistant species. OprD, which is homologous with 43KDa protein, is associated with imipenem resistance. CarO, a 29KDa protein, is also known to be involved in imipenem resistance *A. baumannii* (Dupont *et al.*, 2005; Limansky *et al.*, 2002; Mussi *et al.*, 2005; Siroy *et al.*, 2005).

2.4.2 Efflux pump

The efflux pump is another mechanism against antibacterial compounds. This pump transports toxic compounds to the outside of bacteria. Different types of efflux pump are determined. The AdeABC efflux pump is known to be one of the most important efflux pumps and plays an important role in the antibiotic resistance of *A. baumannii (Magnet et al., 2001)*. It is known that point mutation in the *adeR* and *adeS* genes (Marchand *et al.*, 2004) involved in AdeABC increases the expression, and, consequently, increases the efflux. However, AbeM is known to be another efflux pump concerned with the multidrug resistance of *A. baumannii* (Su *et al.*, 2005).

2.5 Genetic

Acinetobacter baumannii contains a double stranded, circular chromosome, which is usually accompanied by two plasmids (pACICU1 and pACICU2) (Iacono *et al.*, 2008). The genome contains 3,946,442 bp including 3830 opening reading frames. Putative alien islands encompass 17.2% of the opening reading frames, which help the ability of *Acinetobacter baumannii* to incorporate foreign DNA into its genome (Michael G. Smith *et al.*, 2007).

Similar to the other bacteria, *Acinetobacter baumannii* has several genes, such as *pilQ*, *comP* and *pilF*, which enable it to pick up foreign DNA from the environment. Interestingly, the genome of *Acinetobacter baumannii* lacks a gene for *comP*, a gene for the membrane transporter that is important for foreign DNA uptake in many species. Nonetheless, the *comEA* gene of *A. baumannii* codes for a transmembrane protein that can bind to foreign DNA in the environment and transport it to the internal environment of the cell where it can be incorporated into the genome. One study has suggested that the *comEA* gene or possibly even the type IV pilus of *A. baumannii* allows it to effectively obtain foreign DNA from the environment, thus eliminating the need for the *comP* gene found in many other species (Michael G. Smith *et al.*, 2007).

Foreign DNA obtained by *A. baumannii* for integration into its genome. These foreign genes located on the putative alien islands (pAs). These areas distinguish *A. baumannii* from the other species of *Acinetobacter.* Preserving pAs from the environment or other microorganisms within *A. baumannii* might be responsible for the virulence and pathogenic factors and considered as PAIs. Of the 28 pAs in *A. baumannii,* 12 share a significant sequence identity with virulence genes in other species of bacteria. The largest size of the PAIs is a133,740 base pair, which contains several genes, such as transposons, integrases and genes with the homology sequence of Legionella/Coxiella. Type IV virulence/secretion apparatus and seven out of the twelve PAIs contain genes that encode proteins or efflux

pumps that confer drug resistance to *A. baumannii*. In addition, PAIs carry out virulence genes. pAs gained from the other bacterium consist of genes for heavy metal resistance, iron metabolism, quorum sensing capabilities, lipid metabolism, amino acid uptake, and genes for the breakdown of xenobiotics (Michael G. Smith *et al.*, 2007).

2.6 General secretion pathway

Most of the studies on *A. baumannii,* have focused on the drug resistant genes (Barbe *et al.*, 2004; Busch *et al.*, 1999; Fournier *et al.*, 2006; Herzberg *et al.*, 2000; Link *et al.*, 1998). The factors that influence other genes of this bacteria are still unknown. The proposed study focuses on genes based on the previous study concerning the drug resistance of *A. baumannii,* which was sent to space and used as a model microorganism for growth in a microgravity environment (Pang, 2012) . The results from the microarray show that 190 genes were up-regulated from the total of 282 obtained for the ground control and 92 genes were down-regulated. On the other hand, the samples obtained from space showed that 292 genes were expressed differently in response to antibiotics under space conditions. Of these, 227 genes were up-regulated and 65 genes were down-regulated. ABAYE3501 (general secretion pathway protein gene) was highly up-regulated in space and ABAYE0161 (hypothetical protein) was highly down-regulated in space. Approximately 20% of the proteins produced by bacteria are located completely or partially outside of the cell (Pugsley, 1993). These polypeptides reach their destination by intermediating general secretion pathways. In gram-positive bacteria, transportation is a single step since gram-positive bacteria do not have a periplasmic membrane. Therefore, transportation in gram-positive bacteria is limited to just passing the polypeptides from the cytoplasmic membrane. In contrast, Gram-negative bacteria proteins are released into the periplasmic membrane or integrate into and are

transported across the outer membrane by one of several terminal branches of GSP (Pugsley, 1993).

The five soluble protein secretion pathways in Gram-negative bacteria are:

Type I or ATP-binding cassette (ABC): This type of exporter is widely used by Gram-negative bacteria. Toxins, protease and lipases are usually transported by this type of protein secretion pathway. The ABC system is able to transport a variety of molecules from toxic compounds and antibiotics. The Type I pathway is Sec independent and secretes proteins directly from the cytoplasm across the OM without a periplasmic intermediate. The compound that passes through this pathway possesses a carboxy-terminal amino acid secretion signals. The apparatus exports by intermediating three types of protein: ABC exporter located in the inner membrane; membrane fusion protein (MFP), which spans through the periplasm; and the outer membrane protein (Binet *et al.*, 1997).

Type II: This pathway possesses two steps. First, the translocase exports proteins to periplasmic space, which are respectively transported across the outer membrane by intermediation of the secretion apparatus. This pathway requires 12-16 accessory proteins (Desvaux *et al.*, 2004). The type II secretion pathway is responsible for secretion of the extra-cellular enzymes and toxins (Russel, 1998). The proteins involved in this pathway contain GspsD and GspS in the outer membrane. GspD is an integral outer membrane protein and GspS is a small lipoprotein that is required in some of the type II systems for proper targeting and insertion of GspD in the outer membrane (Nouwen *et al.*, 1999). In the inner membrane GspG,H,I and J are processed by GspO (Strom *et al.*, 1991). GspC, F, K, L, M and N are also present in the inner membrane and have extensive periplasmic domains. GspE interacts with GspL and is located in the inner membrane (Possot & Pugsley, 1997). This cytoplasmic protein has autokinase activity. GspE may regulate secretion or energize the secretion process and the assembly of the secretion (Py *et al.*, 1999).

GspC spans the periplasmic space and is swallowed in both the outer membrane and inner membrane. This protein might be responsible for energy transduction to GspD in the outer membrane (Bleves *et al.*, 1999). In *A. baumannii,* the gene that is responsible for synthesising the GspC protein is ABAYE3501 (Kanehisa & Goto, 2000; Kanehisa *et al.*, 2014).

Type III: This system contains complex proteins that span both membranes of the bacteria. Almost 20 compounds collaborate in this system. Some of these proteins act as a syringe and direct proteins to the host eukaryotic cells (Galán & Bliska, 1996). In order to export proteins by using type III secretion, the proteins should be carried by two amino terminal secretion signals. One signal may target the RNA-ribosome complex. The second signal will bind to the cytoplasmic chaperones (Cheng & Schneewind, 1999).

Type IV: This secretion pathway is especially for the conjugative transfer of DNA. Nucleoprotein DNA conjugation intermediates multi-subunit toxins, and monomeric proteins are allowed to transfer by this system. Proteins secreted by this pathway can be secreted either into the extracellular milieu or directly into the host cell (Christie, 2001).

Type V: This system is responsible for presenting outer membrane porins to the outside of the bacteria. Proteins are passed through the outer membrane via a transmembrane pore formed by a self-encoded β-barrel structure. Type Va is used for autotransporters, type Vb corresponds to the two-partner secretion system and type Vc to the Oca (oligomeric coiled-coils adhesin) family (Roggenkamp *et al.*, 2003).

2.7 Quantitative Reverse Transcriptase PCR (q-RT-PCR)

Quantitative Reverse Transcriptase PCR (q-RT-PCR) is a method that can be used to determine the gene expression level. In qRT-PCR, the accumulated amplicon is detected

during the reaction. In real time PCR, the RNA level is measured at the exponential phase rather than the end-point plateau (Wong & Medrano, 2005). Through the q-RT-PCR method, the increase in fluorescent signals is directly proportional to the number of generated amplicons. The small differences in gene expression between samples can be detected by q-RT-PCR for which a smaller amount of the mRNA template is required (Wong & Medrano, 2005).

The quantification of mRNA by q-RT-PCR can be achieved by one of the following methods. The first is the one-step method in which the whole reaction from cDNA synthesis up to PCR amplification is carried out in one tube, while in the two-step method, as the second procedure for synthesis of cDNA and amplification, two different tubes are used, which allows the storage of cDNA for a longer time (Wong & Medrano, 2005). Therefore, in this study, the two-step PCR was used.

In both above-mentioned methods, quantification can be either absolute or relative. In absolute quantification, a standard curve is generated by preparing serial dilution of a known concentration of the positive control and the concentration of the unknown sample is determined. In relative quantification, the variation in gene expression is measured based on the standard or reference sample (Livak & Schmittgen, 2001).

Fluorescent dye is used in the q-RT- PCR method to detect the amplified product. Two types of chemistry are used as the detector using the Sequence Detection System (SDS) instrument as follows:

SYBR Green I dye chemistry: This dye binds to the double-strand DNA and emits fluorescence. This is the cheapest method for q-RT-PCR and cannot differentiate between the desired and undesired product.

The TaqMan system as the second chemistry (known as “fluorogenic 5′ nuclease chemistry”) is a gold standard for real-time PCR. In this method, three sets of primers are

used including two sets for amplification. The third set is specific for the internal product, and contains a reporter and quencher as fluorescent compounds. All these three sets of primer come together in one vial in which the exonuclease activity of the Taq polymerase cleaves the third set. In TaqMan assay each PCR reaction contains exponential, linear and plateau phases. In the first 10-15 cycles, the fluorescence emission does not rise above the background. Data collection and analyses are carried out during the exponential phase in which the product is accumulated and the reaction is very specific. While in the linear phase, the reaction is slow and most compounds are consumed, in the plateau phase, the reaction stops and no more product is produced (Bustin, 2000).

Standard curve drawing

The real-time PCR data from the standard curve plots can be used to identify if inhibition occurs in the RNA. The standard curve is also used to examine PCR efficiency. The slope of the standard curve is commonly used to estimate the PCR amplification efficiency of a real-time PCR reaction. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of CT (cycle threshold) value vs. log of input nucleic acid.Thus, purified RNA that qualifies for real time PCR must contain less than 0.005% of genomic DNA by weight. It should be free of inhibitors for reverse transcription and PCR reactions with solubility in a PCR-compatible buffer. Indeed, it must be non-denatured and RNase free.

3 CHAPTER III: MATERIALS AND METHODS

3.1 Bacterial culture

Acinetobacter baumannii is considered as an importantnosocomial infectious agent, especially in ICU wards, over the last decade. This bacterium has become resistant to several antibiotics, such as aminopenicillins, first and second-generation cephalosporin and choloramphenicol. Recent study in our lab, based on the effect of gravity on the gene expression of *A. baumannii* in space, indicated that two genes; namely, ABAYE 3501 and ABAYE0161, were highly up-regulated and down-regulated, respectively. The present study demonstrated the gene expression of these two genes in the presence and absence of imipenem in imipenem-resistant *A. baumannii*. *Acinetobacter baumannii* M26 23-24, which is an imipenem resistant strain, was used in this study and the antibiotic susceptibility of this strain of bacterium was previously confirmed. MIC level of this bacteria is 32mg/l. The strain was initially isolated from the skin of a patient treated at the University of Malaya Medical Centre (UMMC), in 1996 (Misbah *et al.*, 2005a). This strain was used in microgravity study and effect of gravity has evaluated on gene expression profile. Bacteria had been cultured on Mueller Hinton (Becton Dickinson, USA) (with and without imipenem) agar and incubated for 24 hours to check the strain purity.

3.2 Sample preparation for PCR

In order to separate the bacterial nucleic acids from the bacterial cell wall bacterial colony was harvested from Mueller Hinton agar. A single colony of bacteria was suspended in 20µl of miliQ water and boiled for 5 minutes before centrifuging for 1 minute in 12000xg.

The clarified supernatant containing bacterial DNA was carefully separated from the bacterial pellet and was stored at -20 ºC until the day of use.

3.3 Amplification of 16S rDNA

The amplification of 16S ribosomal of *Acinetobacter baumannii* was performed as follows. The bacterial DNA was amplified using three sets of primers (Table 3.1), synthesized by Research Biolabs pte. Ltd., Singapore (Misbah *et al.*, 2005b). All reactions were performed in 25µl volume; containing 15pmol of each primer, dNTPs, 5X buffer, magnesium chloride and Taq DNA polymerase (Promega, USA). Amplification was conducted using a PTC-200 Peltier Thermal Cycler (MJ Research, USA) with the following thermocycling programme: Initial denaturation was done at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 5 minutes.

Table 3.1: Oligonucleotide primers used for amplification and sequencing of the *Acinetobacter* spp. 16S rDNA gene

Primers	Sequence (5' to 3')	Position
27F'	AGAGTTTGATCCTGGCTCAG	8 – 27
780R'	TACCAGGGTATCTAATCCTGTT	780 – 801
529F'	GTGCCAGCMGCCGCGG	514 – 529
1099R'	GGGTTGCGCTCGTTG	1099 - 1113
925F'	AAACTYAAAKGAATTGACGG	906 – 925
1491R'	ACGGCTACCTTGTTACGACTT	1491 – 1511

3.4 Nucleic Acid Agarose Gel Electrophoresis

The amplified truncated region was separated by 1.2% agarose gel (Sigma, Missouri, USA) electrophoresis. The agarose had been resolved in 0.5 TAE buffer (1st base,

Singapore). Resolving was completed by heating to which 1µl of cyber safe (Invitrogene, California, USA) was added. After the gel solidified the whole sample and 8µl of loading dye (Promega, Wisconsin, USA) was added, before 12 µl of 100bp ladder was added to one of the wells. The container was filled with 0.5 TAE buffer and connected to the electrophoresis machine EC105. The machine was set to a voltage of 100 V for 45 minutes. The gel was cut and kept in an Eppendorf tube for further experiments.

3.5 PCR Product Purification

Prior to purification, the QIAquick Miniprep kit (Qiagen, Germany) was used in accordance with the manufacturer's protocol, as stated in the book provided. Briefly, 500µl of QG buffer was added to each tube containing the excised PCR product. The tubes had been incubated at 50^{o}C for 10 minutes to dissolve the gel. In order to increase the process of dissolving, this step could be carried out by vortexing. The QIAquick spin column was placed in a 2ml collection tube. The sample was applied to the QIAquick column and centrifuged for 1 minute at 13000xg. The flow through was discarded and centrifuged for 1 minute at 13000xg. Then, 0.5 ml of QG buffer was added to the column and centrifuged for 1 minute at 13000xg. Subsequently, 0.75 ml of PE buffer was used for washing before centrifuging for 1 minute at 13000xg. The flow through was discarded and centrifuged for 1 additional minute at 17000xg. The QIA quick column was placed into a 1.5ml microcentrifuge tube and 30µl nuclease free water was added to the centre of the QIA quick membrane. The column stood for 10 mins, and was then centrifuged for 1 min at 13000xg.

3.6 16S rDNA Sequencing

Sequencing is the method used to determine the exact and precise order of nucleotide in a desired DNA. DNA sequencing can be done using two different methods:

1- Chemical degradation

2- Chain termination

In the chemical degradation or Maxam Gilbert method, double strand DNA is synthesised, and 5' end of DNA is labelled with radioactive phosphorus. The DNA is cleaved by using chemical reagents that specifically affect a particular nucleotide. For example, dimethyl sulphate and piperidine cut the DNA near the location of guanine. If acid is combined with piperidine instead of dimethyl sulphate, the DNA breaks down in the location of the purine (adenine and guanine) bases. For pyrimidine bases, a combination of piperidine and hydrasine is needed, and, if NaCl is added to this combination, this reaction would be specific for cytosine. Each reaction is in a separate tube. The contents of each tube are loaded in an electrophoresis well, and each band is detected via their radioactive phosphorus using X-ray.

The chain reaction method was invented by Sanger. In this method, a single strand of DNA is used. For this method, dideoxyNTPs, which is a modified nucleate, is used. In the dideoxyNTPs group -H is replaced with -OH in deoxyribose. Therefore, the formation of a phosphodiester bond between two nucleotides does not occur and elongation is terminated. For this method, four separate tubes are used, each of which contain the primer, Klenow DNA polymerase I, and all four of the standard deoxynucleotides. For each reaction, only one of the four dideoxynucleotides is added. The DNA fragment is separated by gel electrophoresis and four reactions are run individually. The DNA bands may be visualised and directly read by X-ray film. Automated DNA-sequencing instruments (DNA sequencers) have the ability to sequence up to 384 DNA samples in a single run. They can detect and record fluorescent dye. Before the samples are run in the sequencer machine, they should be cleaned and suspended in a buffer solution. Low quality DNA traces should be trimmed by the software, before proceeding for further analysis.

The purity of the strain was confirmed by 16S rDNA sequencing. The extracted DNA was then sequenced by the previously described ethanol precipitation method (Wallis & Morrell, 2011). Briefly, cycle sequencing was performed in 5µl volumes consisting of 2µl of 16S rDNA, 0.5µl of Big Dye Terminator V3.1 (Biosystem, California, USA), 0.5 µl of sequencing buffer (Biosystem, California, USA), 0.5 µl of 10pmol/ul of primer and 1.5µl of nuclease free water for each reaction. This was done using a thermal cycler ABI 3730 XL DNA with the following protocol:

Initial denaturation at 96^{o}C for 2 minutes followed by 29 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and final extension at 72°C for 4 minutes. The plate was then centrifuged (5810R Eppendorf) at 100xg for 1 minute.

To this product, 80ul of precipitation reagent was then added consisting of 2.55µl of 3M sodium acetate (Sigma, Missouri, USA) pH4.6, 54.74µl of 95% ethanol and 22.71 µl of nuclease free water. The sample was properly mixed using a vortex and centrifuged at 100xg for 1 minute. It was then left at room temperature for 30 minutes to precipitate the sequencing products. The sample was centrifuged at 3000xg for 30 minutes at 4^{o}C. The supernatant was removed from the well and the pellet was then rinsed with 150µl of 70% ethanol, vortexed and centrifuged at 3000xg for 10 minutes at 4^{o}C. The ethanol was discarded and the washing step was repeated. Then, the plate was inverted on a piece of paper towel and centrifuged at 50xg for 1 minute. The clean pellet was dried at 65^{o}C for 10 minutes using a thermo cycler. The Pellet was re-suspended in 10µl of Hi-Di formamide (Life Technologies California, USA) and the plate was then covered with septa and centrifuged at 100xg for 1 minute. Heat denature was then performed at 95^{o}C for 2 minutes before cooling immediately to 4^{o}C. The plate was centrifuged at 200xg for 1 minute. It was placed on the plate deck and the injection was done using an ABI3730 XL DNA Analyser (Applied Biosystems, Renton, USA).

3.7 DNA Sequencing Analyses

The sequencing results were analysed using Sequencer version 4.0.5 software (Gene Codes Corporation, USA). The results were assembled with the reference gene (accession number: JX035953).

3.8 Bacterial Culture Condition for genes expression study

One colony of *Acinetobacter baumannii* M26 23-24 strain was inoculated into 5ml Mueller Hinton (Becton Dickinson, USA) broth. It was then incubated for 16 hours (overnight) with constant agitation of 150rpm at 33ºC. The overnight culture was inoculated into the fresh Mueller Hinton broth at the ratio of 1:100 and grown at 37ºC with constant agitation of 150rpm until the optical density (OD) 600 nm reached 0.3. The bacterial culture was then divided in two conical flasks and the freshly prepared Imipenem solution (final concentration should be 32µg/ml) was added to one of the flasks. As the vehicle control, an equal volume of PBS as the solvent for imipenem was added to the other flask. Both flasks were then incubated at 37ºC with constant agitation of 150rpm for 2 hours. The reduction of bacterial number due to penetration of antibiotic to the cells is starting after 2 hours (Pang, 2012). The cultured bacteria were harvested by centrifugation at 3000xg for 20 minutes. The supernatant was discarded and the pellet was stored at -80ºC in a fridge.

3.9 Total RNA extraction

All the RNA from the bacterial culture was extracted using a TRI reagent (Molecular Research Centre, Inc., USA) (Amin *et al.*, 2013) Briefly, the TRI Reagent was added to the bacterial pellet to lyse the bacterial cells before it was incubated for 5 minutes at room temperature. Then, 200µl/ml of chloroform was added and shaken vigorously for 15 seconds. The tube was incubated at room temperature for 15 minutes and centrifuged at 12,000xg for 15 minutes in a 4ºC centrifuge. The aqueous phase containing RNA was separated from the

phenol-chloroform phase, and 5µl of RNase-free DNase (5U/ml TRI Reagent) was added to the aqueous phase and incubated for 30 minutes at 37ºC to digest any DNA contamination within the aqueous phase. This step was followed by incubation at 37ºC for 30 minutes. The RNA was precipitated by adding 0.5ml isopropyl alcohol, and then incubated at room temperature for 10 minutes followed by centrifuging at 12000xg for 10 minutes in a 4ºC centrifuge. The pellet was washed with 1ml of cold 75% ethanol, and centrifuged at 7500xg for 5 minutes at 4ºC. Subsequently, it was air-dried and solubilised in RNase-free water.

Purification of RNA and determination of total RNA integrity: The extracted total RNA was further purified using the RNeasy Kit (Qiagen, Germany) in accordance with the manufacturer's protocol. Briefly, 350µl of buffer RLT was mixed with the RNA followed by the addition of 250µl of ethanol. The total mixture was transferred to the RNeasy spin column and centrifuged at 8000xg for 15 seconds. The flow through was discarded, and 700µl of buffer RW1 was added into the column and the membrane within the column was washed by centrifugation at 8000xg for 15 seconds; the flow through was again discarded. Then, the membrane was rewashed by adding 500µl of buffer RPE containing ethanol followed by centrifugation at 8000xg for 15 seconds. The RNA on the membrane was finally washed with 500µl buffer RPE containing ethanol followed by centrifugation at 8000xg for 2 minutes. The ethanol and the remaining buffer on the membrane were dried by long centrifugation at 8000xg for 2 minutes. Finally, the RNA was eluted out by adding 30µl of nuclease free water followed by centrifugation at 8000xg for 1 minute. The integrity of RNA was then determined by using micro-fluidic capillary electrophoresis (Agilent RNA 6000 Nano LabChip) according to the manufacturer's instructions followed by separation using the Agilent Bioanalyzer 2100 system (Agilent Technologies, California, USA). A gel image of sample was analysed and the RNA integrity number (RIN) of each sample was generated by the Agilent Bioanalyzer software. The ratio of rRNA 23S/16S and the RNA concentration of

the sample were examined. The purity and concentration of the RNA was further assessed using a nanospectrophotometer (Implen, Germany). After RNA extraction, the quality and quantity of RNA has been checked, as explained before. Then the extracted RNA was converted to cDNA for 2-step real time PCR, or stored at -80 ºC for 1-step qPCR. In this study, the 2-step was used to investigate the gene expression through the application of the double-dye probe kit (Primer design, UK).

3.10 Purification of RNA and determination of total RNA integrity:

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of rRNA 23S/16S and the RNA concentration of the sample were examined. The purity and concentration of the RNA was further assessed using a nanospectrophotometer (Implen, Germany). After RNA extraction, the quality and quantity of RNA has been checked out, as explained before. Then the extracted RNA converted to cDNA for 2-step real time PCR, or stored at -80ºC for 1-step qPCR. In this study, the 2-step was used to investigate the gene expression through the application of the double-dye probe kit (Primer design, UK).

3.11 cDNA Synthesis

RNA is converted to cDNA using the Precision nanoScriptTM ReverseTranscription Kit (Primer design, UK). To prepare the cocktail for this reaction 2ng of total RNA was used as a template, to which 1µl of random nonamer primer was added as the RT primer and topped up with RNAse/DNAse free water to reach the final volume of 10µl. The mixture was heated at 65ºC for 5 minutes. The samples were placed on ice immediately. The master mix mixture was prepared in 10µl final volume containing 2µl of nanoScript10 Buffer, 1µl of dNTP mix, 2µl DTT 100mM, 4Ul of RNAse/DNAse free water and 1µl of nanoScript enzyme (Primer design, UK). Subsequently, 10µl of the master mix was added to each of the samples, and incubated at 25ºC for 5 minutes, and then at 55ºC for 20 minutes. Heat inactivation was done by incubation at 75ºC for 15 minutes.

3.12 Real time RT-PCR

In the second step, real-time RT-PCR was performed to check the expression level of the genes by real-time PCR assay using a double-dye probe kit. To prepare the reaction cocktail, each reaction tube contained 1µl of re-suspended primer/probe mix, 10µl of Primer Design 2X Precision Master mix and 4µl of RNAse/DNAse free water. To each well, 5µl of cDNA with a concentration of 5ng/µl was added.

In the reaction plate, three replicates of each sample (hypothetical protein) with the official gene symbol ABAYE0161 and accession number *000180, and general secretion pathway protein CDS with the official symbol name ABAYE3501 and accession number *000164, along with three replicates of endogenous control were used. The expression of endogenous control is not affected by different experimental conditions, and, thus, can be used to normalize the fluorescence signals for the target assay. In this research, 16S ribosomal RNA gene (16S rRNA) with the accession number EF031068 was used as the reference gene. The prepared reaction plate was loaded into Agilent technologies startagene MX3000P in order to do the q-RT-PCR.

Table 3.2: Real-time PCR running program

Stage	**Step**	**Temp**	**Time**
	Enzyme activation	95°C	10min
Cycling (50 cycle)	Denature	95°C	15sec
	Data collection	60°C	1 min

Gene expression of ABAYE 3501 and ABAYE 0161 genes was estimated and the fold change was calculated using the formulae below:

$[delta]Ct_{(treated)} = Ct_{(gene)} - Ct_{(16srRNA)}$

$[delta]Ct_{(untreated)} = Ct_{(gene)} - Ct_{(16srRNA)}$

$[delta][delta]Ct = [delta]Ct_{(treated)} - [delta]Ct_{(untreated)}$

$\text{Fold change} = 2^{-[delta][delta]Ct}$

4 CHAPTER IV: RESULTS AND DISCUSSION

4.1 Bacterial culture

Acinetobacter baumannii M26 23-24 strain was revived and its purity has been evaluated by culturing MH agar (Figure 4.1). The strain was allowed to grow in Mueller Hinton agar. After overnight incubation, the colonies observed were smooth and mucoid. The colour of colonies were whitish-grey. The culture was pure as smooth, mucoid and whitish-grey in colour colonies could observed on agar media.

Figure 4.1: *Acinetobacter baumannii* culture in Mueller Hinton agar. Strain Purity was checked out on agar media. Colonies were smooth and mucoid. The colour of colonies were whitish-grey. Size, shape, and colour of colonies were same

4.2 PCR and DNA purification

The 16S rDNA genome contains approximately 1500 nucleotides. This region was amplified using three sets of primer. The first set of primer generates a single DNA fragment with 750bp while the other two sets of primer generate a distinguishable DNA band with 580bp (Figure 4.2).

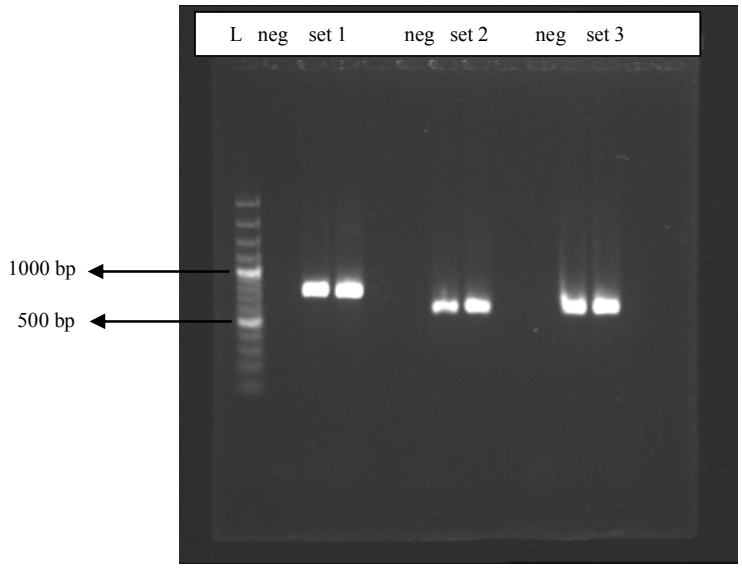


Figure 4.2: Electrophoresis results for 16S rDNA PCR
(From left to right: negative control, first set of primers, without antibiotic, negative control, second set of primers, negative control, third set of primers). The first set of primer generates a single DNA fragment with 750bp while the other two sets of primer generate a distinguishable DNA band with 580bp

The PCR reaction was done using these three sets of primers, as explained earlier. 16S rDNA was chosen for identification of the bacterium, which is a house keeping gene that is present in prokaryotes. The 16S rDNA sequence analysis is currently considered as one of the best methods for the identification of bacteria. The method has some benefits for both clinicians and researchers in terms of choosing appropriate antibiotics, controlling infectious diseases and the infectious disease pathway.

4.3 Sequencing and analysis

In this study, we performed the Sangar chain termination method for DNA sequencing. Currently, this method can read 1000bp with an accuracy of 99%. The BigDye terminator was chosen as the dye terminator since it was one the cheapest and also most commonly used method in the stages of fluorescent cycle sequencing.

The complete sequences of the 16S rDNA gene from the tested sample were amplified by PCR amplification (Figure 4.3). To confirm the identity of the tested strains, the obtained 16S rDNA sequence data were compared to the data available in the GenBank database using the BLAST algorithm (http//www.ncbi.nih.gov). The blast results of the 16S rDNA sequences gave at least 98% similarity to the *Acinetobacter baumannii* partial 16S rDNA gene (Accession number: HE978267).

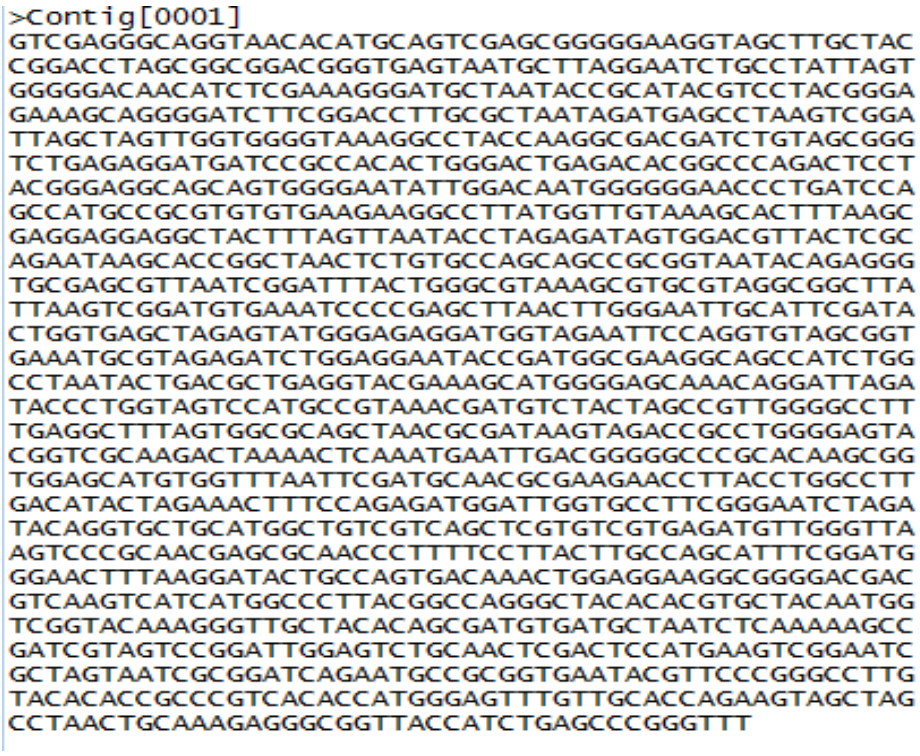


Figure 4.3: Sequencing result for 16S rDNA of the isolate for identity confirmation. Identity of tested strain was confirmed by GenBank database. 98% similarity was reported for 16SrDNA gene through the BLAST algorithm

4.4 RNA extraction and purification

The RNA of the cultured bacteria was extracted using the conventional method (Amin *et al.*, 2013) . The quantities of *A. baumannii* total RNA were evaluated using nano-spectrophotometry (Table 4.3). One of the most common methods for checking the integrity

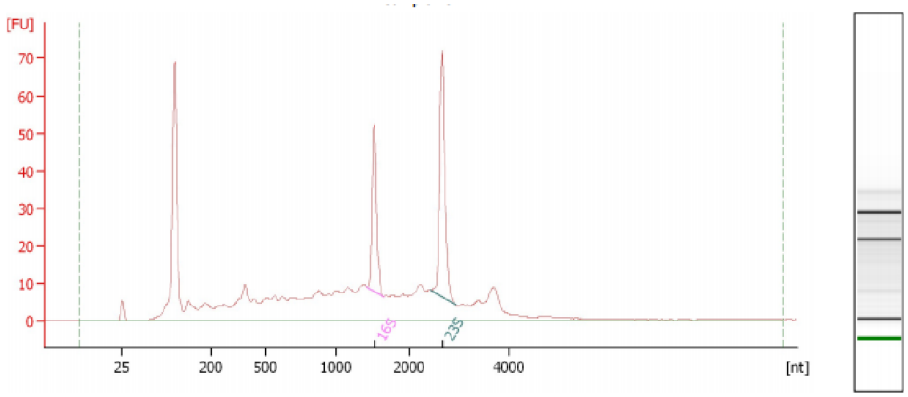
of extracted RNA is inspection of prominent band of the ribosomal RNA (rRNA). The quality of the samples was estimated using the Agilent Bioanalyzer 2100 system (Agilent Technologies, USA). Large rRNAs (16S and 23S) are particularly analysed in prokaryotes. The integrity number (RIN) of the samples was measured using software. The band sizes of 16S and 23S, and the ratio of rRNA 23S/16S were examined. (Figures 4.4 and 4.5). Sharpness of 16S and 23S peaks shows the integrity of RNA.

The absorption of some compounds, such as Phenolate ions, and other organic compounds, was measured at 230nm. Therefore, low absorption in the measurement ratio of A260:A230 shows contamination with one of these reagents. Usually, a pure RNA sample has 2.0-2.2 absorption of A260:A230. Another ratio that is used for the qualification of RNA is A260:A280. The acceptable range for showing the purity of RNA is between 1.8 and 2.2.

A bioanalyzer is another tool used for estimating the quality of RNA. Usually it is used to measure the degradation of RNA. The Agilent 2100 bioanalyzer detects small amounts of RNA by using laser-induced fluorescence detection. The output is reported as a RIN value. Intact RNA approaches a RIN value of 10 while highly degraded RNA approaches a RIN value close to 1. An acceptable RIN value is based on the purpose of the experiment. It is mentioned that an acceptable RIN value for microarray is more than 7 whereas a RIN value of 5 would be appropriate for qRT-PCR.

Table 4.3: Results from nano-spectrophotometry

	concentration(ng/µl)	**A260/A280**	**A260/A230**
With imipenem	448	2.093	2.196
Without imipenem	412	2.096	2.403



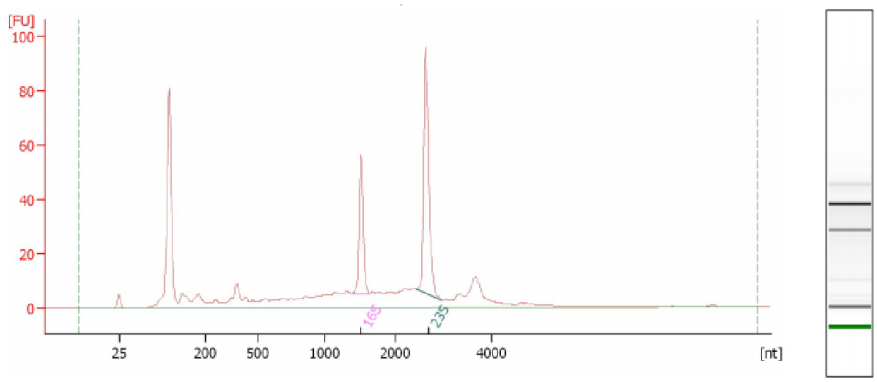
Overall Results for sample 10 : **Sample 10**

RNA Area:	588.6	rRNA Ratio [23s / 16s]:	1.5
RNA Concentration:	441 ng/µl	RNA Integrity Number (RIN):	6.4 (B.02.07)

Fragment table for sample 10 : **Sample 10**

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,430	1,655	40.6	6.9
23S	2,432	2,924	61.9	10.5

Figure 4.4: Level of mRNA synthesis in presence of antibiotic. The band sizes of 16S and 23S, and the ratio of rRNA 23S/16S were examined. Sharpness of 16S and 23S peaks shows the integrity of RNA. RIN number was 6.4



Overall Results for sample 11 : **Sample 11**

RNA Area:	521.7	rRNA Ratio [23s / 16s]:	1.8
RNA Concentration:	391 ng/µl	RNA Integrity Number (RIN):	7.7 (B.02.07)

Fragment table for sample 11 : **Sample 11**

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,379	1,633	46.0	8.8
23S	2,432	2,967	83.7	16.0

Figure 4.5: Level of mRNA in absence of antibiotic. The band sizes of 16S and 23S, and the ratio of rRNA 23S/16S were examined. Sharpness of 16S and 23S peaks shows the integrity of RNA. RIN number was 7.7

4.5 Real Time PCR

Since real-time quantification is based on the relationship between initial template amount and obtained CT (cycle threshold) value during amplification, an optimal qPCR assay is essential for accurate quantification of samples. A powerful way to determine the optimization of a qPCR assay is to run serial dilutions of a template and use the results to generate a standard curve. The amplification curve is built up by plotting the log of the starting quantity of template against the CT value obtained during amplification of each dilution. The amplification plot shows the fluorescent signals (dRn) against the cycle number. In this experiment 5 dilutions were prepared with the dilution factor of 2 for selected genes (ABAYE 3501 and ABAYE 0161) and housekeeping gene (16S rRNA) as it shown in Figures: 4.6, 4.8 and 4.10. The highest chosen concentration was 5 ng/µl for this purpose. In perfect amplification cycle, spacing of fluorescence curves is determine by equation 2^n =dilution factor where n is the number of cycles between curves at the fluorescence threshold. In this experiment dilution factor was 2 therefore based on above formula the number of cycles should be 1 (Figures: 4.6, 4.8 and 4.10). Each dots in amplification plots shows fluorescent signals (dRn) for each cycles. According to the figure 4.6, 4.8 and 4.10 amplification starts at cycle 23 and 22 for genes ABAYE 3501 and ABAYE 0161 respectively whereas amplification starts at cycle 12 for 16S rRNA (housekeeping gene). Figures 4.7, 4.9 and 4.11 represent CT plotted against the log of the starting quantity of template for each dilution. The equation of linear regression line was used to evaluate qPCR assay optimisation. The coefficient of determination (R^2) of standard curve represents that whether the experimental data is fit the regression line. Linearity, gives a measure of variability across replicates and whether the amplification efficiency is the same for different starting template. Acceptable plot has R^2 value > 0.98 and slop of -3.3%±10%, which demonstrates 90%-110% efficiency. In this study, gene ABAYE 3501 represents R^2 value =

0.999, efficiency = 92.5%, slop = -3.515 (Figure: 4.7), 16S rRNA as a housekeeping gene shows R^2 value = 0.999, efficiency = 105%, slop = -3.189 (Figure: 4.9) and ABAYE 0161demonstrate R^2 value = 0.992, efficiency = 93.4%, slop = -3.491 (Figure: 4.11). In each graph five spots demonstrated which stand for each dilution. As it shown in figures 4.7, 4.9 and 4.11 the highest concentration has the lowest CT value which means that amplification starts earlier compare to the lower concentration.

Figure 4.6: Amplification plot for ABAYE 3501 gene. A standard curve was generated using 2 fold dilution of a gene ABAYE 3501 amplified on real time system. Each dilution assayed in triplicate.