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Acinetobacter baumannii: A Study on Prevalence, Detection and Virulence

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Acinetobacter baumannii has emerged as an important, multidrug resistant (MDR) pathogen causing a wide spectrum of disease conditions in humans. The emergence of multi-drug, pan-drug resistant, and extended drug-resistant strains of *A. baumannii*, which are resistant to almost all classes of antibiotics, makes the treatment of *A. baumannii* infections difficult. The ubiquitous presence of *A. baumannii* in water and soil along with its ability to persist under various environmental conditions aids in the potential contamination of foods, especially fresh produce. Further, the genotypic and phenotypic similarities among various species of *Acinetobacter* impede the specific detection of *A. baumannii* in clinical and environmental samples.

In this dissertation, the prevalence of *A. baumannii* on fresh vegetables collected from farmers' markets in Connecticut was studied. The results showed the presence of a variety of MDR opportunistic pathogens, including *A. baumannii* on vegetables. In addition, the effect of chlorine, a commonly used disinfectant in water, was tested for its efficacy in killing *A. baumannii*, besides determining its effect on the antibiotic resistance genes in the bacterium. Chlorine was not only found ineffective to inactivate significant populations of *A. baumannii* in water, but also induced the expression of major antibiotic resistance genes in the pathogen.

A real-time quantitative PCR targeting a highly conserved 102-bp DNA sequence in *adeT*, an efflux pump gene found only in *A. baumannii*, was developed for rapid and specific detection of the bacterium in water and blood. The PCR detected all the *A. baumannii* isolates by

amplifying a 107-bp DNA fragment, however, none of the tested negative isolates, including other closely related *Acinetobacter* species produced any amplification. The sensitivity of PCR for detecting *A. baumannii* in blood and water was 3 $\log_{10}$ CFU/ml or 0.1 ng/ml of DNA. However, with an enrichment step, the PCR was able to detect 2 $\log_{10}$ and 1 $\log_{10}$ CFU/ml of *A. baumannii* in water after 6 h and 14 h of incubation at 37^{o}C, respectively.

Because of *A. baumannii*'s resistance to most of the currently used antibiotics, this dissertation also investigated the efficacy of two plant-derived antimicrobials (PDAs), *trans*-cinnamaldehyde (TC) and eugenol (EG) in reducing its resistance to seven commonly used β-lactam antibiotics. Both TC and EG were effective in significantly increasing the sensitivity of *A. baumannii* to the selected beta-lactam antibiotics. Further, it was revealed that TC and EG down-regulated the expression of major genes conferring antibiotic resistance in the bacterium.

A. baumannii has been increasingly linked to persistent wound infections, especially in burn victims and combat-related injuries. *A. baumannii*'s multi-drug resistance and ability to form biofilms and colonize skin epithelial cells limit the efficacy of currently used wound infection treatments. Therefore, this dissertation determined the efficacy of TC and EG in decreasing *A. baumannii* adhesion to and invasion of human keratinocytes, in addition to inhibiting *A. baumannii* biofilm formation using an *in vitro* wound model. Both TC and EG significantly reduced *A. baumannii* colonization of skin cells, and biofilm production, suggesting their potential application in treating *A. baumannii* wound infections.

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***Acinetobacter baumannii*: A Study on Prevalence, Detection and Virulence**

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ATCC	American Type Culture Collection
CCCP	Carbony cyanide m-chlorophenylhydrazone
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CR	Carvacrol
CSPI	Center for Science in the Public Interest
EDTA	Ethylene diamine tetraacetic acid
EG	Eugenol
EPA	Environmental Protection Agency
EPS	Extracellular polymeric substance
EtBr	Ethidium bromide
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
ICU	Intensive Care Unit
MBC	Minimum bactericidal concentration
MDR	Multi drug Resistant Bacteria
MIC	Minimum inhibitory concentration
MPN	Most Probable Number
NPN	1-N-phenylnapthylamine
PBS	Phosphate buffer saline

PDA	Plant-derived antimicrobial
PPM	Parts Per Million
RT-qPCR	Real-time quantitative polymerase chain reaction
SAS	Statistical analysis software
SIC	Sub-inhibitory concentration
TC	Trans-cinnamaldehyde
TSA	Tryptic soy agar
TSB	Tryptic soy broth
USDA	United States Department of Agriculture
USDH	United States Department of Health
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1. INTRODUCTION

Bacterial antimicrobial resistance has emerged as one of the most serious public health threats globally. The Centers for Disease Control and Prevention (CDC) reported that antibiotic-resistant infections account for two million illnesses and 23,000 deaths annually in the US, with over $20 billion as direct health-care costs and $35 billion in lost productivity (CDC, 2013). The problem is further exacerbated by the lack of new and effective antibiotics under development against multi-drug resistant (MDR) pathogens (Maragakis and Perl, 2008; Manchanda et al., 2010). *Acinetobacter baumannii* has emerged into a significant MDR pathogen, especially linked to infections associated with hospitals and intensive care units (Giamarellou, 2010; Medell et al, 2013). The Infectious Diseases Society of America has ranked *A. baumannii* as one of the top priority, antibiotic-resistant pathogens to target, due to its rapid propensity to develop drug resistance, and the restricted choice of antibiotics to treat infections caused by the bacterium (Talbot et al., 2006). In humans, MDR *A. baumannii* causes a variety of infections, including pneumonia (Leung et al., 2006), blood-stream infections (CDC, 2004; Wisplingoff et al., 2004), meningitis (Metan et al., 2007), and urinary tract infections (Gaynes and Edwards, 2005). In addition, MDR *A. baumannii* has been increasingly linked to wound infections in burn victims (Bergogne-Berezin, 1995; Santucci et al., 2003; Sharma, 2007), and combat-related wound injuries (Scott et al., 2007; Murray and Hospenthal, 2008).

A. baumannii is ubiquitously distributed in nature because of its ability to persist under a wide variety of environmental conditions for extended periods of time (Maragakis and Perl, 2008). *Acinetobacter* spp., including *A. baumannii* are frequently isolated from soil and water (Baumann et al; 1968; Berlau et al., 1999; Houang et al., 2001; Dijkshoorn et al., 2007;Cateau et al., 2011), thereby potentially serving as a source of food contamination, especially fruits and

vegetables. Protozoans such as *Acanthamoeba* are reported to support *A. baumannii* growth in aquatic ecosystems, and act as a reservoir of the pathogen (Cateau et al., 2011). A limited number of studies conducted outside the United States have isolated *A. baumannii* and other *Acinetobacter* spp. from a variety of foods, including bulk tank milk (Gurung et al., 2013), raw meat (Houang et al., 2001; Lupo et al., 2014), fresh produce (Berlau et al.,1999), and fish (Houang et al., 2001). Since fruits and vegetables are more vulnerable to contamination by antibiotic resistant bacteria from soil and water due to their cultivation patterns, and are not generally subjected to any terminal lethal process or cooking, the potential for transfer of resistant determinants to commensal or pathogenic bacteria in the human gut is greater.

The detection of *A. baumannii* by conventional bacteriological methods requires at least 48 to 72 h for obtaining accurate results (Levi et al., 2003; Jamal et al., 2014). Furthermore, the four *Acinetobacter* species grouped under *A. baumannii -A. calcoaceticus* complex (ABC),including *A. calcoaceticus*, *A. baumannii*, *A. pitti* and *A. nosocomalis*, are phenotypically and genotypically closely related, and are difficult to be distinguished from each other. Further, species identification using biochemical tests fails to differentiate *A. calcoaceticus* from *A. baumannii*, and specific detection needs to be followed up by further tests, which could be time consuming in clinical diagnosis. Thus, there is a need for rapid and accurate assays for specific detection of *A.baumannii* in clinical and environmental samples.

A. baumannii is resistant to most of the currently available antibiotics (Hsueh et al., 2002; Lin et al 2014). Further, *A. baumannii* possesses almost all known antibacterial resistance mechanisms reported in bacteria, including the ability to acquire multidrug-resistance from other bacteria through plasmids, transposons and integrons, reduced permeability of outer membrane to some antibiotics, and the constitutive expression of efflux pumps (Vila and Pachon, 2008).

Since there are limited therapeutic options against *A. baumannii*, there is a critical need to explore novel strategies for controlling antibiotic resistance in the bacterium.

For centuries, plants have served as a basis for the development of novel drugs, thereby contributing to human health. Plants are capable of synthesizing a large number of molecules, most of which are phenolic compounds or their derivatives (Geissman, 1963; Burt 2004; Holley and Patel, 2005; Savoia, 2012). Plants produce a majority of these compounds as a defense mechanism against predation by microorganisms and insects (Cowan, 1999). A variety of plant-derived polyphenols serve as dietary ingredients as well as active components in a number of herbal and traditional medicines (Wollenweber, 1988). Several plant-derived essential oils have demonstrated antimicrobial activity towards a wide range of microorganisms, and several active components of these oils have been identified (Burt, 2004; Holley and Patel, 2005).

Although considerable literature exists on the antimicrobial properties of several plant derived antimicrobials (PDAs) on a wide range of microorganisms, relatively limited information is available on their effect on bacterial antibiotic resistance, especially in Gram-negative pathogens. Previous research conducted in our laboratory revealed that several PDAs, including *trans*-cinnamaldehyde, carvacrol, eugenol and thymol decreased antibiotic resistance in MDR *S.* Typhimurium DT 104 (Kollanoor-Johny et al., 2010). Similarly, Chusri et al. (2009) observed that two plant molecules, ellagic acid and tannic acid when used as adjuvant enhanced the antimicrobial activity of novobiocin, coumermycin, chlorobiocin, rifampicin and fusidic acid against *A. baumannii*.

Based on the published literature and preliminary research conducted in our laboratory, it was hypothesized that fresh vegetables could be a potential source of MDR *A. baumannii*, and chlorine is ineffective as a water disinfectant for killing the bacterium. In addition, it was

hypothesized that *trans*-cinnamaldehyde and eugenol reduce MDR *A. baumannii*'s resistance to selected β-lactam antibiotics, and could be used for controlling wound infections of *A. baumannii*.

The specific objectives include of this dissertation are to

1. Investigate the prevalence of MDR *A. baumannii* on fresh vegetables collected from farmers' markets in Connecticut.

2. Determine the effect of chlorine on *A. baumannii* survival in water and expression of antibiotic resistance genes

3. Develop a real-time quantitative PCR for specific detection of *A. baumannii* in water and blood.

4. Determine the effect of TC and EG on decreasing *A. baumannii* resistance to β-lactam antibiotics, and

5. Investigate the efficacy of TC and EG in reducing *A. baumannii* adhesion to and invasion of human keratinocytes and controlling wound infections *in vitro*.

CHAPTER II

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

1. Taxonomy of Acinetobacter

In 1911, a Dutch microbiologist named Beijerinck M.W. isolated a bacterium from soil using a minimal medium enriched with calcium acetate, and named it as *Micrococcus calcoaceticus* (Howard et al., 2012). After 43 years, Brisou and Prevott in 1954, proposed the genus *Acinetobacter* to differentiate this bacterium from the motile organisms within the genus, *Achromobacter* (Brisou and Prevot, 1954; Towner, 1992). Subsequently extensive acceptance for the genus *Acinetobacter* was given following a comprehensive study of *Micrococcus calco-aceticus, Alcaligenes hemolysans, Mima polymorpha, Moraxella lwoffi, Herellea vaginicola* and *Bacterium anitratum* by Baumann et al. (1968). The conclusion of this study was that the above organisms belonged to a single genus, and further sub-classification into different species based on phenotypical characteristics was not possible. The results of Baumann's 1968 publication regarding the genus *Acinetobacter* was later officially acknowledged by the sub-committee on the Taxonomy of Moraxella and Allied Bacteria (Lessel, 1971).

In the Bergey's Manual of Systematic Bacteriology (Lautrop,1974), the genus *Acinetobacter* was listed with the description of a single species *Acinetobacter calcoaceticus*, the type strain being *A. calcoaceticus* ATCC 23055. However, in the "Approved list of bacterial names" two different species, *A. calcoaceticus* and *A. lwoffii* were included considering the ability of some *Acinetobacter*s to acidify glucose, whereas others did not (Skerman, 1980). The current definition of the genus *Acinetobacter* is Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative bacteria with a DNA G + C content of 39 to 47% (Albrecht et al., 2006). Currently, based on the DNA-DNA hybridization studies made by Bouvet and Grimnot in 1986, the genus *Acinetobacter* consists of 26 named

species and nine genomic species (Di Nocera, 2011). *Acinetobacter* species have phenotypic similarities and are difficult to differentiate among *A. calcoaceticus, A. baumannii, Acinetobacter* genomic species 3 and *Acinetobacter* genomic species 13TU (Gerner-Smidt, et al 1991).

The genus *Acinetobacter* is now classified in the family Moraxillaceae. The important genera within Moraxillaceae include Moraxella, *Acinetobacter*, *Psychrobacter*, and related organisms (Rossau et al., 1991). *Acinetobacter* genomospecies 2 (*A. baumannii*) is the principal pathogen associated with human diseases (Seifert et al., 1993; Gales et al., 2001). In addition, less frequently reported pathogenic species such as *Acinetobacter calcoaceticus* (formerly *Acinetobacter* genomospecies 1), *Acinetobacter pittii* (formerly *Acinetobacter* genomospecies 3) and *Acinetobacter nosocomalis* (formerly *Acinetobacter* genomospecies 13TU) are included in the genus. These bacteria together with *A. baumannii* are commonly referred to as *A. baumannii-calcoaceticus* complex due to their close genotypic similarity and the difficulty in differentiating by normal phenotypic methods (Seifert et al., 1993; Houang et al., 2001),

Although substantial progress has been achieved in the species level identification of *Acinetobacter*, several problems and inconsistencies still exist in the process. In the currently described DNA–DNA hybridization groups, only 17 of them have valid names. The remaining hybridization groups were considered too small (< 10 strains) to describe their characteristics as a group, and the criteria for phenotypic differentiation were inadequate (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989; Gerner Smidt and Tjernberg, 1993; Peleg et al., 2008). Therefore, no definite species names were provided (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989; Gerner- Smidt and Tjernberg, 1993). These limitations in species identification methods of *Acinetobacter* are

considered an impediment for elucidating the biology, pathogenicity and ecology of the organism at the species level.

2. Morphology, physiological and biochemical characteristics

The *Acinetobacter* is identified as Gram-negative, catalase-positive, oxidase-negative, non-motile, non-fermenting coccobacilli. Although *Acinetobacter* is considered as non-motile, there are reports on differential motility in response to factors, including illumination (Mussi et al., 2010), quorum sensing (Clemmer et al., 2011), and iron chelation (Eijkelkamp et al., 2011). Although not decisively proven, *Acinetobacter* spp. can move on semi-solid surfaces by expressing twitching motility rather than gliding, sliding, swimming, or swarming motility (Barker and Maxted, 1975; Henrichsen, 1984; Eijkelkamp et al., 2011). When subjected to Gram staining, the bacteria are often difficult to de-stain and are incorrectly identified as Gram-positive. Currently there are no well-defined and definite metabolic tests for distinguishing *Acinetobacter* from other non-fermenting Gram-negative bacteria (Peleg et al., 2008). However, for the differentiation of *Acinetobacter* from other similar non-fermenting organisms, the negative oxidase test is used for rapid presumptive identification.

Acinetobacter can be easily grown on simple microbiological media and the colony characteristics include smooth, dome shaped, pale yellow to grayish, and with entire edge (Doi et al 2011). The colonies of *A. calcoaceticus-A. baumannii* complex can grow to a size of 1.5 to 3 mm in diameter in an overnight culture similar to that of Enterobacteriaceae, whereas majority of other *Acinetobacter* species produce smaller colonies (Peleg et al., 2008). Although most *Acinetobacter* species can grow at ambient temperatures, the pathogenic species, *A. baumannii* grows very well at 37℃. For recovering *Acinetobacter* isolates from complex samples, enrichment medium such as Leeds selective medium is used (Doi et al., 2011). However,

Baumann et al (1968) reported that *Acinetobacter*s from environmental and clinical specimens could be recovered by growing aerobically in an acidic enrichment medium containing acetate as a carbon source and nitrate as nitrogen source. *A. baumannii* has been reported to possess a thick cell wall which protects the organism from dry conditions, besides providing high tolerance to temperature, pH and nutrient changes (Vila et al., 2007; Kramer et al., 2006; Houang et al., 1998).

3. Natural Habitats and Sources

Water and soil are considered as major habitats of *A. baumannii* although the pathogen has been isolated from other sources, including foods, arthropods, animals, and humans (Baumann et al 1968; Berlau et al., 1999; Mussi et al., 2005; Fournier and Richet, 2006; Munoz-Price and Weinstein, 2008). Moreover, protozoans such as *Acanthamoeba* are reported to support the growth of *A. baumannii*, and act as its reservoir in water (Cateau et al 2011). Recently, studies indicated the potential presence of *A. baumannii* in water systems from multiple parts of the world (Catel-Ferreira et al., 2011; Zhang et al., 2013).

Although a limited number of studies have revealed the presence of *A. baumannii* on a variety of foods, the pathogen has not been implicated in any food-borne disease outbreaks. In a study conducted in South Korea, *A. baumannii* strains showing different antimicrobial resistance patterns were isolated from bulk tank milk (Gurung et al., 2013). Out of the 2,287 bulk tank milk (BTM) sampled from different provinces in Korea, *Acinetobacter* spp. were isolated from 176 BTM samples, of which 57 isolates were identified as *A. baumannii*. The presence of *Acinetobacter* spp. in BTM has been attributed to the contamination from environmental sources such as teat, udder surfaces, milking machines, and contaminated water used for cleaning. This could represent a potential source for resistance genes as they may survive pasteurization (Jain

and Danziger, 2004). Similarly, the isolation of *Acinetobacter* spp. from goat milk samples from Kenya at a rate of 5% was reported (Ndegwa et al., 2001).

Berlau and colleagues (1999), while studying the prevalence of *Acinetobacter* spp. in fresh produce in the United Kingdom, reported that 17% of the samples tested positive for the bacteria, including *A. baumannii*. These investigators observed that 56% of the *Acinetobacter* belonged to *A.baumannii-calcoaceticus* group, which were found on apple, melon, bean, cabbage, cauliflower, carrot, potato, radish, lettuce, cucumber, pepper, mushroom, and sweet corn. Houang et al. (2001) found *A. baumannii* and genomospecies 3 on vegetables, pork, beef, and freshwater fish procured from local markets in Hong Kong. *Acinetobacter* spp. was present in 27 of the 36 meat samples, 21 of 41 vegetable samples, and 26 of 38 fish samples screened. In another study, Gennari and Lombardi (1993) observed that out of 170 strains isolated from foods, including fresh and spoiled meat and fish, vegetables, raw milk and cheese, *A. lwoffii* was confirmed in over half of the isolates, whereas 11% and 5% of the isolates were *A. johnsonii* and *A. baumannii*, respectively indicating that foods could be a potential source of *A. baumannii*. In a recent study from Switzerland, Lupo et al. (2014) isolated *A. baumannii* from 25% of the meat samples, where 48 % of the positive food samples were poultry meat. The isolated strains displayed resistance to third and fourth generation cephalosporins, which are currently used for treating human infections.

A. baumannii has been reported to colonize the skin, nostrils, and throat of healthy people (Seifert, et al., 1997; Chu et al., 1999; Dijkshoorn et al., 2005; Montefour et al., 2008). A common route for spread of *A. baumannii* in the hospital environment has been reported to be unclean hands of the hospital staff (Jalalpour and Abdol, 2012). Further, it has been indicated that *A. baumannii* strains once colonized can remain for weeks in the body of patients

(Dijkshoorn et al., 2007).Therefore, stringent personal hygiene and cleanliness are critical for preventing the pathogen transmission (Allegranzi et al., 2009). Besides humans, La Scola and Raoult (2004) reported that 22% of body lice sampled from homeless people tested positive for *A. baumannii*, thereby suggesting that these parasites could also act as a potent reservoir of the pathogen.

Another major source of *A. baumannii* is the hospital environment, where its persistence is attributed to the ability to grow under various temperatures and pH conditions, and more importantly to tide over unfavourable conditions such as desiccation, nutrient starvation and treatment with disinfectants (Wendt et al., 1997; Perez et al., 2007; Wisplinghoff et al., 2007; Gaddy and Actis, 2009). This is achieved by the ability of *A. baumannii* to form biofilms on abiotic surfaces in the hospital environment, which protects the pathogen from disinfectants, thereby helping it to persist for a long duration and act as a permanent source of infection (Gaddy et al., 2009; Shin et al., 2009; Moultrie et al., 2011; Pour et al., 2011). In health-care environments, a variety of surfaces, including tabletops, bed rails, sinks, door handles, floors, mattresses, and pillows are implicated as potential sources of *A. baumannii* (Wilks et al., 2006; Karageorgopoulos and Falagas, 2008). It was reported that the use of contaminated medical devices such as catheters, sutures, ventilators can also serve as *A. baumannii* sources in hospitals (Montefour et al., 2008; Rao et al., 2008; Lee et al., 2008), and lead to subsequent colonization of the organism in the respiratory tract, blood, pleural fluid, urinary tract, surgical wounds, skin and eyes of patients (Bayuga et al., 2002; Gusten et al., 2002; Lorente et al., 2004). Thus the persistence of *A. baumannii* in the hospital environment is regarded as the most important factor aiding in the transmission of infection in nursing homes and nosocomial settings (Denton et al., 2005; Rastogi et al., 2007; Karageorgopoulos and Falagas, 2008).

4. Pathogenicity of Acinetobacter infections and virulence

The pathogenesis of *A. baumannii* infections is not completely understood, and hence the mechanisms involved in its colonization, infection and systemic spread are yet to be fully elucidated. A number of putative mechanisms having specific roles in colonization, infection and epidemic spread have been illustrated. These mechanisms include adherence to host cells, resistance to desiccation, disinfectants, and antibiotics, biofilm formation, quorum sensing, and elicitation of inflammatory responses and cytotoxicity (Dijkshoorn et al., 2007).

Bacterial adherence to epithelial cells is a critical initial step in the process of colonization and infection (Beachey, 1981). The study conducted by Choi et al. (2008) found that *A. baumannii* has the potential to invade epithelial cells and its invasion of cells varies with the type of host cells. It was observed that respiratory tract epithelial cells were the most susceptible to invasion by *A. baumannii* compared to other epithelial cells. The various known virulence factors potentially contributing to *A. baumannii* pathogenesis are discussed below.

4.1. Cell surface hydrophobicity and enzymes

A. baumannii possesses significant cell hydrophobicity, which helps it adhere to host cells and evade phagocytosis, besides helping in successful attachment to plastic or polymer surfaces such as catheters (Doughari et al., 2011). The presence of a rough cell surface and protein protrusion on the surface of *Acinetobacter* reportedly confers hydrophobicity (Phuong et al., 2009; Rosenberg et al., 1980). In addition, cell surface enzymes have been found to influence the hydrophobicity in *A. baumannii*. Boujaafar et al. (1990) observed that *A. baumannii* isolates from catheters and tracheal devices possessed a greater hydrophobicity than environmental isolates.

4.2. Toxic slime polysaccharides

The production of toxic slime polysaccharides in *Acinetobacter* spp. have been reported by Hoštacká (2002). These polysaccharides are composed of glucose building blocks, glucuronic acid, D-mannose, L-ramnose and D-glucose, which are produced during the exponential stage of *A. baumannii* growth. Besides exerting a toxic effect on neutrophils, the slime inhibits their migration and phagocytosis. Moreover, capsular polysaccharides produced by *A. baumannii* help the bacterium in evading the host immune response, and growing in serum (Russo et al., 2011). In general, slime producing *Acinetobacter* strains have been found to be more virulent than non-producers (Obana, 1986).

4.3. Outer membrane protein

Outer membrane proteins (Omp) play an important in the pathogenesis of many Gram-negative bacteria. Outer membrane protein A (OmpA, 38 kDa) has been identified in several strains of *Acinetobacter*, especially *A. baumannii* (Dijkshoorn, et al., 2007; Gordon and Wareham, 2010). During infection, *A. baumannii* OmpA binds to eukaryotic cells and subsequently gets translocated into the nucleus, causing cell death (Dorsey et al., 2003; Dijkshoorn et al., 2007; Choi et al., 2008). It was also shown that OmpA of *A. baumannii* induces early onset of apoptosis and delays necrosis in dendritic cells. In addition, OmpA has been reported to play a role in biofilm formation, surface motility and serum resistance in *A. baumannii* (McConnell et al., 2013). Besides OmpA, $Omp_{33\text{-}36}$ is another outer membrane protein shown to be critical for *A. baumannii* virulence by inducing apoptosis of host cells by activation of caspases and modulation of autophagy (Rumbo et al., 2014). In addition, cell surface components such as adhesins and fimbriae (pili) have been reported to play a role in *A.*

baumannii attachment to host cell as well as inanimate surfaces (Smith et al., 2007; Braun, 2008; Musafer and Essa, 2013; Eijkelkamp et al., 2013).

4.4. Surface and mitochondrial porins

Porins are proteins that form channels enabling the transport of molecules across the lipid bilayer membrane of bacterial cells. Depending upon the bacterial species, porins play an important role in the maintenance of cellular structural integrity, bacterial conjugation, bacteriophage binding and antimicrobial resistance (Vaara et al., 1992). Moreover, pore formation facilitates the passage of small molecules into the bacterial cell (Braun, 2008). Pores as outer membrane structures, are an important part of efflux pumps, thereby aiding in the exclusion of toxic materials from the cell. Vila et al. (2007) indicated that porins in *A. baumannii* coupled with efflux pumps create an important barrier for the uptake of antibiotics.

4.5. Siderophores

The capacity of a bacterium to acquire iron for its growth in a host is considered as a critical virulence determinant. Siderophores are host iron-binding proteins which assist in iron uptake by the producing bacteria. *Acinetobacter* siderophores are called as acinetobactins (Mihara et al., 2004). *Acinetobacters* are capable of synthesizing siderophores, which can convert polymeric ferric oxy-hydroxides to soluble iron chelates under low iron stress (Neilands, 1995). *A. baumannii* can acquire ferric ions under limited iron conditions through siderophores, and this is reported to play a critical role in its virulence. Gaddy et al. (2012) observed that acinetobactin mediated iron acquisition system in *A. baumannii* resulted in human epithelial cell damage and mortality in *Galleria mellonella* caterpillars and infected mice. Further, clinical strains of *A. baumannii* have been found to grow under iron deficient conditions, and were

shown to excrete an iron-regulated catechol siderophore into culture supernatants (Echenique et al., 1992; Actis et al., 1993).

In addition to the aforementioned virulence factors, a variety of enzymes produced by *A. baumannii* have been reported to a play role in its virulence. For example, mutants of *A. baumannii* in phopsholipases were found to possess decreased serum resistance and invasion of host cells, and reduced pathogenesis in a murine model of pneumonia (Jacobs et al., 2010; Stahl et al., 2015). Similarly, enzymes such as butyrate esterase, caprylate esterase, and leucine arylamidase are believed to be involved in the hydrolysis of short chain fatty acids, thereby causing damage to host tissue lipids (Jolly-Guillou and Brun Buisson, 1996; Rathinavelu et al., 2003; Towner, 2006). Besides these factors, biofilm formation and antibiotic resistance contribute to *A. baumannii* virulence.

5. Biofilm Formation

Biofilm is a complex material, composed of microorganisms attached to surfaces and enclosed in a hydrated polymeric matrix of three dimensional structures. The matrix comprises of polysaccharides, proteins and nucleic acids (Vidal et al., 1996; Costerton et al., 1999; Sauer et al., 2007). *A. baumannii* can persist for prolonged periods of time in the hospital environment in biofilms, thereby insulating it from disinfectants, and serving as a continuous source of infection. Besides increasing the potential for nosocomial spread, biofilm production in *A. baumannii* also contributes to antibiotic resistance. The adherence of *A. baumannii* to biotic and abiotic surfaces facilitates the development of biofilms, which is responsible for the persistence of the pathogen in medical settings as well as development of resistance to antibiotics (Donlan and Costerton, 2002; Gaddy and Actis, 2009). The major factors that regulate biofilm synthesis in *A. baumannii* include nutrient availability, surface structures such as pili, outer membrane proteins, quorum

sensing, and macromolecular substances such as DNA and polysaccharides (Gaddy and Actis, 2009; Cerqueira and Peleg, 2011).

A. baumannii pili are encoded by the *csu*/BABCDE chaperone-usher assembly system, which is controlled by a two-component regulatory system encoded by *bfmS* and *bfmR* (Cerqueira and Peleg, 2011; Luo et al., 2015). Transposon mutagenesis revealed that *csuA* and *csuE* are critical for bacterial attachment, biofilm production and pili synthesis (Tomoaras et al., 2003). Subsequent research by the same researchers indicated that inactivation of *bfmR* response regulator led to absence of *csu* expression, with a lack of pili production and biofilm formation in *A. baumannii*. However, mutation in *bfmS* was found to impair biofilm synthesis only partially. Another surface protein called biofilm-associated protein (Bap) is also associated with biofilm production in *A. baumannii*; mutation of this gene reduced biofilm thickness by more than 50% compared to wild type (Loehfelm, 2008). Similarly, OmpA has been found to be critical for forming thick biofilms, especially on polystyrene surface (Gaddy et al., 2009). In addition, exopolysaccharides, particularly poly-β-(1-6)-N-acetylglucosamine (PNAG) have been reported to be necessary for the integrity of *A. baumannii* biofilms formed under dynamic and stressful environments (Choi et al., 2009). Further, PNAG is believed to play a role in cell-to-cell adhesion (Cramton et al., 1999). Besides the aforementioned factors, quorum sensing, a well-studied cell-to-cell communication mechanism in bacteria, has also been observed to regulate biofilm synthesis in *A. baumannii*. The quorum sensing molecule, 3-hydroxy-C_{12}-HSL, encoded by the autoinducer synthase gene, *abaI* is critical for later stages of biofilm production, especially on abiotic surfaces (Niu et al., 2008).

Several researchers have observed that the clinical isolates of *A. baumannii* are found to be strong biofilm formers (Rodriguez-Baño et al., 2008; King et al., 2009; Eijkelkamp et al.,

2011; Gurung et al., 2013). Besides increasing the potential for nosocomial spread, biofilm production in *A. baumannii* contributes to antibiotic resistance and increased virulence. For example, clinical isolates of MDR *A. baumannii* demonstrated a strong ability to produce biofilms, which correlated to increased attachment to human bronchial epithelial cells (Lee et al., 2008; Rao et al., 2008; Shin et al., 2009; Gordon et al., 2010). Therefore, inhibiting biofilm production in *A. baumannii* is crucial for controlling its transmission in hospitals as well as reducing its antibiotic resistance and virulence.

6. Clinical manifestations of A. baumannii infections

Being an opportunistic pathogen, *A.baumannii* generally affects critically ill patients in intensive care units (ICUs), specifically those depended on mechanical ventilation or in patients with wound or burn injuries. It causes infections like ventilator-associated pneumonia, skin and soft-tissue wound infections, urinary tract infections, peritonitis, secondary meningitis and bloodstream infections (Bergogne-Bérézin and Towner, 1996; Peleg et al., 2008). The major risk factors that predispose to *A. baumannii* infection include prolonged period of hospitalization, residence in an intensive care unit, receipt of mechanical ventilation, use of central venous or urinary catheters, exposure to broad-spectrum antimicrobial agents, recent surgery, invasive procedures, and underlying disease conditions (Saulnier et al., 2001; Fournier et al., 2006; Playford et al., 2007; Montefour et al., 2008). However, the persistence of *A. baumannii* in the hospital environment is the most important factor aiding in the transmission of infection in nursing homes and nosocomial settings (Simor et al., 2002; Denton et al., 2005; Rastogi et al., 2007). In addition, community-acquired *A. baumannii* pneumonia Guner et al., 2011; Silva et al., 2012), bacteremia (Obaro et al., 2011), urinary tract infection (Solak et al., 2011), and meningitis (Ozaki et al., 2009) have been reported.

Hospital acquired pneumonia was reported to be 15% of all nosocomial infections, with *A. baumannii* being considered as an important pathogen associated with high mortality in patients suffering from pneumonia (Fagon et al., 1989; Timsit et al., 1996). A report from the Centers for Disease Control and Prevention (CDC) showed that the percentage of incidence of *Acinetobacter*-associated ICU-acquired pneumonia increased from 4% in 1986 to 7% in 2003 (Gaynes and Edwards, 2005). This is of concern especially in immunocompromised patients admitted to ICUs and in surgical wards. The high incidence of hospital-acquired pneumonia is attributed to the use of *A. baumannii* contaminated ventilators and endotracheal tubes (Cefai et al., 1990; Bernards et al., 2004). Pneumonia caused by *Acinetobacter* are similar to those caused by Gram-negative bacteria, with symptoms such as fever, leukocytosis, purulent sputum production and appearance of new infiltrates on radiograph or CT scan. Further, the pathogen could be isolated from bronchial brushings (Chaste et al., 1996).

A. baumannii has been reported as the tenth most common etiologic agent causing nosocomial ICU-acquired bloodstream infections (BSI) in the United States (Wisplinghoff, 2000). The overall mortality from *A. baumannii* BSIs ranges from 34 to 43% in the ICU and 16% in patients outside the ICU (Peleg et al., 2008). In a study investigating the clinical and epidemiological features of nosocomial BSIs caused by *Acinetobacter* species at 49 US hospitals during the period from March 1995 through February 1998, Wisplingoff et al. (2000) reported that *Acinetobacter* species were found in 24 hospitals (49%) accounting for 1.5% of all nosocomial BSIs. Of the 129 *Acinetobacter* isolates, 111 were *A. baumannii* and 18 belonged to other *Acinetobacter* species. In addition, 30% of the isolates were resistant to multiple antibiotics. A report from South Korea indicated that *A. baumannii* was responsible for 112 cases of bacteremia in patients, whereas other *Acinetobacter* species such as *A. lwoffii*, *A.*

haemolyticus, and *A. calcoaceticus* were the causative agents of BSI in 28 patients (Choi et al., 2006).

Although *A. baumannii* rarely causes meningitis in community settings, it is increasingly reported to be associated with post-neurosurgical meningitis, especially with mortality rates as high as 64% (Garcia-Garmendia et al., 2001). In a study involving 281 adult patients with nosocomial bacterial meningitis, 3.6% were diagnosed with *Acinetobacter* spp. meningitis (Kim et al., 2009). Likewise, two studies from Turkey reported *Acinetobacter* spp. as the leading cause of Gram-negative post-neurosurgical meningitis (Sacar et al., 2006; Metan et al., 2007).

Frequent distribution of *Acinetobacter* species on human skin has been reported to predispose infections involving skin and soft tissues (Seifert et al., 1997). *A. baumannii* was found responsible for 2.1% of ICU–acquired skin/soft tissue infections (Gaynes and Edwards, 2005). *A. baumannii* was also isolated from wounds of combat casualties from Iraq and Afghanistan (Scott et al., 2007; Murray and Hospenthal, 2008; Peleg et al., 2008). In addition, *A. baumannii* is associated with persistent wound infections in burn victims with delayed wound healing (Bergogne-Berezin, 1995; Santucci et al., 2003; Sharma, 2007).Besides the above conditions, *A. baumannii* has been implicated in catheter-associated urinary tract infections (Gaynes and Edwards, 2005), endocarditis (Olut and Erkek, 2005; Menon et al., 2006), and eye infections (Kau et al., 2002; Levy et al., 2005).

7. Antibiotic resistance

Acinetobacter is considered multi-drug resistant (MDR) if it is resistant to at least three classes of antimicrobial agents. *A.baumannii* has emerged into a MDR bacterium difficult to treat due to its resistance to most of the currently available antibiotics. In fact, it is often viewed as the most difficult antimicrobial-resistant Gram-negative bacillus to control and treat (Maragakis and

Perl, 2008). Carbapenems were once the first choice antibiotics for treating *A. baumannii*, but are no longer completely effective due to resistance development by the bacterium (Falagas and Karaveli 2007; Bassetti et al., 2008). Similarly, polymyxins were successfully used to treat *A. baumannii* infections a few years ago, however, strains resistant to these drugs have also evolved (Hernan et al., 2009). Thus, the resistance of *A. baumannii* to multiple antibiotics presents a great challenge for health-care professionals for controlling infections associated with this pathogen.

A. baumannii possesses a variety of antibacterial resistance mechanisms. These mechanisms include intrinsic and extrinsic ones, and multiple factors are reported to favour the acquisition of multidrug-resistance in *A. baumannii* (Vila and Pachon, 2008). These include the ability of the bacterium to persist in environmental and human reservoirs, which aids in the transfer of genetic materials between other microorganisms and *A. baumannii*, capacity to acquire a wide variety of genetic elements, including plasmids, transposons and integrons (Vila and Pachon, 2008).

Besides the intrinsic mechanisms conferring resistance to aminopenicillins, cephalosporins and chloramphenicol, *A. baumannii* has acquired mechanisms that impart resistance to β-lactam antibiotics, aminoglycosides, tetracyclines and fluoroquinolones (Dijkshoorn, et al., 2007). In addition, expression of β-lactam hydrolyzing enzymes, induced changes in the outer membrane proteins, up-regulation of multiple drug efflux pumps, altered penicillin binding proteins (PBP), antibiotic modifying enzymes, machineries for modification of antibiotic target binding sites, ribosomal protection, and porin channel deletions have also been reported in *A. baumannii* (Gordon and Wareham., 2010).

The transportation of antimicrobial agents into the bacterial cell and their subsequent effect are controlled by porin channels and other outer membrane proteins in bacteria. The lack

of these outer membrane proteins and channels, thus preventing antimicrobials from reaching their targets resulting in resistance has been reported in *Acinetobacter* spp. (Thomson and Bonomo, 2005; Bou et al 2000; Mussi et al., 2005). Li et al. (2005) reported the development of colistin resistance in *Acinetobacter* due to changes in the bacterial membrane that hinder the action of the antibiotic on the bacterial targets. In addition, the efflux pumps which aid *Acinetobacter* spp. in the removal of antimicrobial agents from bacterial cells have been observed (Bonomo and Szabo, 2006). The efflux pumps are membranes embedded proteins which function by excluding the antibiotic out of the cell. The reported efflux pump systems include AdeABC (Magnet et al., 2001), AdeIJK (Damier-Piolle et al., 2008), AdeXYZ (Roca et al., 2011), AdeM (Su et al., 2005),Tet(A) and Tet(B) (Guardabassi et al., 2000; Ribera et al., 2003a; Ribera et al., 2003b). Schaack et al. (2010) reported that the over expression of AdeABC efflux pump causes resistance to cephalosporins, fluoroquinolones, and tigecycline. AbeM efflux pump confers resistance aminoglycoside and fluoroquinolone (Gootz and Marra, 2008), whereas Tet(A) and Tet(B) and CmlA encode for resistance to tetracyclines and chloramphenicols, respectively (Bergogne-Berezin et al., 2008).

Exceeding 50 different degrading enzymes or their allelic forms have been found to confer resistance to β–lactam antibiotics, including extended spectrum β-lactamases, oxacillinases and metallo-β-lactamases (Bergogne-Be're'zini and Towner., 1996). These multiple β-lactamases confer resistance to different classes of β-lactam antibiotics such as penicillin derivatives, cephalosporins and carbapenems in *A. baumannii*. The various classes of β-lactamases include class A β-lactamases (Nagano et al., 2004; alNaiemi et al.,2005; Pasteran et al., 2006), class B β-lactamases (metallo β-lactamases) (Chu et al., 2001;Lee et al.,2003; 2005), class C β-lactamases (eg. *Acinetobacter*-derived cephalosporinases or the chromosomally-

encoded AmpC enzymes) (Bou et al 2000) and class D β-lactamases responsible for resistance of carbapenems through the production of oxacillinases (Peleg et al., 2008). In addition, class A extended spectrum beta lactamases (ESBLs) are also present in *A. baumannii* (Vila et al., 1993). Additionally, the presence of PER-1, an ESBL in *A. baumannii* which imparts a high level of resistance to penicillins and extended-spectrum cephalosporins, but not to carbapenems has been detected (Yong et al., 2003; Sechi et al., 2004; Farajnia et al., 2013).

A few *Acinetobacter* species expressing VIM and IMP β-lactamases capable of hydrolyzing different antimicrobial agents, including carbapenems have been detected (Thomson and Bonomo, 2005). Researchers commented that since the metallo-β-lactamases are located on mobile genetic elements, the potential for their transfer from one bacterium to another is high (Thomson and Bonomo, 2005; Bonomo and Szabo, 2006). AmpC cephalosporinases which come under class C β-lactamases are chromosomally encoded and confer resistance to broad-spectrum cephalosporins (Thomson and Bonomo, 2005; Bonomo and Szabo, 2006). Similarly, class D OXA type enzymes (β-lactamases with carbapenem-hydrolysing properties) were also reported in *A. baumannii* from various countries (Brown and Amyes, 2006; Afzal-Shah et al., 1999; Opazo et al., 2011; 2012).

Resistance to aminoglycoside antibiotics through their modification by integron-associated or plasmid-encoded genes, which encode acetyltransferases, nucleotidyltransferases and phosphotransferases is documented in MDR *A. baumannii*. Moreover, 16S rRNA methylation causing impaired binding of gentamicin, tobramycin and amikacin to their target sites, thereby leading to their resistance is documented (Beceiro et al., 2013). Besides these machineries, gentamicin and kanamycin can be effluxed out by AbeM efflux pump in *A. baumannii* (Vila et al., 2007). Mutations in *gyrA* and *parC* resulting in the modification of DNA

gyrase or topoisomerase IV is the major underlying mechanism for quinolone resistance in *A. baumannii* (Park et al., 2011). Chromosomal mutations in the quinolone resistance determining region (QRDR) of the *gyrA* and *parC*, and resulting production of modified bacterial DNA gyrase and topoisomerase IV enzymes has been reported to confer resistance to quinolones in *A. baumannii* (Peleg et al., 2008). A decreased influx and/or increased efflux of quinolones can also contribute to quinolone resistance (Peleg et al., 2008). However, as an adjunct, involvement of AdeABC and AdeM pumps also play a role in quinolone resistance (Chopra and Galande., 2011).

8. Plant-derived antimicrobials

Plant-derived antimicrobials (PDAs) are secondary metabolites produced by plants possessing potential antibacterial activity against a wide range of microbial pathogens and food spoilage bacteria (Burt, 2004; Holley and Patel; 2005; Upadhyay et al., 2014). These secondary metabolites are generally aromatic compounds comprising alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones and coumarins (Das et al., 2010). A majority of these compounds are produced by plants as a defence mechanism against by microorganisms and insects (Cowan, 1999). In many cultures, PDAs have been used as dietary constituents and ingredients in herbal and traditional medicines (Wollenweber and Kinzel, 1988). The general mechanisms of antimicrobial effect of PDAs include increasing permeability of the bacterial membrane, disintegration of bacterial cytoplasmic membrane, and destabilization of the proton motive force, electron flow, active transport and coagulation of the cell content (Burt, 2004).

Trans-cinnamaldehyde (TC) is an aldehyde present in the bark extract of cinnamon. Trans-cinnamaldehyde has a wide margin of safety between conservative estimates of intake, with no observed adverse effects, including genotoxic and mutagenic effects from sub chronic

and chronic studies (Adams et al., 2004). Trans-cinnamaldehyde possesses antimicrobial activity towards a wide range of Gram-positive and Gram-negative bacteria (Bowles and Miller, 1993; Bowles et al., 1995; Friedman et al., 2002). Besides the deleterious effect exerted on the bacterial membrane, TC acts by depleting intracellular ATP by inhibiting ATPase supported energy metabolism along with inhibition of glucose uptake and its utilization (Gill and Holley,2004; Oussalah et al.,2006; Negi, 2012). Eugenol (EG) is a natural molecule present as an active ingredient in the oil from cloves (*Eugenia caryophillis*) (Ali et al., 2005; Yadav et al., 2013). The antibacterial activity of clove oil and eugenol has been documented by many researchers (Menon and Garg, 2001; Hyldgaard et al., 2012). Eugenol has been found to cause cell wall degradation and cell lysis in bacteria (Thoroski, 1989). Both TC and EG are classified as GRAS by the FDA (TC, 21 CFR 182.60 (CFR, 2011a); EG, 21 CFR 582.60 (CFR, 2011b).

Although considerable amount of literature exists on the antimicrobial properties of several PDAs on a wide range of microorganisms, comparatively limited information is available on their effect on bacterial antibiotic resistance, especially in Gram-negative pathogens. A handful of studies have revealed that PDAs could be used to potentiate the effect of antibiotics in bacteria. Previously, Chusri et al. (2009) observed that two plant molecules, ellagic acid and tannic acid when used as adjuvants enhanced the antimicrobial activity of several antibiotics against *A. baumannii*. These investigators reported that ellagic and tannic acids at 40 µM, increased the activity of novobiocin, coumermycin, chlorobiocin, rifampicin and fusidic acid against *A. baumannii*. Likewise, Duarte et al. (2012) reported that the essential oil from coriander exerted synergistic or additive effects with antibiotics such as tetracycline, chloroamphenicol, ciprofloxacin, gentamicin, piperacillin and cefoperazone against *A. baumannii*. In addition, a recent study by Saghi et al. (2015) reported that the essential oil from *Origanum vulgare*

possessed potent antimicrobial activity against MDR *A. baumannii*. In yet another study, Kollanoor-Johny et al. (2010) reported that sub-inhibitory concentrations of several PDAs, including TC and EG decreased antibiotic resistance ($P < 0.05$) in MDR *S.* Typhimurium DT 104. Follow up mechanistic studies using RT-qPCR revealed that TC decreased antibiotic resistance in DT 104 by down-regulating several antibiotic resistance genes.

In Gram-positive bacteria, Blanco et al. (2005) reported that epigallocatechin-gallate, an ingredient in green tea, neutralized tetracycline resistance in *Staphylococcus aureus* by inhibiting the efflux pump, TetK. Similarly, biacalein, a flavalone present in thyme extract potentiated the effect of tetracyclines and β-lactams on methicillin-resistant *S. aureus* (Fujita et al., 2005). In another study, two ingredients in the medicinal plant Berberis, namely berberine and 5'-methoxyhydnocarpin, inhibited the multi-drug efflux pump, NorA in *S. aureus* (Sternmitz et al., 2000). Likewise, TC was reported to reduce clindamycin resistance in the human pathogen, *Clostridium difficile* (Shahverdi et al., 2007).

9. Summary

Being ubiquitous in distribution, *A. baumannii* has been isolated from soil, water and a variety of foods, including fruits and vegetables, thereby raising concerns that fresh produce could be a potential source of infection to humans, particularly in health-care settings. In addition, the isolation of genotypically similar strains of *Acinetobacter* from food, environment and clinical specimens further underscores the concerns on the role of food and water as potential sources of human infections. Being resistant to most of the currently available antibiotics, identification of effective antimicrobials or molecules that potentiate the efficacy of antibiotics against *A. baumannii* is critical for controlling infections caused by this bacterium.

Based on published literature and preliminary research conducted in our laboratory, it was hypothesized that MDR *A. baumannii* is potentially present on fresh vegetables, and chlorine is ineffective as a water disinfectant forkilling the bacterium. In addition, it was hypothesized that TC and EG reduce antibiotic resistance and virulence in *A. baumannii*, and may be useful in treating wound infections caused by the bacterium.

The specific objectives of this dissertation were

1: To investigate the prevalence of MDR *A. baumannii* on fresh vegetables collected from farmers' market in Connecticut.

2: To determine the effect of chlorine on *A. baumannii* survival in water and expression of its antibiotic resistance genes

3: To develop a real-time quantitative PCR for specific detection of *A. baumannii* in water and blood.

4: To determine the effect of TC and EG on decreasing *A. baumannii* resistance to β-lactam antibiotics.

5: To investigate the efficacy of TC and EG in reducing *A. baumannii* adhesion to and invasion of human keratinocytes and controlling wound infections *in vitro.*

REFERENCES:

Actis, L.A., Tolmasky, M.E., Crosa, L.M., Crosa, J.H.,1993. Effect of iron-limiting conditions on growth of clinical isolates of Acinetobacter baumannii. Journal of clinical microbiology 31, 2812-2815.

Adams, T.B., Cohen, S.M., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Munro, I.C., Portoghese, P.S., Smith, R.L., Waddell, W.J.,2004. The FEMA GRAS assessment of cinnamyl derivatives used as flavor ingredients. Food and chemical toxicology 42, 157-185.

Afzal-Shah, M., Villar, H.E., Livermore, D.M.,1999. Biochemical characteristics of a carbapenemase from an Acinetobacter baumannii isolate collected in Buenos Aires, Argentina. The Journal of antimicrobial chemotherapy 43, 127-131.

Albrecht, M. C., M. E. Griffith, C. K. Murray, K. K. Chung, E. E. Horvath, J. A. Ward, D. R. Hospenthal, J. B. Holcomb, and S. E. Wolf. 2006. Impact of *Acinetobacter* infection on the mortality of burn patients. J. Am. Coll. Surg. 203**:**546-550

al Naiemi, N., Duim, B., Bart, A.,2006. A CTX-M extended-spectrum beta-lactamase in Pseudomonas aeruginosa and Stenotrophomonas maltophilia. Journal of medical microbiology 55, 1607-1608.

Ali, S.M., Khan, A.A., Ahmed, I., Musaddiq, M., Ahmed, K.S., Polasa, H., Rao, L.V., Habibullah, C.M., Sechi, L.A., Ahmed, N.,2005. Antimicrobial activities of Eugenol and Cinnamaldehyde against the human gastric pathogen Helicobacter pylori. Annals of clinical microbiology and antimicrobials 4, 20.

Allegranzi, B., Memish, Z.A., Donaldson, L., Pittet, D.,2009 Safety, World Health Organization Global Patient, on Religious, Challenge Task Force, Religion and culture: potential undercurrents influencing hand hygiene promotion in health care. American Journal of Infection Control 37, 28-34.

Barker, J., Maxted, H.,1975. Observations on the growth and movement of Acinetobacter on semi-solid media. Journal of medical microbiology 8, 443-446.

Bassetti, M., Righi, E., Esposito, S., Petrosillo, N., Nicolini, L.,2008. Drug treatment for multidrug-resistant Acinetobacter baumannii infections.

Baumann, P., Doudoroff, M., Stanier, R.Y.,1968. A study of the Moraxella group. II. Oxidative-negative species (genus Acinetobacter). Journal of Bacteriology 95, 1520-1541.

Bayuga, S., Zeana, C., Sahni, J., Della-Latta, P., El-Sadr, W., Larson, E.,2002. Prevalence and antimicrobial patterns of Acinetobacter baumannii on hands and nares of hospital personnel and patients: the iceberg phenomenon again. Heart & Lung: The Journal of Acute and Critical Care 31, 382-390.

Beachey, E.H.,1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface. The Journal of infectious diseases 143, 325-345.

Beceiro, A., Tomas, M., Bou, G.,2013. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clinical microbiology reviews 26, 185-230.

Bergogne-Berezin, E.,1995. The increasing significance of outbreaks of Acinetobacter spp.: the need for control and new agents. journal of Hospital Infection 30, 441-452.

Bergogne-Bérézin, E., Friedman, H., Bendinelli, M., 2008. Acinetobacter: Biology and Pathogenesis, Springer Science & Business Media,

Bergogne-Berezin, E., Towner, K.J.,1996. Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clinical microbiology reviews 9, 148-165.

Berlau, J., Aucken, H., Houang, E., Pitt, T.,1999. Isolation of Acinetobacter spp including A. baumannii from vegetables: implications for hospital-acquired infections. Journal of Hospital Infection 42, 201-204.

Bernards, A., Harinck, H., Dijkshoorn, L., van der Reijden, T., Van den Broek, P.,2004. Persistent Acinetobacter baumannii? Look inside your medical equipment. Infection Control 25, 1002-1004.

Blanco, A.R., Sudano-Roccaro, A., Spoto, G.C., Nostro, A., Rusciano, D.,2005. Epigallocatechin gallate inhibits biofilm formation by ocular staphylococcal isolates. Antimicrobial Agents and Chemotherapy 49, 4339-4343.

Bonomo, R.A., Szabo, D.,2006. Mechanisms of multidrug resistance in Acinetobacter species and Pseudomonas aeruginosa. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 43 Suppl 2, S49-56.

Bou, G., Cervero, G., Dominguez, M.A., Quereda, C., Martinez-Beltran, J.,2000. Characterization of a nosocomial outbreak caused by a multiresistant Acinetobacter baumannii strain with a carbapenem-hydrolyzing enzyme: high-level carbapenem resistance in A. baumannii is not due solely to the presence of beta-lactamases. Journal of clinical microbiology 38, 3299-3305.

Bou, G., Oliver, A., Martinez-Beltran, J.,2000. OXA-24, a novel class D beta-lactamase with carbapenemase activity in an Acinetobacter baumannii clinical strain. Antimicrobial Agents and Chemotherapy 44, 1556-1561.

Boujaafar, N., Freney, J., Bouvet, P., Jeddi, M.,1990. Cell surface hydrophobicity of 88 clinical strains of Acinetobacter baumannii. Research in microbiology 141, 477-482.

Bouvet, P.J., Grimont, P.A.,1986. Taxonomy of the genus Acinetobacter with the recognition of Acinetobacter baumannii sp. nov., Acinetobacter haemolyticus sp. nov., Acinetobacter johnsonii sp. nov., and Acinetobacter junii sp. nov. and emended descriptions of Acinetobacter calcoaceticus and Acinetobacter lwoffii. International Journal of Systematic Bacteriology 36, 228-240.

Bouvet, P., Jeanjean, S.,1989. Delineation of new proteolytic genomic species in the genus Acinetobacter. Research in microbiology 140, 291-299.

Bowles, B.L., Miller, A.J.,1993. Antibotulinal properties of selected aromatic and aliphatic aldehydes. Journal of Food Protection® 56, 788-794.

Bowles, B.L., Sackitey, S.K., Williams, A.C.,1995. Inhibitory effects of flavor compounds on Staphylococcus aureus WRRC. Journal of Food Safety 15, 337-347.

Braun, G., 2008. Virulence mechanisms of Acinetobacter, Acinetobacter Biology and Pathogenesis. Springer,pp. 145-154.

Brisou, J., Prevot, A.R.,1954. Studies on bacterial taxonomy. X. The revision of species under Acromobacter group. Annales de l'Institut Pasteur 86, 722-728.

Brown, S., Amyes, S.,2006. OXA (beta)-lactamases in Acinetobacter: the story so far. The Journal of antimicrobial chemotherapy 57, 1-3.

Burt, S.,2004. Essential oils: their antibacterial properties and potential applications in foods—a review. International journal of food microbiology 94, 223-253.

Cateau, E., Verdon, J., Fernandez, B., Hechard, Y., Rodier, M.H.,2011. *Acanthamoeba* sp. promotes the survival and growth of Acinetobacter baumanii. FEMS microbiology letters 319, 19-25.

Catel-Ferreira, M., Coadou, G., Molle, V., Mugnier, P., Nordmann, P., Siroy, A., Jouenne, T., De, E.,2011. Structure-function relationships of CarO, the carbapenem resistance-associated outer membrane protein of Acinetobacter baumannii. The Journal of antimicrobial chemotherapy 66, 2053-2056.

CDC, 2004. Centers for Disease Control and Prevention. Acinetobacter baumannii infections among patients at military medical facilities treating injured U.S. service members, 2002–2004. MMWR Morb Mortal Wkly Rep 2004; 53:1063–6.

CDC, 2013.Centers for Disease Control and Prevention. Antibiotic resistance threats in the United States, 2013

Cefai, C., Richards, J., Gould, F., McPeake, P.,1990. An outbreak of Acinetobacter respiratory tract infection resulting from incomplete disinfection of ventilatory equipment. Journal of Hospital Infection 15, 177-182.

Cerqueira, G.M., Peleg, A.Y.,2011. Insights into Acinetobacter baumannii pathogenicity. IUBMB life 63, 1055-1060.

Chaste J., Trouillet J.L., Vuagnat A., and Joly-Guillou M.L. 1996. Nosocomial pneumonia caused by Acinetobacter spp. In Bergogne-berezin E., Joly-Guillou M.L., and Towner K.J. (eds) Acinetobacter – Microbiology, Epidemiology , Infection, Management. CRC Press, New York, Chap. 6, pp.117-132

Choi, S., Choo, E.J., Kwak, Y.G., Kim, M., Jun, J., Kim, M., Kim, N.J., Jeong, J., Kim, Y.S., Woo, J.H.,2006. Clinical characteristics and outcomes of bacteremia caused by Acinetobacter species other than A. baumannii: comparison with A. baumannii bacteremia. Journal of infection and chemotherapy 12, 380-386.

Choi, A.H., Slamti, L., Avci, F.Y., Pier, G.B., Maira-Litran, T.,2009. The pgaABCD locus of Acinetobacter baumannii encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. Journal of Bacteriology 191, 5953-5963.

Choi, C.H., Lee, J.S., Lee, Y.C., Park, T.I., Lee, J.C.,2008. Acinetobacter baumannii invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. BMC microbiology 8, 216-2180-8-216.

Chopra, I., Roberts, M.,2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and molecular biology reviews : MMBR 65, 232-60 ; second page, table of contents.

Chopra, S., Galande, A.,2011. A fluoroquinolone-resistant Acinetobacter baumannii without the quinolone resistance-determining region mutations. The Journal of antimicrobial chemotherapy 66, 2668-2670.

Chu, Y.W., Afzal-Shah, M., Houang, E.T., Palepou, M.I., Lyon, D.J., Woodford, N., Livermore, D.M.,2001. IMP-4, a novel metallo-beta-lactamase from nosocomial Acinetobacter spp. collected in Hong Kong between 1994 and 1998. Antimicrobial Agents and Chemotherapy 45, 710-714.

Chu, Y.W., Leung, C.M., Houang, E.T., Ng, K.C., Leung, C.B., Leung, H.Y., Cheng, A.F.,1999. Skin carriage of acinetobacters in Hong Kong. Journal of clinical microbiology 37, 2962-2967.

Chusri, S., Villanueva, I., Voravuthikunchai, S.P., Davies, J.,2009. Enhancing antibiotic activity: a strategy to control Acinetobacter infections. The Journal of antimicrobial chemotherapy 64, 1203-1211.

Clemmer, K.M., Bonomo, R.A., Rather, P.N.,2011. Genetic analysis of surface motility in Acinetobacter baumannii. Microbiology (Reading, England) 157, 2534-2544.

Costerton, J.W., Stewart, P.S., Greenberg, E.P.,1999. Bacterial biofilms: a common cause of persistent infections. Science (New York, N.Y.) 284, 1318-1322.

Cowan, M.M.,1999. Plant products as antimicrobial agents. Clinical microbiology reviews 12, 564-582.

Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W., Gotz, F.,1999. The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. Infection and immunity 67, 5427-5433.

Crowe, M., Towner, K.J., Humphreys, H.,1995. Clinical and epidemiological features of an outbreak of acinetobacter infection in an intensive therapy unit. Journal of medical microbiology 43, 55-62.

Damier-Piolle, L., Magnet, S., Bremont, S., Lambert, T., Courvalin, P.,2008. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy 52, 557-562.

Das, K., Tiwari, R., Shrivastava, D.,2010. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. Journal of medicinal plants research 4, 104-111.

Denton, M., Wilcox, M., Parnell, P., Green, D., Keer, V., Hawkey, P., Evans, I., Murphy, P.,2005. Role of environmental cleaning in controlling an outbreak of Acinetobacter baumannii on a neurosurgical intensive care unit. Intensive and Critical Care Nursing 21, 94-98.

Di Nocera, P.P., Rocco, F., Giannouli, M., Triassi, M., Zarrilli, R.,2011. Genome organization of epidemic Acinetobacter baumannii strains. BMC microbiology 11, 224-2180-11-224.

Dijkshoorn, L., Van Aken, E., Shunburne, L., Van Der Reijden, T., Bernards, A., Nemec, A., Towner, K.,2005. Prevalence of Acinetobacter baumannii and other Acinetobacter spp. in faecal samples from non- hospitalised individuals. Clinical microbiology and infection 11, 329-332.

Dijkshoorn, L., Nemec, A., Seifert, H.,2007. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. Nature Reviews Microbiology 5, 939-951.

Doi, Y., Onuoha, E.O., Adams-Haduch, J.M., Pakstis, D.L., McGaha, T.L., Werner, C.A., Parker, B.N., Brooks, M.M., Shutt, K.A., Pasculle, A.W., Muto, C.A., Harrison, L.H.,2011. Screening for Acinetobacter baumannii colonization by use of sponges. Journal of clinical microbiology 49, 154-158.

Donlan, R.M., Costerton, J.W.,2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical microbiology reviews 15, 167-193.

Dorsey, C.W., Beglin, M.S., Actis, L.A.,2003. Detection and analysis of iron uptake components expressed by Acinetobacter baumannii clinical isolates. Journal of clinical microbiology 41, 4188-4193.

Doughari, H.J., Ndakidemi, P.A., Human, I.S., Benade, S.,2011. The ecology, biology and pathogenesis of Acinetobacter spp.: an overview. Microbes and environments 26, 101-112.

Duarte, A., Ferreira, S., Silva, F., Domingues, F.,2012. Synergistic activity of coriander oil and conventional antibiotics against Acinetobacter baumannii. Phytomedicine 19, 236-238.

Echenique, J.R., Arienti, H., Tolmasky, M.E., Read, R.R., Staneloni, R.J., Crosa, J.H., Actis, L.A.,1992. Characterization of a high-affinity iron transport system in Acinetobacter baumannii. Journal of Bacteriology 174, 7670-7679.

Eijkelkamp, B.A., Hassan, K.A., Paulsen, I.T., Brown, M.H.,2011. Investigation of the human pathogen Acinetobacter baumannii under iron limiting conditions. BMC genomics 12, 126-2164-12-126.

Eijkelkamp, B.A., Stroeher, U.H., Hassan, K.A., Elbourne, L.D., Paulsen, I.T., Brown, M.H.,2013. H-NS plays a role in expression of Acinetobacter baumannii virulence features. Infection and immunity 81, 2574-2583.

Fagon, J., Chastre, J., Domart, Y., Trouillet, J., Pierre, J., Darne, C., Gibert, C.,1989. Nosocomial pneumonia in patients receiving continuous mechanical ventilation: prospective analysis of 52 episodes with use of a protected specimen brush and quantitative culture techniques. American Review of Respiratory Disease 139, 877-884.

Falagas, M., Karveli, E.,2007. The changing global epidemiology of Acinetobacter baumannii infections: a development with major public health implications. Clinical microbiology and infection 13, 117-119.

Farajnia, S., Azhari, F., Alikhani, M.Y., Hosseini, M.K., Peymani, A., Sohrabi, N.,2013. Prevalence of PER and VEB Type Extended Spectrum Betalactamases among Multidrug Resistant Acinetobacter baumannii Isolates in North-West of Iran. Iranian journal of basic medical sciences 16, 751-755.

Fournier, P.E., Richet, H.,2006. The epidemiology and control of Acinetobacter baumannii in health care facilities. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 42, 692-699.

Friedman, M., Henika, P.R., Mandrell, R.E.,2002. Bactericidal activities of plant essential oils and some of their isolated constituents against Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enterica. Journal of Food Protection® 65, 1545-1560.

Fujita, M., Shiota, S., Kuroda, T., Hatano, T., Yoshida, T., Mizushima, T., Tsuchiya, T.,2005. Remarkable Synergies between Baicalein and Tetracycline, and Baicalein and β- Lactams

against Methicillin- Resistant Staphylococcus aureus. Microbiology and immunology 49, 391-396.

Gaddy, J.A., Actis, L.A.,2009. Regulation of Acinetobacter baumannii biofilm formation.

Gaddy, J.A., Arivett, B.A., McConnell, M.J., Lopez-Rojas, R., Pachon, J., Actis, L.A.,2012. Role of acinetobactin-mediated iron acquisition functions in the interaction of Acinetobacter baumannii strain ATCC 19606T with human lung epithelial cells, Galleria mellonella caterpillars, and mice. Infection and immunity 80, 1015-1024.

Gaddy, J.A., Tomaras, A.P., Actis, L.A.,2009. The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infection and immunity 77, 3150-3160.

Gales, A.C., Jones, R.N., Forward, K.R., Linares, J., Sader, H.S., Verhoef, J.,2001. Emerging importance of multidrug-resistant Acinetobacter species and Stenotrophomonas maltophilia as pathogens in seriously ill patients: geographic patterns, epidemiological features, and trends in the SENTRY Antimicrobial Surveillance Program (1997-1999). Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 32 Suppl 2, S104-13.

Garcia-Garmendia, J.L., Ortiz-Leyba, C., Garnacho-Montero, J., Jimenez-Jimenez, F.J., Perez-Paredes, C., Barrero-Almodovar, A.E., Gili-Miner, M.,2001. Risk factors for Acinetobacter baumannii nosocomial bacteremia in critically ill patients: a cohort study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 33, 939-946.

Gaynes, R., Edwards, J.R., National Nosocomial Infections Surveillance System,2005. Overview of nosocomial infections caused by gram-negative bacilli. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 41, 848-854.

Geissman TA.,1963 Flavonoid compounds: tannins, lignins and related compounds. Vol. 9. Elsevier, New York. 1963, 265

Gennari, M., Lombardi, P.,1993. Comparative characterization of Acinetobacter strains isolated from different foods and clinical sources. Zentralblatt für Bakteriologie 279, 553-564.

Gerner-Smidt, P., Tjernberg, I.,1993. Acinetobacter in Denmark: II. Molecular studies of the Acinetobacter calcoaceticus- Acinetobacter baumannii complex. Apmis 101, 826-832.

Gerner-Smidt, P., Tjernberg, I., Ursing, J.,1991. Reliability of phenotypic tests for identification of Acinetobacter species. Journal of clinical microbiology 29, 277-282.

Giamarellou, H, 2010. Multidrug-resistant Gram-negative bacteria: how to treat and for how long.Intenational Journal of Antimicrobial agents . 36, S50–S54

Gill, A.O., Holley, R.A.,2004. Mechanisms of bactericidal action of cinnamaldehyde against Listeria monocytogenes and of eugenol against L. monocytogenes and Lactobacillus sakei. Applied and Environmental Microbiology 70, 5750-5755.

Gootz, T.D., Marra, A.,2008. Acinetobacter baumannii: an emerging multidrug-resistant threat.

Gordon, N.C., Wareham, D.W.,2010. Multidrug-resistant Acinetobacter baumannii: mechanisms of virulence and resistance. International journal of antimicrobial agents 35, 219-226.

Guardabassi, L., Dijkshoorn, L., Collard, J.M., Olsen, J.E., Dalsgaard, A.,2000. Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic Acinetobacter strains. Journal of medical microbiology 49, 929-936.

Guner, R., Hasanoglu, I., Keske, S., Kalem, A., Tasyaran, M.,2011. Outcomes in patients infected with carbapenem-resistant Acinetobacter baumannii and treated with tigecycline alone or in combination therapy. Infection 39, 515-518.

Gurung, J., Khyriem, A.B., Banik, A., Lyngdoh, W.V., Choudhury, B., Bhattacharyya, P.,2013. Association of biofilm production with multidrug resistance among clinical isolates of Acinetobacter baumannii and Pseudomonas aeruginosa from intensive care unit. Indian journal of critical care medicine : peer-reviewed, official publication of Indian Society of Critical Care Medicine 17, 214-218.

Gusten, W.M., Dorsainvil, P.A., Cunha, B.A.,2002. Acinetobacter lwoffi pseudomeningitis. Antimicrobics and Infectious Diseases Newsletter 18, 86-88.

Henrichsen, J.,1984. Not gliding but twitching motility of Acinetobacter calcoaceticus. Journal of clinical pathology 37, 102-103.

Hernan, R.C., Karina, B., Gabriela, G., Marcela, N., Carlos, V., Angela, F.,2009. Selection of colistin-resistant Acinetobacter baumannii isolates in postneurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. Diagnostic microbiology and infectious disease 65, 188-191.

Holley, R.A., Patel, D.,2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Food Microbiology 22, 273-292.

Hoštacká, A.,2002. Characteristics of clinicalAcinetobacter spp. strains. Folia microbiologica 47, 579-582.

Houang, E.T., Chu, Y.W., Leung, C.M., Chu, K.Y., Berlau, J., Ng, K.C., Cheng, A.F.,2001. Epidemiology and infection control implications of Acinetobacter spp. in Hong Kong. Journal of clinical microbiology 39, 228-234.

Houang, E.T., Sormunen, R.T., Lai, L., Chan, C.Y., Leong, A.S.,1998. Effect of desiccation on the ultrastructural appearances of Acinetobacter baumannii and Acinetobacter lwoffii. Journal of clinical pathology 51, 786-788.

Howard, A., O'Donoghue, M., Feeney, A., Sleator, R.D.,2012. Acinetobacter baumannii: an emerging opportunistic pathogen. Virulence 3, 243-250.

Hsueh PR, Teng LJ, Chen CY, Chen WH, Yu CJ, Ho SW,Luh KT. Pandrug-resistant Acinetobacter baumannii causingnosocomial infections in a university hospital, Taiwan. *Emerg Infect Dis* 2002; **8**: 827-832 [

Hyldgaard, M., Mygind, T., Meyer, R.L.,2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Frontiers in microbiology 3, 12.

Jacobs, A.C., Hood, I., Boyd, K.L., Olson, P.D., Morrison, J.M., Carson, S., Sayood, K., Iwen, P.C., Skaar, E.P., Dunman, P.M.,2010. Inactivation of phospholipase D diminishes Acinetobacter baumannii pathogenesis. Infection and immunity 78, 1952-1962.

Jain, R., Danziger, L.H.,2004. Multidrug-resistant Acinetobacter infections: an emerging challenge to clinicians. The Annals of Pharmacotherapy 38, 1449-1459.

Jalalpour, S., Ebadi, A.G., 2012. Frequency of isolated antibiotic resistant bacterial strains from nursing staff (Azzahra hospital, Iran). African journal of Pharmacy and Pharmacology 6.2:108-112.

Jamal,W., Al Roomi,E., AbdulAziz,L.R., Rotimia,V.O.,2014. Evaluation of Curetis Unyvero, a Multiplex PCR-Based Testing System, for Rapid Detection of Bacteria and Antibiotic Resistance and Impact of the Assay on Management of Severe Nosocomial Pneumonia. Journal of Clinical Microbiology. 52, 2487–2492

Joly-Guillou M. L. and Brun-Buisson C., 1996 Epidemiology of Acinetobacter spp.: surveillance and management of outbreaks. In: Acinetobacter, Microbiology, Epidemiology, Infections, Management (eds E. Bergogne-Bérézin, M. L. Joly-Guillou, and K. J. Towner) CRC Press, Boca Raton

Karageorgopoulos, D.E., Falagas, M.E.,2008. Current control and treatment of multidrug-resistant Acinetobacter baumannii infections. The Lancet infectious diseases 8, 751-762.

Kau, H., Tsai, C., Kao, S., Hsu, W., Liu, J.,2002. Corneal ulcer of the side port after phacoemulsification induced by Acinetobacter baumannii. Journal of Cataract & Refractive Surgery 28, 895-897.

Kim, B., Peleg, A.Y., Lodise, T.P., Lipman, J., Li, J., Nation, R., Paterson, D.L.,2009. Management of meningitis due to antibiotic-resistant Acinetobacter species. The Lancet infectious diseases 9, 245-255.

King, L.B., Swiatlo, E., Swiatlo, A., McDaniel, L.S.,2009. Serum resistance and biofilm formation in clinical isolates of Acinetobacter baumannii. FEMS Immunology & Medical Microbiology 55, 414-421.

Johny, A.K., Hoagland, T., Venkitanarayanan, K.,2010. Effect of subinhibitory concentrations of plant-derived molecules in increasing the sensitivity of multidrug-resistant Salmonella enterica serovar Typhimurium DT104 to antibiotics. Foodborne pathogens and disease 7, 1165-1170.

Kramer, A., Schwebke, I., Kampf, G.,2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC infectious diseases 6, 130.

Lautrop, H. 1974. Bergey's manual of determinative bacteriology.Williams & Wilkins Co., Baltimore, MD.

La Scola, B., Raoult, D.,2004. Acinetobacter baumannii in human body louse. Emerging infectious diseases 10, 1671-1673.

Lee, H., Koh, Y., Kim, J., Lee, J., Lee, Y., Seol, S., Cho, D.,2008. Capacity of multidrug- resistant clinical isolates of Acinetobacter baumannii to form biofilm and adhere to epithelial cell surfaces. Clinical microbiology and infection 14, 49-54.

Lee, K., Lim, Y.S., Yong, D., Yum, J.H., Chong, Y.,2003. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of Pseudomonas spp. and Acinetobacter spp. Journal of clinical microbiology 41, 4623-4629.

Lee, S., Park, Y.J., Kim, M., Lee, H.K., Han, K., Kang, C.S., Kang, M.W.,2005. Prevalence of Ambler class A and D beta-lactamases among clinical isolates of Pseudomonas aeruginosa in Korea. The Journal of antimicrobial chemotherapy 56, 122-127.

Leung, W.S., Chu, C.M., Tsang, K.Y., Lo, F.H., Lo, K.F., Ho, P.L.,2006. Fulminant community-acquired Acinetobacter baumannii pneumonia as a distinct clinical syndrome. Chest 129, 102-109

Levi, K., Bailey, C., Bennett, A., Marsh, P., Cardy, D.L., Towner, K.J.,2003. Evaluation of an isothermal signal amplification method for rapid detection of methicillin-resistant Staphylococcus aureus from patient-screening swabs. Journal of clinical microbiology 41, 3187-3191

Levy, J., Oshry, T., Rabinowitz, R., Lifshitz, T.,2005. Acinetobacter corneal graft ulcer and endophthalmitis: report of two cases. Canadian Journal of Ophthalmology/Journal Canadien d'Ophtalmologie 40, 79-82.

Lessel, E. F. 1971. Subcommittee on nomenclature of Moraxella and allied bacteria. Int. J. Syst. Bacteriol. 21:213-214.

Lupo, A., Vogt, D., Seiffert, S.N., Endimiani, A., Perreten, V.,2014. Antibiotic Resistance and Phylogenetic Characterization of Acinetobacter baumannii Strains Isolated from Commercial Raw Meat in Switzerland. Journal of Food Protection® 77, 1976-1981

Li, C., Chen, S., Cheng, C., Chen, T.,2005. Production of Acinetobacter radioresistens lipase with repeated fed-batch culture. Biochemical engineering journal 25, 195-199.

Lin, M.F., Lin,Y.Y., Yeh, H.W, and Lan,C.Y, 2014. Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii adeAB* genes and its correlation with tigecycline susceptibility. ***BMC Microbiology*** **2014,** 14**:119**

Livermore et al., 2008 Livermore,D.M ., Mushtaq, S., Warner, M.,Miossec, C and Woodford, N. 2008 NXL104 combinations versus Enterobacteriaceae with CTX-M extended-spectrum β-lactamases and carbapenemases. J. Antimicrob. Chemother. (2008)62 (5): 1053-1056.

Loehfelm, T.W., Luke, N.R., Campagnari, A.A.,2008. Identification and characterization of an Acinetobacter baumannii biofilm-associated protein. Journal of Bacteriology 190, 1036-1044.

Lorente, L., Lecuona, M., Galván, R., Ramos, M.J., Mora, M.L., Sierra, A.,2004. Periodically changing ventilator circuits is not necessary to prevent ventilator-associated pneumonia when a heat and moisture exchanger is used. Infection Control 25, 1077-1082.

Luo, L., Wu, L., Xiao, Y., Zhao, D., Chen, Z., Kang, M., Zhang, Q., Xie, Y.,2015. Enhancing pili assembly and biofilm formation in Acinetobacter baumannii ATCC19606 using non-native acyl-homoserine lactones. BMC microbiology 15, 62.

Magnet, S., Courvalin, P., Lambert, T.,2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454. Antimicrobial Agents and Chemotherapy 45, 3375-3380.

Maragakis, L.L., Perl, T.M.,2008. Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 46, 1254-1263.

McConnell, M.J., Actis, L., Pachon, J.,2013. Acinetobacter baumannii: human infections, factors contributing to pathogenesis and animal models. FEMS microbiology reviews 37, 130-155.

Medell, M., Hart, M., Duquesne, A., Espinosa, F., Valdes, R.,2013. Nosocomial ventilator-associated pneumonia in Cuban intensive care units: bacterial species and antibiotic resistance. MEDICC review 15, 26-29.

Menon, K.V., Garg, S.,2001. Inhibitory effect of clove oil on Listeria monocytogenes in meat and cheese. Food Microbiology 18, 647-650.

Menon, T., Shanmugasundaram, S., Nandhakumar, B., Nalina, K., Balasubramaniam,2006. Infective endocarditis due to Acinetobacter baumannii complex--a case report. Indian journal of pathology & microbiology 49, 576-578.

Metan, G., Alp, E., Aygen, B., Sumerkan, B.,2007. Acinetobacter baumannii meningitis in post-neurosurgical patients: clinical outcome and impact of carbapenem resistance. The Journal of antimicrobial chemotherapy 60, 197-199.

Mihara, K., Tanabe, T., Yamakawa, Y., Funahashi, T., Nakao, H., Narimatsu, S., Yamamoto, S.,2004. Identification and transcriptional organization of a gene cluster involved in biosynthesis and transport of acinetobactin, a siderophore produced by Acinetobacter baumannii ATCC 19606T. Microbiology (Reading, England) 150, 2587-2597.

Montefour, K., Frieden, J., Hurst, S., Helmich, C., Headley, D., Martin, M., Boyle, D.A.,2008. Acinetobacter baumannii: an emerging multidrug-resistant pathogen in critical care. Critical Care Nurse 28, 15-25; quiz 26.

Moultrie et al., 2011 Moultrie,D., Hawker,J., and Cole,2011. Factors Associated with Multidrug-Resistant Acinetobacter Transmission: An Integrative Review of the Literature . AORN Journal.94,27-36

Munoz-Price, L.S., Weinstein, R.A.,2008. Acinetobacter infection. The New England journal of medicine 358, 1271-1281.

Murray, C.K., Hospenthal, D.R.,2008. Acinetobacter infection in the ICU. Critical Care Clinics 24, 237-248.

Musafer, H.K., Essa, R.H.,2013. Inhibition of Acinetobacter baumannii adhesion by anti-fimbrial antibody: the fimbrial antigen effectiveness.Journal Details

Mussi et al., 2005 Mussi, M.A.,. Limansky,A.S and Viale, A.M. 2005. Acquisition of Resistance to Carbapenems in Multidrug-Resistant Clinical Strains of *Acinetobacter baumannii*: Natural Insertional Inactivation of a Gene Encoding a Member of a Novel Family of β-Barrel Outer Membrane Proteins. *Antimicrob. Agents Chemother*.April 2005 vol. 49 no. 4 1432-1440

Mussi, M.A., Gaddy, J.A., Cabruja, M., Arivett, B.A., Viale, A.M., Rasia, R., Actis, L.A.,2010. The opportunistic human pathogen Acinetobacter baumannii senses and responds to light. Journal of Bacteriology 192, 6336-6345.

Nagano, N., Nagano, Y., Cordevant, C., Shibata, N., Arakawa, Y.,2004. Nosocomial transmission of CTX-M-2 beta-lactamase-producing Acinetobacter baumannii in a neurosurgery ward. Journal of clinical microbiology 42, 3978-3984.

Ndegwa, E., Munyua, S., Mulei, C.,2001. Prevalence of microorganisms associated with udder infections in dairy goats on small-scale farms in Kenya: short communication. Journal of the South African Veterinary Association 72, p. 97-98.

Negi, P.S.,2012. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International journal of food microbiology 156, 7-17.

Neilands, J.B.,1995. Siderophores: structure and function of microbial iron transport compounds. The Journal of biological chemistry 270, 26723-26726.

Niu, C., Clemmer, K.M., Bonomo, R.A., Rather, P.N.,2008. Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii. Journal of Bacteriology 190, 3386-3392.

Obana, Y.,1986. Pathogenic Significance of Acinetobacter calcoaceticus. Microbiology and immunology 30, 645-657.

Obaro, S., Lawson, L., Essen, U., Ibrahim, K., Brooks, K., Otuneye, A., Shetima, D., Ahmed, P., Ajose, T., Olugbile, M., Idiong, D., Ogundeji, D., Ochigbo, C., Olanipekun, G., Khalife, W., Adegbola, R.,2011. Community acquired bacteremia in young children from central Nigeria--a pilot study. BMC infectious diseases 11, 137-2334-11-137.

Olut, A.I., Erkek, E.,2005. Early prosthetic valve endocarditis due to Acinetobacter baumannii: a case report and brief review of the literature. Scandinavian Journal of Infectious Diseases 37, 919-921.

Opazo, A., Domínguez, M., Bello, H., Amyes, S.G., González-Rocha, G.,2011. OXA-type carbapenemases in Acinetobacter baumannii in South America. The Journal of Infection in Developing Countries 6, 311-316.

Opazo, A., Sonnevend, A., Lopes, B., Hamouda, A., Ghazawi, A., Pal, T., Amyes, S.G.,2012. Plasmid-encoded PER-7 beta-lactamase responsible for ceftazidime resistance in Acinetobacter baumannii isolated in the United Arab Emirates. The Journal of antimicrobial chemotherapy 67, 1619-1622.

Oussalah, M., Caillet, S., Lacroix, M.,2006. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of Escherichia coli O157: H7 and Listeria monocytogenes. Journal of Food Protection® 69, 1046-1055.

Ozaki, T., Nishimura, N., Arakawa, Y., Suzuki, M., Narita, A., Yamamoto, Y., Koyama, N., Nakane, K., Yasuda, N., Funahashi, K.,2009. Community-acquired Acinetobacter baumannii meningitis in a previously healthy 14-month-old boy. Journal of infection and chemotherapy 15, 322-324.

Park, Y.K., Choi, J.Y., Shin, D., Ko, K.S.,2011. Correlation between overexpression and amino acid substitution of the PmrAB locus and colistin resistance in Acinetobacter baumannii. International journal of antimicrobial agents 37, 525-530.

Pasteran, F., Rapoport, M., Petroni, A., Faccone, D., Corso, A., Galas, M., Vazquez, M., Procopio, A., Tokumoto, M., Cagnoni, V.,2006. Emergence of PER-2 and VEB-1a in

Acinetobacter baumannii Strains in the Americas. Antimicrobial Agents and Chemotherapy 50, 3222-3224.

Peleg, A.Y., Potoski, B.A., Rea, R., Adams, J., Sethi, J., Capitano, B., Husain, S., Kwak, E.J., Bhat, S.V., Paterson, D.L.,2007. Acinetobacter baumannii bloodstream infection while receiving tigecycline: a cautionary report. The Journal of antimicrobial chemotherapy 59, 128-131.

Peleg, A.Y., Seifert, H., Paterson, D.L.,2008. Acinetobacter baumannii: emergence of a successful pathogen. Clinical microbiology reviews 21, 538-582.

Perez, F., A. M. Hujer, K. M. Hujer, B. K. Decker, P. N. Rather, and R. A. Bonomo. 2007. Global challenge of multidrug-resistant Acinetobacter baumannii. Antimicrob. Agents Chemother. 51:3471-3484

Phuong, K., Kakii, K., Nikata, T.,2009. Intergeneric coaggregation of non-flocculating Acinetobacter spp. isolates with other sludge-constituting bacteria. Journal of bioscience and bioengineering 107, 394-400.

Playford, E., Craig, J., Iredell, J.,2007. Carbapenem-resistant Acinetobacter baumannii in intensive care unit patients: risk factors for acquisition, infection and their consequences. Journal of Hospital Infection 65, 204-211.

Pour, N.K., Dusane, D.H., Dhakephalkar, P.K., Zamin, F.R., Zinjarde, S.S., Chopade, B.A.,2011. Biofilm formation by Acinetobacter baumannii strains isolated from urinary tract infection and urinary catheters. FEMS Immunology & Medical Microbiology 62, 328-338.

Rastogi, V.K., Wallace, L., Smith, L.S.,2007. Disinfection of Acinetobacter baumannii-contaminated surfaces relevant to medical treatment facilities with ultraviolet C light. Military medicine 172, 1166-1169.

Rathinavelu, S., Zavros, Y., Merchant, J.L.,2003. Acinetobacter lwoffii infection and gastritis. Microbes and Infection 5, 651-657.

Rao, R.S., Karthika, R.U., Singh, S.P., Shashikala, P., Kanungo, R., Jayachandran, S., Prashanth, K.,2008. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of Acinetobacter baumannii. Indian journal of medical microbiology 26, 333-337.

Ribera, A., Roca, I., Ruiz, J., Gibert, I., Vila, J.,2003. Partial characterization of a transposon containing the tet(A) determinant in a clinical isolate of Acinetobacter baumannii. The Journal of antimicrobial chemotherapy 52, 477-480.

Ribera, A., Ruiz, J., Vila, J.,2003. Presence of the Tet M determinant in a clinical isolate of Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy 47, 2310-2312.

Roca, I., Espinal, P., Marti, S., Vila, J.,2011. First identification and characterization of an AdeABC-like efflux pump in Acinetobacter genomospecies 13TU. Antimicrobial Agents and Chemotherapy 55, 1285-1286.

Rodríguez- Baño, J., Marti, S., Soto, S., Fernández- Cuenca, F., Cisneros, J.M., Pachón, J., Pascual, A., Martínez- Martínez, L., McQueary, C., Actis, L.,2008. Biofilm formation in Acinetobacter baumannii: associated features and clinical implications. Clinical Microbiology and Infection 14, 276-278.

Rosenberg, M., Gutnik,D. and Rosenberg,E., 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. FEMS Microbiol. Lett., 9, 29–33

Rossau, R., Van Landschoot, A., Gillis, M., De Ley, J.,1991. Taxonomy of Moraxellaceae fam. nov., a new bacterial family to accommodate the genera Moraxella, Acinetobacter, and Psychrobacter and related organisms. International Journal of Systematic Bacteriology 41, 310-319.

Rumbo, C., Tomas, M., Fernandez Moreira, E., Soares, N.C., Carvajal, M., Santillana, E., Beceiro, A., Romero, A., Bou, G.,2014. The Acinetobacter baumannii Omp33-36 porin is a virulence factor that induces apoptosis and modulates autophagy in human cells. Infection and immunity 82, 4666-4680. Russo et al., 2011.

Ruzin, A., Keeney, D., Bradford, P.A.,2007. AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in Acinetobacter calcoaceticus-Acinetobacter baumannii complex. The Journal of antimicrobial chemotherapy 59, 1001-1004.

Sacar, S., Turgut, H., Toprak, S., Cirak, B., Coskun, E., Yilmaz, O., Tekin, K.,2006. A retrospective study of central nervous system shunt infections diagnosed in a university hospital during a 4-year period. BMC infectious diseases 6, 43.

Saghi, H., Bahador, A., Khaledi, A., Kachoei, R.A., Dastjerdi, F.A., Esmaeili, D.,2015. Antibacterial Effects of Origanum vulgareEssence Against Multidrug-Resistant.

Santucci, S., Gobara, S., Santos, C., Fontana, C., Levin, A.,2003. Infections in a burn intensive care unit: experience of seven years. Journal of Hospital Infection 53, 6-13.

Sauer, K., Rickard, A.H., Davies, D.G.,2007. Biofilms and biocomplexity. Microbe- American society for microbiology. 2, 347.

Savoia,S 2012.Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiology.7, 979-990

Saulnier, F.F., Hubert, H., Onimus, T.M., Beague, S., Nseir, S., Grandbastien, B., Renault, C.Y., Idzik, M., Erb, M.P., Durocher, A.V.,2001. Assessing excess nurse work load generated by multiresistant nosocomial bacteria in intensive care. Infection Control 22, 273-278.

Schaack, S., Gilbert, C., Feschotte, C.,2010. Promiscuous DNA: horizontal transfer of transposable elements and why it matters for eukaryotic evolution. Trends in ecology & evolution 25, 537-546.

Scott, P., Deye, G., Srinivasan, A., Murray, C., Moran, K., Hulten, E., Fishbain, J., Craft, D., Riddell, S., Lindler, L., Mancuso, J., Milstrey, E., Bautista, C.T., Patel, J., Ewell, A., Hamilton, T., Gaddy, C., Tenney, M., Christopher, G., Petersen, K., Endy, T., Petruccelli, B.,2007. An outbreak of multidrug-resistant Acinetobacter baumannii-calcoaceticus complex infection in the US military health care system associated with military operations in Iraq. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 44, 1577-1584.

Sechi, L.A., Karadenizli, A., Deriu, A., Zanetti, S., Kolayli, F., Balikci, E., Vahaboglu, H.,2004. PER-1 type beta-lactamase production in Acinetobacter baumannii is related to cell adhesion. Medical science monitor : international medical journal of experimental and clinical research 10, BR180-4.

Seifert, H., Baginski, R., Schulze, A., Pulverer, G.,1993. Antimicrobial susceptibility of Acinetobacter species. Antimicrobial Agents and Chemotherapy 37, 750-753.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Tjernberg, I., Vaneechoutte, M.,1997. Distribution of Acinetobacter species on human skin: comparison of phenotypic and genotypic identification methods. Journal of clinical microbiology 35, 2819-2825.

Shahverdi, A., Monsef- Esfahani, H., Tavasoli, F., Zaheri, A., Mirjani, R.,2007. Trans- Cinnamaldehyde from Cinnamomum zeylanicum Bark Essential Oil Reduces the Clindamycin Resistance of Clostridium difficile in vitro. Journal of Food Science 72, S055-S058.

Sharma, B.,2007. Infection in patients with severe burns: causes and prevention thereof. Infectious disease clinics of North America 21, 745-759.

Shin, J.H.; Lee, H.W.; Kim, S.M.; Kim, J. Proteomic analysis of Acinetobacter baumannii in biofilm and planktonic growth mode. J. Microbiol. 2009, 47, 728–735.

Silva, G.M., Morais, L., Marques, L., Senra, V.,2012. Acinetobacter community-acquired pneumonia in a healthy child. Revista Portuguesa de Pneumologia (English Edition) 18, 96-98.

Simor, A.E., Lee, M., Vearncombe, M., Jones-Paul, L., Barry, C., Gomez, M., Fish, J.S., Cartotto, R.C., Palmer, R., Louie, M.,2002. An outbreak due to multiresistant Acinetobacter baumannii in a burn unit: risk factors for acquisition and management. Infection Control 23, 261-267.

Skerman, 1980. Skerman, V. B. D., V. McGowan, and P. H. A. Sneath. 1980. Approved list of bacterial names. Int. J. Syst. Bacteriol. 30:225–420

Smith, M.G., Gianoulis, T.A., Pukatzki, S., Mekalanos, J.J., Ornston, L.N., Gerstein, M., Snyder, M.,2007. New insights into Acinetobacter baumannii pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. Genes & development 21, 601-614.

Solak Y, Atalay H, Turkmen K, Biyik Z, Genc N, Yeksan M.2011 Community-acquired carbapenem-resistant Acinetobacter baumannii urinary tract infection just after marriage in a renal transplant recipient. *Transpl Infect Dis* 2011; **13**: 638-640

Stahl, J., Bergmann, H., Göttig, S., Ebersberger, I., Averhoff, B.,2015. Acinetobacter baumannii Virulence Is Mediated by the Concerted Action of Three Phospholipases D. PloS one 10, e0138360.

Stermitz, F.R., Lorenz, P., Tawara, J.N., Zenewicz, L.A., Lewis, K.,2000. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. Proceedings of the National Academy of Sciences of the United States of America 97, 1433-1437.

Su, X.Z., Chen, J., Mizushima, T., Kuroda, T., Tsuchiya, T.,2005. AbeM, an H+-coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters. Antimicrobial Agents and Chemotherapy 49, 4362-4364.

Talbot, G.H., Bradley, J., Edwards, J.E.,Jr, Gilbert, D., Scheld, M., Bartlett, J.G., Antimicrobial Availability Task Force of the Infectious Diseases Society of America,2006. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 42, 657-668.

Thomson, J.M., Bonomo, R.A.,2005. The threat of antibiotic resistance in Gram-negative pathogenic bacteria: beta-lactams in peril! Current opinion in microbiology 8, 518-524.

Thoroski, J., Blank, G., Biliaderis, C.,1989. Eugenol induced inhibition of extracellular enzyme production by Bacillus subtilis. Journal of Food Protection® 52, 399-403.

Timsit, J.F., Chevret, S., Valcke, J., Misset, B., Renaud, B., Goldstein, F.W., Vaury, P., Carlet, J.,1996. Mortality of nosocomial pneumonia in ventilated patients: influence of diagnostic tools. American journal of respiratory and critical care medicine 154, 116-123.

Tjernberg, I., Ursing, J.,1989. Clinical strains of Acinetobacter classified by DNA-DNA hybridization. APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica 97, 595-605.

Tomaras, A.P., Dorsey, C.W., Edelmann, R.E., Actis, L.A.,2003. Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: involvement of a novel chaperone-usher pili assembly system. Microbiology (Reading, England) 149, 3473-3484.

Towner, 2006 Towner, 2006 Towner, K. 2006.The genus Acinetobacter.Prok. 6:746–758.Hoštacká, A., and L.' Klokočníková. 2002. Characteristics of clinical Acinetobacter spp. Strains. Folia Microbiologica. 47:579– 582

Towner, K.,The Genus Acinetobacter. 1992 pp 3137-3143

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., Venkitanarayanan, K.,2014. Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis. BioMed research international 2014

Vaara, M.,1992. Agents that increase the permeability of the outer membrane. Microbiological reviews 56, 395-411.

Vidal R, Dominguez M, Urrutia H, Bello H, Gonzalez G, Garcia A, et al. Biofilm formation by Acinetobacter baumannii. Microbios 1996;86:49–58

Vidal, R., Dominguez, M., Urrutia, H., Bello, H., Garcia, A., Gonzalez, G., Zemelman, R.,1997. Effect of imipenem and sulbactam on sessile cells of Acinetobacter baumannii growing in biofilm. Microbios 91, 79-87.

Vila, J., Pachón, J.,2008. Therapeutic options for Acinetobacter baumannii infections. Expert Opinion on Pharmacotherapy.9,587-599

Vila, J., Marcos, A., Marco, F., Abdalla, S., Vergara, Y., Reig, R., Gomez-Lus, R., Jimenez de Anta, T.,1993. In vitro antimicrobial production of beta-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferase by and susceptibility of clinical isolates of Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy 37, 138-141.

Vila, J., Marti, S., Sanchez-Cespedes, J.,2007. Porins, efflux pumps and multidrug resistance in Acinetobacter baumannii. The Journal of antimicrobial chemotherapy 59, 1210-1215.

Wendt, C., Dietze, B., Dietz, E., Ruden, H.,1997. Survival of Acinetobacter baumannii on dry surfaces. Journal of clinical microbiology 35, 1394-1397.

Wilks, M, Wilson A, Warwick S, Price E, Kennedy D, Ely A, Millar MR.2006 Control of an outbreak of multidrug-resistant Acinetobacter baumannii-calcoaceticus colonization and infection in an intensive care unit (ICU) without closing the ICU or placing patients in isolation. *Infect Control Hosp Epidemiol* 2006; **27**: 654-658.

Wisplinghoff, H., Schmitt, R., Wöhrmann, A., Stefanik, D., Seifert, H.,2007. Resistance to disinfectants in epidemiologically defined clinical isolates of Acinetobacter baumannii. Journal of Hospital Infection 66, 174-181.

Wisplinghoff, H., Edmond, M.B., Pfaller, M.A., Jones, R.N., Wenzel, R.P., Seifert, H.,2000. Nosocomial bloodstream infections caused by Acinetobacter species in United States hospitals:

clinical features, molecular epidemiology, and antimicrobial susceptibility. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 31, 690-697.

Wisplinghoff, H., Bischoff, T., Tallent, S.M., Seifert, H., Wenzel, R.P., Edmond, M.B.,2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 39, 309-317.

Wollenweber, B., Kinzel, H.,1988. Role of carboxylate in the nitrogen metabolism of plants from different natural habitats. Physiologia Plantarum 72, 321-328.

Yadav, M.K., Park, S., Chae, S., Song, J., Kim, H.C.,2013. Antimicrobial activities of Eugenia caryophyllata extract and its major chemical constituent eugenol against Streptococcus pneumoniae. Apmis 121, 1198-1206.

Yong, D., Shin, J.H., Kim, S., Lim, Y., Yum, J.H., Lee, K., Chong, Y., Bauernfeind, A.,2003. High prevalence of PER-1 extended-spectrum beta-lactamase-producing Acinetobacter spp. in Korea. Antimicrobial Agents and Chemotherapy 47, 1749-1751.

Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z., Li, P., Wang, L., Cui, J., Wang, P., Huang, L., Klena, J.D., Song, H.,2013. Higher isolation of NDM-1 producing Acinetobacter baumannii from the sewage of the hospitals in Beijing. PloS one 8, e64857.

Chapter III

Prevalence of MDR *A. baumannii* on fresh vegetables collected from farmers' market in Connecticut.

ABSTRACT

This study determined the prevalence of multidrug resistant (MDR) *A. baumannii* on fresh vegetables collected from farmers' markets in Connecticut. One hundred samples each of fresh carrots, potatoes and lettuce were collected during the period 2012-13 for one year. The vegetables were sampled and streaked on selective media, namely Leeds *Acinetobacter* and MDR *Acinetobacter* agars. All morphologically distinct colonies from MDR *Acinetobacter* agar were identified using Gram staining, biochemical tests and polymerase chain reaction. In addition, susceptibility of the isolates to ten commonly used antibiotics in humans, namely imipenem, ceftriaxone, cefepime, minocycline, erythromycin, colistin-sulphate, streptomycin, neomycin, doxycycline, rifampcin was determined using antibiotic disc diffusion assay. The results revealed that out of 100 samples of each vegetable tested, only two samples of potato and one sample of lettuce yielded *A. baumannii*. No carrot sample was found to be positive for *A. baumannii*. However, several other opportunistic, MDR human pathogens such as *Burkholderia cepacia* (1% potatoes, 5% carrots and none from lettuce), *Stenotrophomonas maltophilia* (6% potatoes, 2% lettuce and none in carrots) and *Pseudomonas luteola* (9% potato, 3% carrot and none in lettuce) were recovered from the vegetables. Antibiotic susceptibility screening of the isolates revealed high resistance rates for ceftriaxone (100%), colistin-sulphate (83%), erythromycin (83%) and streptomycin (66%) in *B. cepacia*; for colistin-sulphate (100 %) and imipenem (90%) in *P.luteola*; for colistin-sulphate (100%), ceftriaxone (100%), cefepime (87%), erythromycin (62%) and imipenem (50%) in *S. maltophilia*, and for imipenem (100%), ceftriaxone (100%), erythromycin (100%) and streptomycin (100%) in *A. baumannii*. The results revealed the presence of MDR bacteria, including human pathogens on fresh produce, thereby

highlighting the potential health risk in consumers, especially those with a compromised immune system.

1. INTRODUCTION

Acinetobacter baumannii, a Gram-negative, aerobic, opportunistic bacterial pathogen, which has emerged as one of the most problematic bacteria to treat due to its resistance to most of the commonly used antibiotics (Fishbain and Peleg. 2010;Moradi et al., 2015). *A. baumannii* has been reported as the most common multi-drug resistant (MDR) pathogenic bacterium involved in hospital-acquired infections, particularly intensive care units (Dijkshoorn et al., 2007; Marti et al., 2011) causing mortality rates ranging from 34 to 61% (Esterly et al., 2011). In addition, *A. baumannii* is capable of causing a wide spectrum of infections in humans, including ventilator associated pneumonia, bacteremia and meningitis (Joshi et al., 2006; Maragakis and Perl; 2008; Manchanda et al., 2010; Guner et al., 2011; Solak et al., 2011). *Acinetobacter* spp. isfrequently isolated from soil and water (Baumann, 1968; Warskow and Juni, 1972; Eliopoulos, 2008; Krizova, 2014), which are considered to be their major habitats. *Acinetobacter* was reported to be present at a level of 38% in ground water samples (Bifulco et al., 1989), with isolation from water systems in multiple parts of the world (Ferreira et al., 2011; Zhang et al., 2013). Moreover, protozoans such as *Acanthamoeba* were found to support the growth of *A. baumannii,* and act as its reservoir in water (Cateau et al., 2011). In addition, *Acinetobacter* spp., including *A. baumannii,* have been isolated from other sources such as foods, arthropods, animals and humans (Baumann, 1968; Berlau et al., 1999; Mussi et al., 2005; Fournier and Richet, 2006; Munoz-Price and Weinstein, 2008). Although a few investigations conducted overseas have isolated *A. baumannii* and other *Acinetobacter* spp. from a variety of fresh produce, no extensive studies have been undertaken in the United States.

During the last decade, there has been an increased in the demand for local and regional food products, especially fresh produce because of their perceived freshness and quality, which

is believed to be due to a shorter period of time elapsed between harvesting and selling. According to the USDA's Agricultural Marketing Service, there has been a rise in the number and popularity of farmers' markets over the years in the United States, with an increase in number from 1,755 in 1994 to 8,284 in 2014 (USDA-ERS, 2014). However, relatively scant information exists on the microbiological safety and quality of fresh produce sold at farmers' markets as against retail sold produce. In addition, concerns exist about the storage conditions of the produce displayed throughout the day, the farming practices adopted by the farmers, and the pre- and post-harvest handling techniques employed (Kader and Rolle, 2004). A study conducted on the microbiological safety and quality of herbs sold at farmers' markets in Los Angeles, Orange county and Seattle revealed the presence of *Salmonella* and generic *Escherichia coli*, although the sources and extent of contamination were not reported (Levy et al., 2014). Another study compared the microbial populations in lettuce and spinach obtained from grocery stores and farmers' markets in Indiana, and reported greater bacterial (mesophilic plate count), yeast and mold contamination on lettuce from farmers' markets (Soendjojo, 2012). A study conducted in Germany revealed that vegetables from farmers' markets contained more antibiotic resistance bacteria when compared to those sold at retail stores (Schwaiger et al., 2011). The same researchers suggested that since vegetables from farmers' markets are not usually cooled or washed with antimicrobials, the bacteria present have greater chances of carrying and expressing antibiotic resistance genes (Schwaiger et al., 2011) as exposure of bacteria to stresses such as low temperature was found to lower the MIC of antimicrobials as compared to unstressed controls (McMahon et al., 2007). In a recent study conducted in British Columbia, Canada, Wood et al. (2015) tested lettuce (n = 68) collected from five farmers' markets, and found that

13% of the samples contained *E. coli*, and 97% of the *E. coli* isolates displayed resistance to one or more antimicrobials.

The objective of this study was to determine the prevalence of MDR *A. baumannii* in fresh vegetables, specifically carrots, lettuce and potatoes collected from farmers' markets in Connecticut.

2. MATERIALS AND METHODS

2.1. Sample collection and detection of *Acinetobacter* spp.

The prevalence rate of *A. baumannii* in vegetables reported in the literature ranges from 17% (Berlau et al., 1999) to 50% (Houang et al., 2001). Based on a conservative 5% prevalence rate, we conducted a power analysis using the probability theory (Snedecor and Cochran, 1967) to determine the number of vegetable samples required for isolating *A. baumannii* with a confidence level of 95%. Based on the power analysis results, a total of 100 samples each of fresh potatoes, carrots and lettuce were procured from 55 farms distributed over 15 farmers' markets in various counties in Connecticut. All the vegetables were sold loose and unprocessed, and were randomly purchased from different vegetable stands. All samples were immediately brought to the laboratory on ice, and processed within 8 h of collection. The sample collection was made during the period 2012-13 for one year covering two successive cropping seasons. For the detection of *A. baumannii*, approximately fifty grams of each sample was shaken vigorously in 450 ml sterile Tryptic soy broth (TSB) in a Whirlpak bag (Nasco, Fort Atkinson, WI) for 2 min. The samples were then streaked on selective media, namely Leeds *Acinetobacter* medium and MDR *Acinetobacter* medium (Hardy Diagnostics, Santa Maria, CA), which have been used to isolate *Acinetobacter* spp. from both clinical and environmental samples (Jawad et al., 1994).

In addition, the enrichment samples in TSB were incubated at 37ºC for 24 h and streaked again on Leeds and MDR *Acinetobacter* media. The plates were incubated at 37ºC for 24 h.

2.2. Identification and Characterization of the Isolates

All morphologically different colonies from MDR *Acinetobacter* medium were first subjected to Gram staining, followed by presumptive identification by biochemical tests using API 20 NE (bioMérieux, Cambridge, MA), which assays for 61 Gram-negative bacteria (Chang et al., 2005; Bosshard et al., 2006; Dortet et al., 2006). The manufacturers' instructions were followed for performing the biochemical tests. Each biochemical test well was inoculated with an overnight (0.5 McFarland) bacterial suspension, followed by incubation at 37ºC. The tests results were read after 24 and 48 h, and the interpretation of the results was done using the identification software version 6.0 (bioMérieux, Cambridge, MA). All the cultures were also subjected to oxidase test to compliment the biochemical tests in API 20 NE. Following identification by biochemical tests, the isolates were further confirmed by polymerase chain reaction (PCR) using 16s rRNA (Widmer et al., 1998; Clode et al., 1999; LiPuma et al., 1999; Whitby et al., 2000). The chromosomal DNA from each isolateextracted using InstaGene Matix (Bio-Rad, Hercules, CA) was used as a template for PCR. The primers tested for confirming the isolates are provided in Table1. The amplification of the 16S ribosomal RNA in each sample was performed in a thermocycler (PTC 100; MJ Research, Waltham, MA). PCR conditions were initial denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation at 94ºC for 1 min, annealing at 55-62ºC (depending on the target bacteria) for 30 s and extension at 72ºC for 10 min. The resulting PCR amplicons were examined by gel electrophoresis and visualization by gel documentation system (Bio-Rad, Hercules, CA).

2.3. Antibiotic Susceptibility Testing

The susceptibility of the various isolates to 10 antibiotics was tested by disc diffusion method (NCCLS, 1999). The antibiotics screened included Imipenem (10 μg), Ceftriaxone (30 μg), Cefepime (30 μg), Minocycline (30 μg), Erythromycin (15 μg), Colistin-sulphate (10 μg), Streptomycin (10 μg), Neomycin (30 μg), Doxycycline (30 μg), Rifampcin (5 μg) purchased from Oxoid (Hampshire, UK). All the cultures were incubated at 37^{o}C for 24 h. The zone diameter around the discs was measured horizontally and vertically.

3. RESULTS AND DISCUSSION

Fresh produce constitutes the leading cause of foodborne illnesses when compared to other food products (Doyle and Erickson, 2008), and a report from the US Centers for Disease Control and Prevention indicated that ~ 46% of all foodborne illnesses are caused by contaminated produce (Painter et al., 2013). Several pre-harvest factors such as proximity or contact with soil, feces, use of improperly composted manure, contaminated irrigation water, wild and domestic carrier animals and farm workers potentially contribute to contamination of fresh produce with a variety of microorganisms (Beuchat, 1996; Beuchat and Scouten, 2002). Antibiotic usage in food animal production, and simultaneous presence of antibiotic resistant bacteria in manure leads to potential persistence of these bacteria in agricultural soil (Sarmah et al., 2006). Since microorganisms present in soil could be transferred to produce, fresh produce consumption is reported as a potential route of direct human exposure to soil bacteria (Marti et al., 2013). In addition, usage of antibiotics in plant agriculture and contamination of irrigation water with animal feces could also serve as potential sources of antibiotic resistant bacteriaon fresh produce (Holvoet et al., 2013). The presence of antibiotic resistant bacteria on fresh produce is a significant concern to public health because of the potential transfer of antibiotic

resistance determinants to the enteric flora in humans (Sommer et al., 2010; Schjorring and Krogfelt, 2011). Similarly, Falomir et al. (2010) indicated that the presence of antibiotic resistance determinants in normal flora and pathogenic microorganisms on fresh vegetables might contribute to horizontal spread of resistances between different isolates, species and genera. It was also suggested that antibiotic resistance genes on transferable elements such as plasmids facilitates rapid distribution of resistance determinants among bacteria (De la Cruz and Davies, 2000; Heuer and Smalla, 2007). Thus monitoring the prevalence of antibiotic resistant bacteria on fresh produce is critical for risk assessment and devising appropriate food safety interventions.

In this study, we determined the prevalence of MDR *A. baumannii* on fresh vegetables such as lettuce, carrots and potatoes, which are previously reported to harbor *A. baumannii* (Berlau et al., 1999; Falomir et al., 2010). All 100 samples each of potatoes, carrots and lettuce were found to yield colonies on Leeds *Acinetobacter* medium. In addition, 74% potatoes, 86% of carrots, 73% lettuce samples had colonies growing on MDR *Acinetobacter* agar. These isolates were characterized using API 20 NE biochemical tests and confirmed with PCR using specific 16s rRNA primers. As observed in Table 2, out of the 100 samples of each vegetable tested, only two samples of potato and one sample of lettuce were found to be positive for *A. baumannii*. No carrot sample was positive for the pathogen. Previously, Berlau and colleagues (1999), while studying the prevalence of *Acinetobacter* in fresh produce in the United Kingdom, reported that 17% of 177 samples tested positive for the bacteria, including *A baumannii*. These investigators observed that 56% of the *Acinetobacter* belonged to *A.baumannii-A. calcoaceticus* group, which were found on apple, melon, bean, cabbage, cauliflower, carrot, potato, radish, lettuce, cucumber, pepper, mushroom, and sweet corn. In another study, Houang et al. (2001) isolated *A.*

baumannii and genomospecies 3 from 21 of 41 vegetable samples, 53 of 74 fish and meat samples and 22 of 60 soil samples. Similarly, Falomir et al. (2010) detected the presence of *A. baumannii* on fresh lettuce collected from a nursery in Valencia city, Spain, with a most probable number of >2400, where the bacterium was found to exhibit resistance to four different antibiotics, namely amoxicillin/clavulanic, ampicillin, chloramphenicol and nitrofurantoine. In yet another study, Walia et al. (2013) reported the isolation of *A. baumannii* along with other Gram negative bacteria from ready-to-eat baby spinach collected from local retail supermarkets in Rochester, MI. The isolates showed maximum resistance to cefoxitin, ampicillin, chloramphenicol, ceftrizoxime and tetracycline (Walia et al., 2013). Moreover, the resistance was found to be transferable in the strains tested.(Walia et al., 2013).

Although, this study was intended to focus on *A. baumannii*, several other antibiotic resistant bacteria were isolated from the MDR *Acinetobacter* agar plates besides *A. baumannii*. The major bacteria isolated included *Burkholderia cepacia*, *Stenotrophomonas maltophilia* and *Pseudomonas luteola*. Although no lettuce samples tested positive for Burkhoderia, 1% of potatoes and 5% of carrots yielded *B. cepacia*. *B. cepacia* is a plant pathogen causing soft rot of onions (Govan et al., 1996); however, it is now considered an opportunistic human pathogen causing respiratory and urinary tract infections as well as bacteremia in humans (Hutchinson et al., 2004; Mahenthiralingam et al., 2008). In addition, 6% of potatoes and 2% of lettuce tested were found to harbor *S. maltophilia*. *S. maltophilia* is ubiquitously present in the environment, and is commonly found associated with the rhizosphere of plants such as potato, wheat and cucumber (Muder et al., 1996; Denton et al., 1998; Berg et al., 1999); however, the bacterium is increasingly recognized as a nosocomial and opportunistic pathogen in humans (Senol, 2004; Tan et al., 2008; Brooke, 2012), especially with intrinsic resistance to many broad spectrum

antibiotics (Clark et al.,2003; Wang et al.,2004; Meyer et al.,2006). In a study conducted by Berg et al. (1999), several isolates of *S. maltophilia* from rhizospheres of plants exhibited resistance to multiple antibiotics, including Imipenem. In the current study, we also isolated *P. luteola* in 9% of potatoes and 3% of carrots, with no lettuce samples testing positive for this pathogen. *P. luteola*, formerly *Chryseomonas luteola*, is a saprophyte and psychrotrophic bacterium that can be isolated from water, soil, damp environments and vegetables (Membre and Kubaczka, 1998; Doublet et al., 2010). It is considered a rare pathogen, but is known to cause septicemia, meningitis and infections in humans, which are mainly attributed to the use of contaminated catheters and prostheses (Doublet et al., 2010).

All of the aforementioned bacterial isolates were screened for resistance to ten antibiotics, which included two drugs from five major classes of antibiotics commonly used in human medicine (Tice et al., 2004; Karageorgopoulos and Falagas, 2008). The antibiotic resistance profile of the aforementioned isolates is depicted in Table 3. All the isolates of *A. baumannii* were resistant to Imipenem (100%), Ceftriaxone (100%), Erythromycin (100%) and Streptomycin (100%); two of the isolates were resistant to Cefepime, Neomycin and Doxycycline, and one isolate showed resistance to Minocycline, Colistin-sulphate and Rifampcin. It is important to note that although the prevalence of *A. baumannii* on the vegetables was low, the isolates were highly resistant to the commonly used antibiotics such asImipenem, Ceftriaxone, Erythromycin and Streptomycin. In a previous study conducted by Falomir et al. (2010), *A. baumannii* isolated from vegetables were resistant to Amoxicillin/clavulanic, Ampicillin, Chloramphenicol and Nitrofurantoine. Similarly, all *B. cepacia/* isolates were resistant to Ceftriaxone, and five isolates were resistant to Cefepime, Colistin-sulfate and Erythromycin. The isolates showed least or no resistance to Doxycycline, Neomycin and

Minocycline. While all the isolates of *S. maltophilia* showed resistance to Colistin-sulfate and Ceftriaxone, they were susceptible to Minocycline and Doxycycline. *P. luteola* isolates showed maximum resistance to Colistin-sulfate and Imipenem and least resistance to Doxycycline and Minocycline.

To conclude, while results of this study indicate a low prevalence of MDR *A. baumannii* on vegetables, the presence of other opportunistic, MDR pathogens represents a potential health hazard to consumers, especially for immune-compromised and convalescent people. It is also critical to note that the isolates possessed resistance to common clinically used antibiotics in humans. Although risk assessment studies on human exposure to antibiotic resistant bacteria through the consumption of meat (via undercooking or cross-contamination) are available (Presi et al., 2009; Depoorter et al., 2012), such information on fresh produce is sparse. Moreover, since majorities of fresh produce are generally consumed without being subjected to a terminal microbial killing step unlike animal food products, the potential risk for human exposure to antibiotic resistant bacteria is greater, thereby underscoring the significance of good agricultural practices in farming.

REFERENCES

Baumann, P.,1968. Isolation of *Acinetobacter* from soil and water. Journal of Bacteriology 96, 39-42.

Berg, G., Roskot, N., Smalla, K.,1999. Genotypic and phenotypic relationships between clinical and environmental isolates of Stenotrophomonas maltophilia. Journal of clinical microbiology 37, 3594-3600.

Berlau, J., Aucken, H.M., Houang, E., Pitt, T.L.,1999. Isolation of *Acinetobacter* spp. including A. baumannii from vegetables: implications for hospital-acquired infections. The Journal of hospital infection 42, 201-204.

Beuchat, L.R.,1996. Pathogenic microorganisms associated with fresh produce. Journal of Food Protection® 59, 204-216.

Beuchat, L.R., Scouten, A.J.,2002. Combined effects of water activity, temperature and chemical treatments on the survival of Salmonella and Escherichia coli O157:H7 on alfalfa seeds. Journal of applied microbiology 92, 382-395.

Bifulco, J.M., Shirey, J.J., Bissonnette, G.K.,1989. Detection of *Acinetobacter* spp. in rural drinking water supplies. Applied and Environmental Microbiology 55, 2214-2219.

Bosshard, P.P., Zbinden, R., Abels, S., Boddinghaus, B., Altwegg, M., Bottger, E.C.,2006. 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory. Journal of clinical microbiology 44, 1359-1366.

Brooke, J.S.,2012. Stenotrophomonas maltophilia: an emerging global opportunistic pathogen. Clinical microbiology reviews 25, 2-41.

Cateau, E., Verdon, J., Fernandez, B., Hechard, Y., Rodier, M.H.,2011. *Acanthamoeba* sp. promotes the survival and growth of *Acinetobacter* baumanii. FEMS microbiology letters 319, 19-25.

Chang, H.C., Wei, Y.F., Dijkshoorn, L., Vaneechoutte, M., Tang, C.T., Chang, T.C.,2005. Species-level identification of isolates of the *Acinetobacter* calcoaceticus-*Acinetobacter* baumannii complex by sequence analysis of the 16S-23S rRNA gene spacer region. Journal of clinical microbiology 43, 1632-1639.

Clark, N.M., Patterson, J., Lynch, J.P.,3rd,2003. Antimicrobial resistance among gram-negative organisms in the intensive care unit. Current opinion in critical care 9, 413-423.

Clode, F.E., Kaufmann, M.E., Malnick, H., Pitt, T.L.,1999. Evaluation of three oligonucleotide primer sets in PCR for the identification of Burkholderia cepacia and their differentiation from Burkholderia gladioli. Journal of clinical pathology 52, 173-176.

de la Cruz, F., Davies, J.,2000. Horizontal gene transfer and the origin of species: lessons from bacteria. Trends in microbiology 8, 128-133.

Denton, M., Todd, N.J., Kerr, K.G., Hawkey, P.M., Littlewood, J.M.,1998. Molecular epidemiology of Stenotrophomonas maltophilia isolated from clinical specimens from patients with cystic fibrosis and associated environmental samples. Journal of clinical microbiology 36, 1953-1958.

Depoorter, P., Persoons, D., Uyttendaele, M., Butaye, P., De Zutter, L., Dierick, K., Herman, L., Imberechts, H., Van Huffel, X., Dewulf, J.,2012. Assessment of human exposure to 3rd generation cephalosporin resistant E. coli (CREC) through consumption of broiler meat in Belgium. International journal of food microbiology 159, 30-38.

Dijkshoorn, L., Nemec, A., Seifert, H.,2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter* baumannii. Nature Reviews Microbiology 5, 939-951.

Dortet, L., Legrand, P., Soussy, C.J., Cattoir, V.,2006. Bacterial identification, clinical significance, and antimicrobial susceptibilities of *Acinetobacter* ursingii and *Acinetobacter* schindleri, two frequently misidentified opportunistic pathogens. Journal of clinical microbiology 44, 4471-4478.

Doublet, B., Robin, F., Casin, I., Fabre, L., Le Fleche, A., Bonnet, R., Weill, F.X.,2010. Molecular and biochemical characterization of the natural chromosome-encoded class A beta-lactamase from Pseudomonas luteola. Antimicrobial Agents and Chemotherapy 54, 45-51.

Doyle, M., Erickson, M.,2008. Summer meeting 2007–the problems with fresh produce: an overview. Journal of applied microbiology 105, 317-330.

Eliopoulos, 2008 *Acinetobacter baumannii*: Epidemiology, Antimicrobial Resistance, and Treatment Options. Oxford Journals Medicine & Health Clinical Infectious Diseases Volume 46, Issue 8 Pp. 1254-1263

Esterly, J.S., Griffith, M., Qi, C., Malczynski, M., Postelnick, M.J., Scheetz, M.H.,2011. Impact of carbapenem resistance and receipt of active antimicrobial therapy on clinical outcomes of *Acinetobacter* baumannii bloodstream infections. Antimicrobial Agents and Chemotherapy 55, 4844-4849.

Falomir, M., Gozalbo, D., Rico, H.,2010. Coliform bacteria in fresh vegetables: from cultivated lands to consumers. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology 2, 1175-1181.

Ferreira, A.E., Marchetti, D.P., De Oliveira, L.M., Gusatti, C.S., Fuentefria, D.B., Corção, G.,2011. Presence of OXA-23-producing isolates of *Acinetobacter* baumannii in wastewater from hospitals in southern Brazil. Microbial Drug Resistance 17, 221-227.

Fishbain, J., Peleg, A.Y.,2010. Treatment of *Acinetobacter* infections. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 51, 79-84.

Fournier, P.E., Richet, H.,2006. The epidemiology and control of *Acinetobacter* baumannii in health care facilities. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 42, 692-699.

Govan, J.R., Hughes, J.E., Vandamme, P.,1996. Burkholderia cepacia: medical, taxonomic and ecological issues. Journal of medical microbiology 45, 395-407.

Guner, R., Hasanoglu, I., Keske, S., Kalem, A., Tasyaran, M.,2011. Outcomes in patients infected with carbapenem-resistant *Acinetobacter* baumannii and treated with tigecycline alone or in combination therapy. Infection 39, 515-518.

Harris, L., Farber, J., Beuchat, L., Parish, M., Suslow, T., Garrett, E., Busta, F.,2003. Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. Comprehensive reviews in food science and food safety 2, 78-141.

Heuer, H., Smalla, K.,2007. Horizontal gene transfer between bacteria. Environmental biosafety research 6, 3-13.

Holvoet, K., Sampers, I., Callens, B., Dewulf, J., Uyttendaele, M.,2013. Moderate prevalence of antimicrobial resistance in Escherichia coli isolates from lettuce, irrigation water, and soil. Applied and Environmental Microbiology 79, 6677-6683.

Houang, E.T., Chu, Y.W., Leung, C.M., Chu, K.Y., Berlau, J., Ng, K.C., Cheng, A.F.,2001. Epidemiology and infection control implications of *Acinetobacter* spp. in Hong Kong. Journal of clinical microbiology 39, 228-234.

Hutchinson, J., Runge, W., Mulvey, M., Norris, G., Yetman, M., Valkova, N., Villemur, R., Lepine, F.,2004. Burkholderia cepacia infections associated with intrinsically contaminated ultrasound gel: the role of microbial degradation of parabens. Infection Control 25, 291-296.

Jawad,A., Hawkey,P.M., Heritage,J and Snelling,A.M. 1994Description of Leeds Acinetobacter Medium, a new selective and differential medium for isolation of clinically important Acinetobacter spp., and comparison with Herellea agar and Holton's agar. J. Clin. Microbiol. vol. 32 no. 10 2353-2358

Joshi, S.G., Litake, G.M., Satpute, M.G., Telang, N.V., Ghole, V.S., Niphadkar, K.B.,2006. Clinical and demographic features of infection caused by *Acinetobacter* species. Indian journal of medical sciences 60, 351-360.

Kader, A.A., Rolle, R.S., 2004. The role of post-harvest management in assuring the quality and safety of horticultural produce, Food & Agriculture Org.,

Karageorgopoulos, D.E., Falagas, M.E.,2008. Current control and treatment of multidrug-resistant *Acinetobacter* baumannii infections. The Lancet infectious diseases 8, 751-762.

Krizova, L., Maixnerova, M., Sedo, O., Nemec, A.,2014. *Acinetobacter* bohemicus sp. nov. widespread in natural soil and water ecosystems in the Czech Republic. Systematic and applied microbiology 37, 467-473.

Levy.,D.J., Beck,N.K., Kossik, A.L., Patti,T., Meschke,J.S., Calicchia,M., and Hellberg,R.S.(2015) Microbial safety and quality of fresh herbs from Los Angeles, Orange County and Seattle farmers' markets Journal of the Science of Food and Agriculture Volume 95, Issue 13, pages 2641–2645, October 2015

LiPuma, J.J., Dulaney, B.J., McMenamin, J.D., Whitby, P.W., Stull, T.L., Coenye, T., Vandamme, P.,1999. Development of rRNA-based PCR assays for identification of Burkholderia cepacia complex isolates recovered from cystic fibrosis patients. Journal of clinical microbiology 37, 3167-3170.

Mahenthiralingam, E., Baldwin, A., Dowson, C.G.,2008. Burkholderia cepacia complex bacteria: opportunistic pathogens with important natural biology. Journal of applied microbiology 104, 1539-1551.

Manchanda, V., Sanchaita, S., Singh, N.,2010. Multidrug resistant *Acinetobacter*. Journal of global infectious diseases 2, 291-304.

Maragakis, L.L., Perl, T.M.,2008. *Acinetobacter* baumannii: epidemiology, antimicrobial resistance, and treatment options. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 46, 1254-1263.

Marti, S., Nait Chabane, Y., Alexandre, S., Coquet, L., Vila, J., Jouenne, T., Dé, E.,2011. Growth of *Acinetobacter* baumannii in pellicle enhanced the expression of potential virulence factors. PloS one 6, e26030.

Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Topp, E.,2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Applied and Environmental Microbiology 79, 5701-5709.

McMahon, M.A., Xu, J., Moore, J.E., Blair, I.S., McDowell, D.A.,2007. Environmental stress and antibiotic resistance in food-related pathogens. Applied and Environmental Microbiology 73, 211-217.

Membre, J.M., Kubaczka, M.,1998. Degradation of pectic compounds during pasteurised vegetable juice spoilage by Chryseomonas luteola: a predictive microbiology approach. International journal of food microbiology 42, 159-166.

Meyer, E., Schwab, F., Gastmeier, P., Rueden, H., Daschner, F., Jonas, D.,2006. Stenotrophomonas maltophilia and antibiotic use in German intensive care units: data from Project SARI (Surveillance of Antimicrobial Use and Antimicrobial Resistance in German Intensive Care Units). Journal of Hospital Infection 64, 238-243.

Moradi, J., Hashemi, F.B., Bahador, A.,2015. Antibiotic Resistance of *Acinetobacter* baumannii in Iran: A Systemic Review of the Published Literature. Osong public health and research perspectives 6, 79-86.

Muder, R.R., Harris, A.P., Muller, S., Edmond, M., Chow, J.W., Papadakis, K., Wagener, M.W., Bodey, G.P., Steckelberg, J.M.,1996. Bacteremia due to Stenotrophomonas (Xanthomonas) maltophilia: a prospective, multicenter study of 91 episodes. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 22, 508-512.

Munoz-Price, L.S., Weinstein, R.A.,2008. *Acinetobacter* infection. New England Journal of Medicine 358, 1271-1281.

Mussi, M.A., Limansky, A.S., Viale, A.M.,2005. Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter* baumannii: natural insertional inactivation of a gene encoding a member of a novel family of beta-barrel outer membrane proteins. Antimicrobial Agents and Chemotherapy 49, 1432-1440.

NCCLS, 1999 National Committee for Clinical Laboratory Standards (NCCLS) (1999) *Performance Standards for Antimicrobial Susceptibility Testing*, ninth informational Supplement. M100–S9, Vol. 19, no. 1.

Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R.V., Braden, C.R., Angulo, F.J., Griffin, P.M.,2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerging infectious diseases 19, 407-415.

Presi, P., Stärk, K.D., Stephan, R., Breidenbach, E., Frey, J., Regula, G.,2009. Risk scoring for setting priorities in a monitoring of antimicrobial resistance in meat and meat products. International journal of food microbiology 130, 94-100.

Sarmah, A.K., Meyer, M.T., Boxall, A.B.,2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725-759.

Schjorring, S., Krogfelt, K.A.,2011. Assessment of bacterial antibiotic resistance transfer in the gut. International journal of microbiology 2011, 312956.

Schwaiger, K., Helmke, K., Holzel, C.S., Bauer, J.,2011. Antibiotic resistance in bacteria isolated from vegetables with regards to the marketing stage (farm vs. supermarket). International journal of food microbiology 148, 191-196.

Silva, G.M., Morais, L., Marques, L., Senra, V.,2012. Acinetobacter community-acquired pneumonia in a healthy child. Revista Portuguesa de Pneumologia (English Edition) 18, 96-98.

Snedecor, G.W., and Cochran, W.G 1967. Statistical methods. 6th edition. Iowa state university Press, Ames.

Senol, E.,2004. Stenotrophomonas maltophilia: the significance and role as a nosocomial pathogen. Journal of Hospital Infection 57, 1-7.

Soendjojo, E.,2012. Is Local Produce Safer? Microbiological Quality of Fresh Lettuce and Spinach from Grocery Stores and Farmers' Markets. The Journal of Purdue Undergraduate Research 2, 10.

Solak, Y., Atalay, H., Turkmen, K., Biyik, Z., Genc, N., Yeksan, M.,2011. Community-acquired carbapenem-resistant *Acinetobacter* baumannii urinary tract infection just after marriage in a renal transplant recipient. Transplant Infectious Disease 13, 638-640.

Sommer, M.O., Church, G.M., Dantas, G.,2010. The human microbiome harbors a diverse reservoir of antibiotic resistance genes. Virulence 1, 299-303.

Tan, C., Liaw, S., Yu, C., Teng, L., Hsueh, P.,2008. Extensively drug-resistant Stenotrophomonas maltophilia in a tertiary care hospital in Taiwan: microbiologic characteristics, clinical features, and outcomes. Diagnostic microbiology and infectious disease 60, 205-210.

Tice, A.D., Rehm, S.J., Dalovisio, J.R., Bradley, J.S., Martinelli, L.P., Graham, D.R., Gainer, R.B., Kunkel, M.J., Yancey, R.W., Williams, D.N., IDSA,2004. Practice guidelines for outpatient parenteral antimicrobial therapy. IDSA guidelines. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 38, 1651-1672.

USDA-AMS, 2014 United States Department of Agriculture. Agriculture Marketing Service Farmers Market Program Fact Sheet. http://www.ams.usda.gov/about-ams/programs-offices/usda-farmers-market

Walia, S., Rana, S.W., Maue, D., Rana, J., Kumar, A., Walia, S.K.,2013. Prevalence of multiple antibiotic-resistant Gram-negative bacteria on bagged, ready-to-eat baby spinach. International journal of environmental health research 23, 108-118.

Wang, W.S., Liu, C.P., Lee, C.M., Huang, F.Y.,2004. Stenotrophomonas maltophilia bacteremia in adults: four years' experience in a medical center in northern Taiwan. Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi 37, 359-365.

Warskow, A.L., Juni, E.,1972. Nutritional requirements of *Acinetobacter* strains isolated from soil, water, and sewage. Journal of Bacteriology 112, 1014-1016.

Whitby, P.W., Carter, K.B., Burns, J.L., Royall, J.A., LiPuma, J.J., Stull, T.L.,2000. Identification and detection of Stenotrophomonas maltophilia by rRNA-directed PCR. Journal of clinical microbiology 38, 4305-4309.

Widmer, F., Seidler, R.J., Gillevet, P.M., Watrud, L.S., Di Giovanni, G.D.1998. A highly selective PCR protocol for detecting 16S rRNA genes of the genus Pseudomonas (sensu stricto) in environmental samples. Applied and Environmental Microbiology 64, 2545-2553.

Wood, J. L; Jessica C.C; Elsie, F; Pascal,D; Kevin A.J. 2015 Microbiological Survey of Locally Grown Lettuce Sold at Farmers' Markets in Vancouver, British Columbia. Journal of Food Protection, 1, 203-208.

Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z.,2013. Higher isolation of NDM-1 producing *Acinetobacter* baumannii from the sewage of the hospitals in Beijing.

Table 1. List of primers used in confirming bacteria isolated from vegetables from Farmers markets

Bacteria		Primer sequence	Size (bp)
A. baumannii	AB-R	GACGATCTGTAGCGGGTCTG	712
	AB-F	CCAGGTAAGGTTCTTCGCGT	
Burkholderia[a]	RHG-F	GGG ATT CAT TTC CTT AGT AAC	494
	RHG-R	GCG ATT ACT AGC CAT TCC AGA	
B. cepacia[b]	PSR1	TTT CGA GCA CTC CCG CCT CTC AG	209
	PSL1	AAC TAG TTG TTG GGG ATT CAT TTC	
Pseudomonas[c]	PS-for	GGT CTG AGA GGA TGA TCA GT	990
	PS-rev	TTA GCT CCA CCT CGC GGC	
P. luteola[c]	PL- F	AGA TGG ATT GGT GCC TTC GG	304
	PL-R	CAG ACT GCG ATC CGG ACT AC	
S. maltophilia[d]	SM4	TTA AGC TTG CCA CGA ACA G	532
	SM1	CAG CCT GCG AAA AGT A	

[a]LiPuma et al.,1999;; [b]Clode et al., 1999; [c] Widmer et al., 1998; [d]Whitby et al., 2000

Table 2. Prevalence of multi-drug resistant bacteria in vegetables from farmers' markets in Connecticut

Vegetable	***A.baumannii***	***B.cepacia***	***S.maltophilia***	***P. luteola***
Potato(n = 100)	2	1	6	9
Carrot(n=100)	0	5	0	3
Lettuce (n=100)	1	0	2	0
Total	**3**	**6**	**8**	**11**

Table 3. Antibiotic resistance profile of the isolates from vegetables

Antibiotics tested	*A.baumannii* Resistant isolates(%)	*B.cepacia* Resistant isolates(%)	*S.maltophilia* Resistant isolates(%)	*P.luteola*Resistant isolates(%)
Imipenem	100	33	50	90
Ceftriaxone	100	100	100	82
Cefepime	66	83	87	63
Minocycline	33	0	0	27
Doxycycline	66	16	0	9
Colistin-sulfate	33	83	100	100
Streptomycin	100	66	50	54
Neomycin	66	16	25	27.
Erythromycin	100	83	62	63
Rifampcin	33	33	37	36

Chapter IV

Effect of chlorine on *A. baumannii* survival in water and expression of antibiotic resistance genes

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ABSTRACT

Acinetobacter baumannii is a multi-drug resistant pathogen capable of causing a wide spectrum of clinical conditions in humans. Being ubiquitous in distribution, *Acinetobacter* spp. has been found in surface water, ground water, natural spring water and drinking water, thereby underscoring the significance of water disinfection to control human infections. Chlorine is the most commonly used disinfectant in drinking and recreational water. Federal agencies, the CDC and EPA, recommend a maximum free chlorine level of 4 ppm in drinking water and 3 ppm in swimming water. This study investigated the effect of different levels of chlorine on the survivability of *A. baumannii* in water. Additionally, the effect of chlorine exposure on the transcription of genes conferring antibiotic resistance in *A. baumannii* was studied. Eight clinical isolates of *A. baumannii*, including a fatal meningitis isolate (ATCC 17978) at ~10^8 CFU/ml were separately exposed to different chlorine concentrations (0.2, 1, 2, 3 and 4 ppm) with a contact time of 30, 60, 90 and 120 sec. The surviving pathogen counts at each specified contact time were determined using broth dilution assay. In addition, real-time quantitative PCR (RT-qPCR) analysis of the antibiotic resistance genes namely efflux pump genes (*adeA, adeB, adeC, abeM*), and those that code for resistance to antibiotics such as β-lactams (*blaP*), aminoglycoside (*ami*), sulfamethoxazole (*sul1*), tetracycline (*tetA*), and multiple drug resistance protein (*mdrp*), of three selected *A. baumannii* strains following exposure to chlorine was performed. Results revealed that all eight *A. baumannii* isolates survived the tested chlorine levels during all exposure times ($P > 0.05$). The RT-qPCR analysis revealed an up-regulation of all or some of the antibiotic resistance genes in the isolates, indicating a chlorine-associated induction of antibiotic resistance in the pathogen. Our results reveal that chlorine is not only ineffective in reducing *A.*

baumannii in water, but also induces the up-regulation of genes conferring resistance to multiple antibiotics in the pathogen.

1. INTRODUCTION

Multi-drug resistant (MDR) *Acinetobacter baumannii* is a major hospital-borne pathogen causing a wide spectrum of clinical conditions with significant mortality rates (Dijkshoorn et al., 2007; Giamarellou., 2010; Perez et al., 2007; Karageorgopoulos et al.,2008; Peleg et al., 2008;Martí, 2011).*A. baumannii* strains are equipped with a multitude of antibiotic resistance mechanisms rendering them resistant to most of the currently available antibiotics (Dijkshoorn et al., 2007; Karageorgopoulos et al.,2008). *A. baumannii* has a remarkable ability to persist for prolonged periods of time in the hospital environment in biofilms, thereby protecting it from the action of disinfectants, and serving as a continuous source of infection (Urban et al., 2003; Gaddy et al.,2009; Shin et al.,2009; Moultrie et al., 2011; Pour et al., 2011). In health-care environments, a variety of surfaces, including table tops, bed rails, sinks, door handles, floors, mattresses, and pillows have been implicated as potential sources of *A. baumannii*(Wilks et al.,2006; Karageorgopoulos et al., 2008;).

Water and soil are considered as a major habitat of*A. baumannii,* although the pathogen has been isolated from other sources, including foods, anthropods, animals, and humans (Baumann et al., 1968; Berlau et al., 1999; Mussi et al., 2005; Fournier et al., 2006; Munoz-Price et al., 2008). Moreover, protozoans such as *Acanthamoeba* have been reported to support the growth of *A. baumannii,* and act as itsreservoir in water (Cateau et al., 2011). Recently, alarming reports indicating the potential presence of *A. baumannii* in water systems have been reported from several parts of the world (Ferreira et al., 2011; Zhang et al., 2013). The ability of *A. baumannii* to thrive in water may result in fatal infections in all age groups (Kempf et al., 2012).

Chlorine has long been used as a disinfectant in drinking water and in swimming pools to inactivatepathogenic microorganisms, thereby making the water safe for human use (Ngwenya et

al, 2013; Shrivastava et al., 2004; WHO, 1993). In the United States, the Environmental Protection Agency (EPA) recommends a maximum free chlorine level of 4 ppm in drinking water (EPA, 2012). In addition to the standards described for chlorine in drinking water, the Centers for Disease Control and Prevention (CDC) have recommended 1-3 ppm chlorine in swimming pool water for recreational purposes (CDC, 2012). However, a variety of microorganisms have been recovered from drinking water distribution systems that maintained chlorine levels between 0.5 – 1.0 ppm, indicating that the low levels of chlorine may not inactivate harmful microorganisms (Means et al., 1981; Ridgway et al., 1982; LeChevallier et al., 1984). It is also reported that chlorine used in potable water can selectively promote the survival of antibiotic resistant bacteria (Armstrong et al., 1981; 1982; Murray et al., 1984; Shrivastava et al., 2004). For instance, drinking water with suboptimal levels of chlorine selectively promoted the survivalof multidrug resistant *Pseudomonas aeruginosa* (Shrivastava et al., 2004).

Since *A. baumannii* could potentially contaminate drinking or recreational water, and that chlorine at the recommended levels may not be effective in killing the pathogen, the current research investigated the viability of *A. baumannii* in water containing chlorine at the recommended levels for potable and recreational usage. In addition, the effect of chlorine exposure on various antibiotic resistance genes in the pathogen was investigated.

2. MATERIALS AND METHODS

2.1. A. baumannii Strains and Growth Conditions

Eight strains of *A. baumannii*, including 251847, 134882, 173795, 474030, 190451, 163731, and 251352 kindly gifted by the International Health Management Associates (IHMA, City, State), and ATCC 17978 (fatal meningitis isolate) were used in the chlorine survival study. The antibiotic resistance profile of the seven clinical strains was Amikacin (MIC 64 μg/mL),

Amoxicillin (MIC 32 μg/mL), Cefepime (MIC 32 μg/mL), Ceftazidime (MIC 32 μg/mL), Ceftriaxone (MIC 64 μg/mL), Imipenem (MIC 4–32 μg/mL), Levofloxacin (MIC 8 μg/mL), Meropenem (MIC 16 μg/mL), Minocycline (MIC 1–16 μg/mL), and Piperacillin (MIC 128 μg/mL).

Each strain of *A. baumannii* was grown individually on MDR *Acinetobacter* and Leeds *Acinetobacter* agars (Hardy Diagnostics, Santa Maria, CA), and an individual colony from these media was subcultured at least 3 times in tryptic soy broth (TSB; Difco) for 24 h at 37ºC with shaking (200 rpm). After the subcultures, the bacterial cells were harvested from an overnight culture by centrifugation at 3600 x g for 30 min at 4ºC. The cells were washed twice in sterile phosphate buffered saline (PBS, pH=7.2), and the bacterial cell pellet was finally resuspended in PBS to get a final concentration of 10^9 cfu/ml. The bacterial population in the inoculum was confirmed by broth dilution and surface plating on tryptic soy agar (TSA; Difco) plates.

2.2. A. baumannii Survival Assay

The effect of chlorine on *A. baumannii* viability in water was determined using a published protocol (Zhao et al., 2001). Deionized, non-chlorinated (EMD Millipore, Billerica, MA) water was used for the study. For each experiment, different chlorine concentrations (0.2, 1, 2, 3 and 4 ppm) in water were achieved by adding standard chlorine solution (Aqua solutions, Deer Park, Texas) to pre-sterilized deionized water. The final concentration of free chlorine in water was confirmed using a digital titrator (Pocket colorimeterTMII, Hach, City, State). One ml of *A. baumannii* suspension containing 10^9 cfu/ml was added to 99 ml of the sterile deionized water containing chlorine at the specifiedconcentrations in a 200 ml Erlenmeyer flask. After thorough mixing, 0.5 ml samplesweretaken at 30, 60, 90 and 120 s, and transferred to 4.5 ml neutralizing broth for buffering chlorine (NB, Difco). Serial ten-fold dilutions in PBS were made and 0.1 ml

of each dilution was surface plated on duplicate on TSA. The plates were incubated at 37ºC for 24 h. After enumeration of the colonies, the counts were expressed as $\log_{10}$ cfu/ml. The colonies on TSA were confirmed as *A. baumannii* by streaking on MDR and Leeds agar plates. Each experiment was done in duplicates and the experiment replicated three times.

2.3. Antibiotic Resistance Gene Expression

2.3.1. RNA Isolation and cDNA Synthesis

Three selected strains of *A. baumannii* (ATCC 17978, 251847 and 474030) were grown on MDR plates and subcultured in TSB separately at 37ºC as before. The bacterial populations in the cultures were confirmed to contain ~8 $\log_{10}$ CFU/ml by plating appropriate dilutions on TSA. The overnight culture from the tubes was centrifuged, washed twice, and reconstituted in PBS as described before. One ml each of this reconstituted culture was transferred to two sets of tubes containing 9 ml of sterile deionized water. To one set of tubes, chlorine was added to yield a concentration of 2 ppm in water. The other set of tubes served as non-chlorine control. The 2 ppm concentration was chosen for the RT-qPCR analysis since the United States Department of Health (USDH) recommends the free chlorine concentration of 1 and 3 ppm to disinfect swimming pools (CDC, 2012). Both set of tubes were incubated at 25ºC for 15 min. The bacterial culture from each tube was centrifuged at 12,000 x g for 2 min at 4ºC. The supernatant was discarded and the pellet was added with 0.5 ml of sterile water and 1 ml of RNA protect reagent (Qiagen, Valencia, CA). The mixture was then incubated at 25ºCfor 5 min. The RNeasy mini kit (Qiagen) was used for extracting total RNA from the control and chlorine-treated samples. The RNA was quantified using Nanodrop (Biorad) by measuring the absorbance at 260 and 280 nm. Super-script II reverse transcriptase kit (Invitrogen, Carlsbad, CA) was used for cDNA synthesis from the extracted RNA.

2.3.2. Real-Time quantitative PCR (RT-qPCR)

The following *A. baumannii* genes were used for the gene expression analysis: efflux pump genes *adeA, adeB, adeC*, and *abeM*; chloramphenicol resistance gene, *cmr*, β-lactam resistance gene, *blaP*; sulphonamide resistance gene *sul1*; tetracycline resistance gene, *tetA*, and multidrug resistance protein, *mdrp*.

The primers specific for the genes and for the endogenous control (16SrRNA) were designed using the Primer Express software® (Applied Biosystems, Foster City, CA, USA based on *Acinetobacter baumannii* AB0057 genome (CP001182.1) published in the NCBI database (Adams et al., 2008). The primers used in the study are provided in Table 1. Custom synthesized primers for each gene were obtained from Integrated DNA Technologies (Foster City, CA USA). RT-qPCR was done with the ABI Prism 7900 sequence detection system (Applied Biosystems) using the SYBR green assay under custom thermal cycling conditions with the normalized mRNA as template (Kollanoor-Johny et al., 2012). The samples were analyzed in duplicates and standardized against 16S rRNA gene expression. The relative changes in mRNA expression levels were determined using comparative threshold cycle (CT) method (2-ΔΔCT) between the chlorine-exposed and chlorine non-exposed *A. baumannii*.

2.4. Statistical Analysis

The counts of *A. baumannii* in the control and treated samples were logarithmically transformed ($\log_{10}$ cfu/ml) to aid in statistical analysis. Since there was no significant difference in bacterial counts between the strains following exposure to chlorine treatment, the data from the eight strains were pooled and averaged. Data analysis was done using the PROC-MIXED procedure of statistical analysis software (SAS version 9.2; SAS Institute Inc., Cary, NC, USA).

Fisher's least significance test (LSD) was used to determine the differences between the means at a *P* level of ≤ 0.05.

3. RESULTS AND DISCUSSION

In order to determine if *A. baumannii* survived the recommended levels of chlorine, we determined the survival of the pathogen exposed to 0 to 4 ppm of free chlorine for 30, 60, 90 and 120 seconds in deionized water (Table 2). All the eight *A. baumannii* controls, where no chlorine was added had 10^7 CFU/ml bacteria at all the time points tested. When exposed to free chlorine levels ranging from 0.2 to 4 ppm, all the eight strains of *A. baumannii* survived with no significant decrease in their counts throughout the sampling period (P> 0.05).

Chlorine is generally used to disinfect both potable and recreational water with stipulated standards for inclusion as determined by the EPA, FDA and CDC. However, previous studies have indicated that chlorine was not completely effective in inactivating several pathogenic bacteria. For example, *Yersinia enterocolitica*, *Yersinia pestis*, *Pasteurella multocida* and *Hafnia alvei* were isolated from chlorine treated sewage water, indicating the inefficiency of chlorine in killing these pathogens (Murray et al., 1984). In another study, Zhao et al. (2001) reported an isolate of *E.coli* O157:H7 showing tolerance of up to 2-ppm free chlorine treatment after one minute exposure, among six other isolates tested in their study. In yet another study, *E. coli* isolated from a chlorine treated swimming pool were found to be resistant to chlorine for up to 9 passages (Farkas-Himsley, 1964). High tolerance of bacteria to disinfectants could either be intrinsic or resulting from mutation. Additionally, wide spread use of disinfectants has been reported to trigger the selection of resistant strains (McDonnell et al., 1999).

Since we observed that *A. baumannii* could survive all the tested concentrations of chlorine in water, we investigated the effect of chlorine exposure on major antibiotic resistance

determinants using RT-qPCR.The effect of chlorine exposure on the expression of ten major antibiotic resistance genes conferring resistance to multiple antibiotics in *A. baumannii* was studied in ATCC strain 17978, 251847, and 474030. The ATCC strain was selected since it has been widely studied, and was isolated from a 4-month-old infant who died of fatal meningitis resulting from an acute infection. The results on the effect of chlorine exposure on antibiotic resistance genes in theATCC strain are shown in Figure 1. Among the various genes tested, those controlling antibiotic efflux pumps in *A. baumannii*, namely *adeA, adeB,* and *abeM* were significantly up-regulated by more than six folds when compared to the control, while the efflux pump gene, *adeC* and the gene encoding chloramphenicol resistance,*cmr* were up-regulated by about four folds ($P< 0.05$). A three fold up-regulation was noticed in *blaP* that confers resistance to β lactam group of antibiotics upon exposure to chlorine ($P> 0.05$). Exposure to chlorine also resulted in an increase in the expression of aminoglycoside (*ami*) and sulphonamide (*sul1*) resistance genes by three and four folds, respectively ($P<0.05$). In addition, an eight-fold up-regulation ($P< 0.05$) of the gene encoding multiple drug resistance protein (*mdrp*) in *A. baumannii* was observed following exposure to chlorine. However, no significant change in the expression of tetracyclineresistance encoding gene, *tetA* was observed upon exposure to chlorine ($P> 0.05$). The results revealed that the efflux pump genes were more up-regulated compared to the antibiotic resistance genes (Figure 1), highlighting the involvement of efflux pump mechanisms on rapid exposure to chlorine (Xi et al., 2009). Figure 2 shows the antibiotic gene expression in *A. baumannii* 251847, where a significant up-regulation was observed in the expression of *adeC, cmr* and *tetA* by nine, four and five folds, respectively ($P< 0.05$). However, the expression of all the other genes tested was decreased following chlorine exposure ($P< 0.05$). In *A. baumannii* 470430, a significant up-expression of all genes except *tetA* (Figure 3) was

observed after exposure to chlorine ($P < 0.05$), which was similar to trend observed in the ATCC isolate. To summarize, despite variations in the expression levels of specific genes, exposure to chlorine induced the expression of multiple antibiotic resistance genes in all the three isolates of *A. baumannii* studied.

Although preventative chlorine levels are in use, several reports suggest that chlorine at suboptimal levels could induce the expression of critical genes in pathogenic bacteria. Shi and coworkers reported that chlorine enhanced the expression of antibiotic resistance genes in diverse microbial populations isolated from drinking water (Shi et al., 2013). Similarly, an increase in the expression of several antibiotic resistance genes was noted in *E.coli* and *P. aeruginosa* by other research groups (Shrivastava et al., 2004; Krige, 2009). It was observed that exposure to chlorine induced stress tolerance in bacteria making them more resistant to antibiotics (Armstrong et al., 1981; 1982). It is also reported that chlorine exposure could induce over expression of efflux pumps resulting in the pumping out of disinfectants and antibiotics by bacteria (Xi et al., 2009).

In conclusion, the current investigation indicated that free chlorine concentration of up to 4 ppm was not effective in killing multidrug resistant *A. baumannii* isolates. All *A. baumannii* isolates were able to survive the recommended levels of chlorine in water. Further, chlorine exposure was found to increase the expression of efflux pumps and genes conferring resistance to chloramphenicol, sulphonamides, and beta-lactam group of antibiotics. Further studies are required to understand the mechanism behind chlorine-induced gene expression in *A. baumannii*, and its significance to public health.

6. REFERENCES

Adams, M.D.; Goglin, K.; Molyneaux, N.; Hujer, K.M.; Lavender, H.; Jamison, J.J.; MacDonald, I.J.; Martin, K.M.; Russo, T.; Campagnari, A.A.; et al. Comparative genome sequence analysis ofmultidrug-resistant *Acinetobacter* baumannii. J. Bacteriol. 2008, 24, 8053–8064.

Armstrong J.L., Calomiris, J.J., Seidler, R.J., 1982. Selection of antibiotic-resistant standard plate count bacteria during water treatment. Appl. Environ. Microbiol. 44 (2), 308-316.

Armstrong J.L., Shigeno, D.S., Calomiris, J.J., Seidler, R.J., 1981. Antibiotic-resistant bacteria in drinking water. Appl. Environ. Microbiol.42 (2), 277-283.

Baumann P., 1968. Isolation of *Acinetobacter* from soil and water. J. Bacteriol.96 (1), 39-42.

Berlau J., Aucken, H.M., Houang, E., Pitt, T.L., 1999. Isolation of *Acinetobacter* spp. including *A. baumannii* from vegetables: implications for hospital-acquired infections. J Hosp Infect.42 (3), 201-204.

Cateau E., Verdon, J., Fernandez, B., Hechard, Y., Rodier, M.H., 2011. *Acanthamoeba* sp. promotes the survival and growth of *Acinetobacter* baumanii. FEMS Microbiol. Lett.319 (1), 19-25.

Centers for Disease Control and Prevention (CDC)., 2004. *Acinetobacter* baumannii infections among patients at military medical facilities treating injured U.S. service members, 2002-2004. MMWR 53 (45), 1063-1066.

Centers for Disease Control and Prevention. Healthy swimming or recreational water. Retrieved from http://www.cdc.gov/healthywater/swimming/pools/disinfection-team-chlorine-ph.html on 12/13/2012.

Dijkshoorn L., Nemec, A.,Seifert, H., 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter* baumannii. Nat. Rev. Microbiol. 5,939-951.

Environmental Protection Agency (EPA). Basic information about disinfectants in Drinking Water: Chloramine, Chlorine and Chlorine dioxide. Retrieved from http://water.epa.gov/drink/contaminants/basicinformation/disinfectants.cfm on 12/18/2012.

Farkas-himsley H., 1964. Killing of Chlorine-Resistant Bacteria by Chlorine-Bromine Solutions. Appl Microbiol. 12, 1-6.

Ferreira A.E., Marchetti, D.P., De Oliveira, L.M., Gusatti, C.S., Fuentefria, D.B., Corcao, G., 2011. Presence of OXA-23-producing isolates of *Acinetobacter* baumannii in wastewater from hospitals in southern Brazil. Microb Drug Resist. 17 (2), 221-227.

Fournier P.E. and Richet, H., 2006. The epidemiology and control of *Acinetobacter* baumannii in health care facilities. Clin Infect Dis.42 (5), 692-699.

Gaddy J.A., Tomaras, A.P., Actis, L.A., 2009. The *Acinetobacter* baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infect Immun. 77, 3150-60

Giamarellou H., 2010. Multidrug-resistant gram-negative bacteria: how to treat and for how long. Int. J. Antimicrob. Agents. 36(Suppl.), 50-54.

Karageorgopoulos D.E.,Falagas, M.E., 2008. Current control and treatment of multidrug-resistant *Acinetobacter* baumannii infections. Review. Lancet Infect. Dis. 8,751-762.

Kempf M., Rolain, J. M., 2012. Emergence of resistance to carbapenems in *Acinetobacter* baumannii in Europe: clinical impact and therapeutic options. Int J Antimicrob Agents. 39 (2), 105-114.

Kollanoor-Johny, A.; Mattson, T.; Baskaran, S.A.; Amalaradjou, M.A.; Babapoor, S.; March, B.; Valipe, S.; Darre, M.; Hoagland, T.; Schreiber, D.; et al. Reduction of Salmonella enterica serovar enteritidis colonization in 20 day old broiler chickens by the plant-derived compounds trans-cinnamaldehyde and eugenol. Appl. Environ. Microbiol. 2012, 78, 2981–2987.

Krige M., 2009. Molecular investigation of the chlorine and antibiotic resistance mechanisms of Escherchia coli isolated from natural water sources in Western Cape. SUNscholar Research Repository. Retrieved from http://scholar.sun.ac.za/handle/10019.1/2779 on 10/18/2012.

LeChevallier M.W., Babcock, T.M., Lee, R.G., 1987. Examination and characterization of distribution system biofilms. Appl. Environ. Microbiol.53 (12), 2714-2724.

LeChevallier M.W., Hassenauer, T.S., Camper, A.K., McFeters, G.A., 1984. Disinfection of bacteria attached to granular activated carbon. Appl. Environ. Microbiol.48 (5), 918-923.

Martí S., Rodríguez-Baño, J., Catel-Ferreira, M., Jouenne, T.,Vila, J., Seifert, H., Dé, E.,2011. Biofilm formation at the solid-liquid and air-liquid interfaces by *Acinetobacter* species. BMC Res. Notes. 4:5.

McDonnell G., Russell, A.D., 1999. Antiseptics and Disinfectants : Activity, Action and Resistance. Clin Microbiol Rev. 12(1), 147-79

Means E.G., Hanami, L., Ridgway, H.F., Olson, B.H., 1981. Enumeration of bacteria in potable water distribution systems: evaluation of media and plating techniques. J. Am. Water Works Assoc.53, 585-590.

Moultrie D., Hawker, J., Cole, S., 2011. Factors associated with multidrug-resistant *Acinetobacter* transmission: an integrative review of the literature. AORN 94 (1), 27-36.

Munoz-Price L.S. and Weinstein, R.A., 2008. *Acinetobacter* infection. New Eng J Med358 (12), 1271-1281.

Murray G.E., Tobin, R.S., Junkins, B., Kushner, D.J., 1984. Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria. Appl. Environ. Microbiol.48 (1), 73-77.

Mussi M.A., Limansky, A.S., Viale, A.M., 2005. Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter* baumannii: natural insertional inactivation of a gene encoding a member of a novel family of beta-barrel outer membrane proteins. Antimicrob Agents Chemother.49 (4), 1432-1440.

Ngwenya N., Ncube, E.J., Parsons, J., 2013. Recent advances in drinking water disinfection: successes and challenges. Rev Environ Contam Toxicol.222, 111-170.

Peleg A.Y., Seifert, H., Paterson, D.L., 2008. *Acinetobacter* baumannii: emergence of a successful pathogen. Clin Microbiol Rev.21 (3), 538-582.

Perez F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N., Bonomo, R. A., 2007. Global challenge of multidrug-resistant *Acinetobacter* baumannii. Antimicrob Agents Chemother.51 (10), 3471-3484.

Pour N.K., Dusane, D.H., Dhakephalkar, P.K., Zamin, F.R., Zinjarde, S.S.,Chopade, B.A.,2011. Biofilm formation by *Acinetobacter* baumannii strains isolated from urinary tract infection and urinary catheters. Immunol. Med. Microbiol. (Ahead of print) ; doi: 10.1111/j.1574-695X.2011.00818.x.

Ridgway H.F. and Olson, B.H., 1982. Chlorine resistance patterns of bacteria from two drinking water distribution systems. Appl. Environ. Microbiol.44 (4), 972-987.

Shi P., Jia, S., Zhang, X. X., Zhang, T., Cheng, S., Li, A., 2013. Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water. Water Res.47 (1), 111-120.

Shin J.H., Lee, H.W., Kim, S.M., Kim, J.,2009. Proteomic analysis of *Acinetobacter* baumannii in biofilm and planktonic growth mode. J Microbiol. 47,728-735.

Shrivastava R., Upreti, R.K., Jain, S. R., Prasad, K. N., Seth, P. K., Chaturvedi, U. C., 2004. Suboptimal chlorine treatment of drinking water leads to selection of multidrug-resistant Pseudomonas aeruginosa. Ecotoxicol Environ Saf.58 (2), 277-283.

Urban C., Segal-Maurer, S., Rahal, J. J., 2003. Considerations in control and treatment of nosocomial infections due to multidrug-resistant *Acinetobacter* baumannii. Clin Infect Dis.36 (10), 1268-1274.

Vila J., Pachon, J., 2012. Therapeutic options for *Acinetobacter* baumannii infections: an update. Expert Opin Pharmacother.13 (16), 2319-2336.

Wilks, M.,Wilson, A., Warwick, S., Price, E., Kennedy, D., Ely, A., Millar, M.R., 2006. Control of an outbreak of multidrug-resistant *Acinetobacter* baumannii-calcoaceticus colonization and infection in an intensive care unit (ICU) without closing the ICU or placing patients in isolation. Infect.Control Hosp. Epidemiol. 27,654-658.

World Health Organization.,1993. Guidelines for drinking water quality, Geneva: WHO. Retrieved from http://www.who.int/water_sanitation_health/publications/2011/dwq_guidelines/en/index.html on 10/13/2012

Xi C., Zhang, Y., Marrs, C. F., Ye, W., Simon, C., Foxman, B., Nriagu, J., 2009. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. Appl Environ Microbiol.75 (17), 5714-5718.

Zhang C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z., Li, P., Wang, L., Cui, J., Wang, P., Huang, L., Klena, J. D., Song, H., 2013. Higher isolation of NDM-1 producing *Acinetobacter*baumannii from the sewage of the hospitals in Beijing.PloS one 8 (6), e64857.

Zhao T., Doyle, M.P., Zhao, P., Blake, P., Wu, F. M., 2001. Chlorine inactivation of Escherichia coli O157:H7 in water. J Food Prot.64 (10), 1607-1609.

Table 1: List of primers used in determing the effect of chlorine on antibiotic resistance genes in *A. baumannii*

Gene	Sequence (5'→ 3')
16S-rRNA (F)	TCGCTAGTAATCGCGGCATCACGCTGGCGGC
(R)	GACGGGCGGTGTGTACAAG
adeA (F)	TGACCGACCAATGCACCTT
(R)	GCAACAGTTCGAGCGCCTAT
adeB (F)	CCGATGACGTATCGAAGTTAGGA
(R)	CCGATGACGTATCGAAGTTAGGA
adeC (F)	ACGGCCCCAGAAGTCTAGTTC
(R)	CGATTAACCCCAATAACCCAGTT
adeM (F)	GGTACATGGAAGCCCAGTTCTT
(R)	CCACTTTCTCTTGCCATTGCT
blaP (F)	ACACTAGGAGAAGCCATGAAGCTT
(R)	GCATGAGATCAAGACCGATACG
cmr (F)	CTATTTGAATTTGCGGTTTATATTGG
(R)	TGCACTTACACCGAAATCTTCAG
ami (F)	TGATCCCGTAAATGAGTTGAATTG
(R)	GCGGGCAAATGTGATGGTA
sul1 (F)	GGCATGACAATAGGGCAGTTG
(R)	CCAAAAAGTAGATGATAATACCGGTAAA
tetA (F)	CTGCGCGATCTGGTTCACT
(R)	GCATACAGCGCCAGCAGAA
mdrp(F)	GTACGGCTTCTAGACCCACCAT
(R)	ACAAAGAGCCGTGCACAGTTT

(F), forward; (R), reverse

Table 2. Effect of chlorine concentrations on the survival of *A. baumannii* in deionized water*

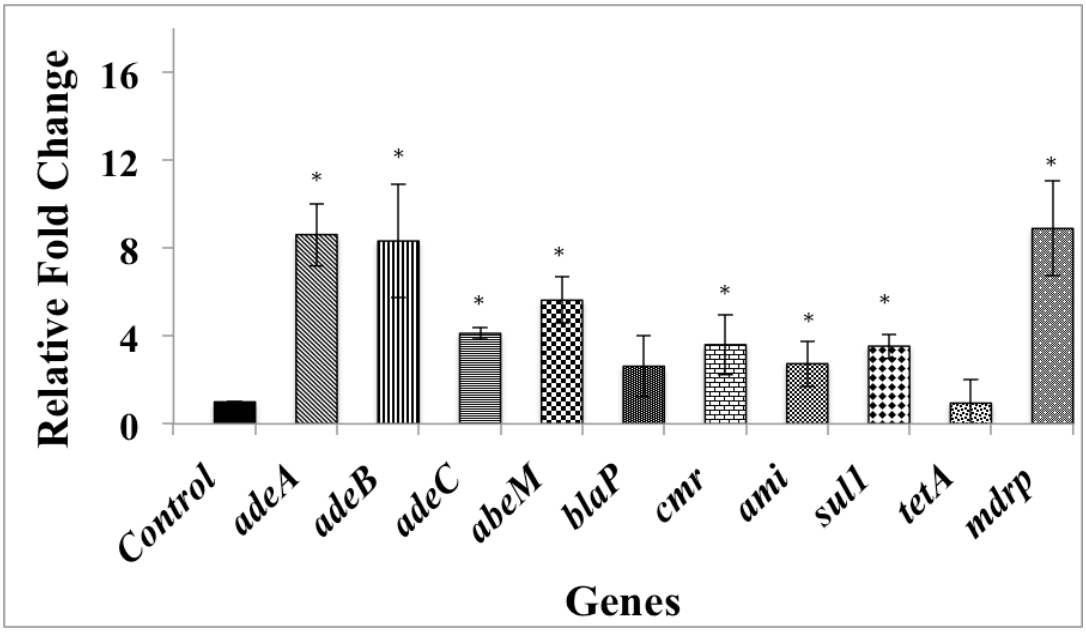
Chlorine(ppm)	Isolates	*A. baumannii* (log_{10} CFU/ml)			
		30 sec	60 sec	90 sec	120 sec
0	ATCC	7.38±0.07	7.42±0.06	7.41±0.06	7.44±0.06
	251847	7.29±0.03	7.33±0.1	7.28±0.04	7.35±0.05
	134882	7.58±0.07	7.56±0.06	7.58±0.05	7.54±0.07
	173795	7.34±0.09	7.38±0.09	7.35±0.1	7.33±0.08
	474030	7.32±0.09	7.37±0.12	7.37±0.11	7.39±0.1
	190451	7.52±0.06	7.63±0.07	7.64±0.05	7.47±0.1
	163731	7.48±0.07	7.48±0.04	7.49±0.09	7.48±0.08
	251352	6.8±0.14	6.86±0.18	6.8±0.15	6.81±0.14
0.2	ATCC	7.42±0.13	7.44±0.09	7.45±0.1	7.47±0.11
	251847	7.33±0.07	7.27±0.08	7.28±0.07	7.32±0.09
	134882	7.53±0.06	7.54±0.05	7.46±0.05	7.57±0.07
	173795	7.21±0.07	7.15±0.03	7.09±0.02	7.1±0.02
	474030	7.31±0.09	7.33±0.1	7.33±0.1	7.25±0.12
	190451	7.58±0.09	7.65±0.1	7.62±0.12	7.57±0.1
	163731	7.45±0.1	7.55±0.09	7.52±0.07	7.64±0.09
	251352	7.15±0.03	7.19±0.05	7.17±0.04	7.23±0.09
1	ATCC	7.34±0.1	7.39±0.09	7.33±0.05	7.46±0.09
	251847	7.27±0.07	7.24±0.09	7.2±0.06	7.16±0.07
	134882	7.3±0.06	7.33±0.05	7.25±0.05	7.38±0.05
	173795	7.22±0.15	7.09±0.01	7.1±0.02	7.09±0.04
	474030	7.31±0.07	7.37±0.11	7.33±0.11	7.29±0.12
	190451	7.71±0.03	7.66±0.04	7.68±0.02	7.74±0.03
	163731	7.53±0.06	7.48±0.06	7.58±0.09	7.49±0.14
	251352	7.13±0.03	7.14±0.04	7.16±0.02	7.12±0.04
2	ATCC	7.45±0.05	7.42±0.05	7.42±0.05	7.37±0.04
	251847	7.14±0.02	7.17±0.05	7.2±0.07	7.19±0.07
	134882	7.44±0.08	7.41±0.09	7.38±0.09	7.36±0.11
	173795	7.62±0.08	7.64±0.08	7.63±0.08	7.57±0.07
	474030	7.39±0.16	7.43±0.12	7.36±0.15	7.2±0.1
	190451	7.64±0.08	7.66±0.05	7.55±0.06	7.55±0.07
	163731	7.43±0.13	7.43±0.1	7.4±0.09	7.47±0.13
	251352	6.06±0.08	6.03±0.05	6.05±0.08	5.89±0.19

- Non significant at P> 0.05

Chlorine(ppm)	Isolates	*A. baumannii* (log_{10} CFU/ml)			
		30 sec	60 sec	90 sec	120 sec
3	ATCC	7.47±0.05	7.44±0.06	7.31±0.05	7.31±0.1
	251847	7.16±0.05	7.21±0.06	7.17±0.06	7.19±0.06
	134882	7.45±0.1	7.41±0.09	7.38±0.04	7.36±0.08
	173795	7.59±0.07	7.61±0.08	7.61±0.09	7.57±0.08
	474030	7.31±0.15	7.48±0.17	7.47±0.16	7.32±0.13
	190451	7.58±0.07	7.56±0.06	7.5±0.1	7.56±0.07
	163731	7.53±0.06	7.46±0.03	7.44±0.06	7.57±0.07
	251352	6.15±0.04	6.16±0.05	6.16±0.07	6.07±0.05
4	ATCC	7.37±0.1	7.31±0.09	7.23±0.09	7.21±0.11
	251847	7.19±0.07	7.17±0.1	7.34±0.16	7.3±0.15
	134882	7.38±0.12	7.62±0.11	7.3±0.07	7.29±0.06
	173795	7.65±0.09	7.61±0.09	7.62±0.09	7.63±0.1
	474030	7.39±0.15	7.34±0.16	7.25±0.11	7.36±0.16
	190451	7.53±0.04	7.54±0.04	7.55±0.05	7.52±0.05
	163731	7.51±0.05	7.61±0.04	7.53±0.06	7.56±0.09
	251352	6.19±0.16	6.14±0.14	6.14±0.16	6.08±0.15

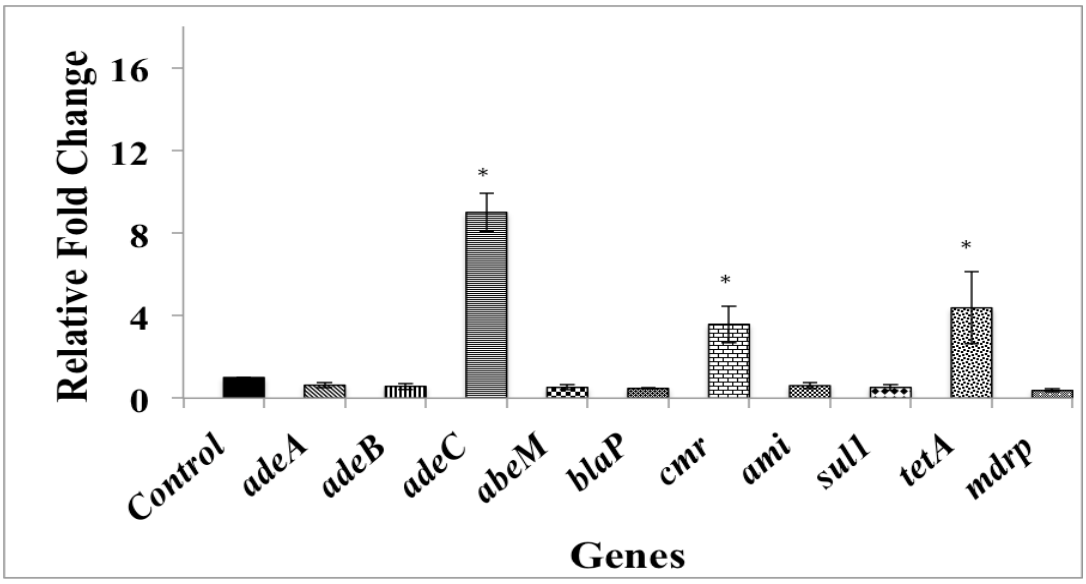
*Non significant at P> 0.05

Fig 1: Effect of chlorine exposure on *A. baumannii*ATCC 17978 antibiotic resistance gene expression



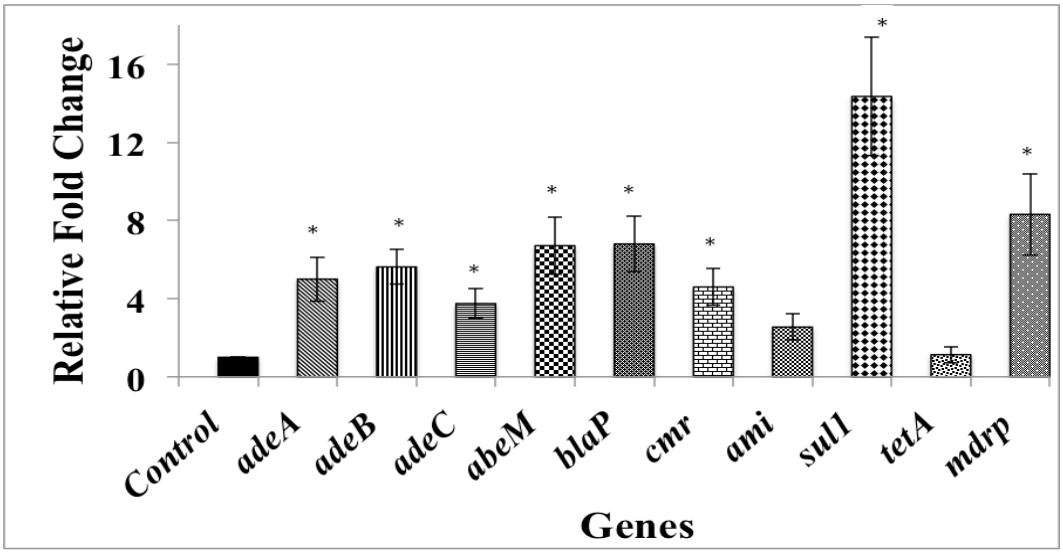
* Genes significantly different from the control atP< 0.05

Fig 2: Effect of chlorine exposure on *A. baumannii* 251847 antibiotic resistance gene expression



* Genes significantly different from the control at P< 0.05.

Fig 3: Effect of chlorine exposure on *A. baumannii* 474030 antibiotic resistance gene expression



* Genes significantly different from the control at P< 0.05.

Chapter V

A Real-Time quantitative PCR for specific detection of *A. baumannii* in water and blood.

ABSTRACT

Multi-drug resistant (MDR) *Acinetobacter baumannii* is a major nosocomial pathogen causing a wide spectrum of clinical conditions with significant mortality rates.Identification of *A. baumannii* by traditional cultural methods requires at least 48 to 72 h for obtaining results. In addition, biochemical methods for the confirmation of *A. baumannii* from other *Acinetobacter* species are not completely accurate. Thus, there is a need for rapid methods for specific identification of *A. baumannii*.

Polymerase chain reaction is a powerful molecular technique, which has been widely used for detecting microorganisms. The objective of this study was to develop a real-time polymerase chain reaction (RT-PCR) for specifically detecting *A. baumannii* in water and blood using TaqMan primer/probe set targeting a highly conserved 102-bp DNA sequence in *adeT*, an efflux pump found in *A. baumannii*. For testing the limit of detection, RT-PCR was done directly with 10-fold dilutions of *A. baumannii* suspension in blood or water (10^6 CFU to 10^1 CFU) and various concentrations of genomic DNA. Further, the sensitivity of the RT-PCR was tested after enrichment of *A. baumannii*in tryptic soy broth. The results revealed that all the *A. baumannii*isolates yielded a 102-bp PCR product, however, none of the tested negative control isolates, including other *Acinetobacter* species produced any amplification. The sensitivity of PCR for detecting *A. baumannii* in blood and water was 3 $\log_{10}$ CFU/ml or 0.1 ng/ml of DNA. However, upon enrichment, the PCR was able to detect 2 $\log_{10}$ and 1 $\log_{10}$ CFU/ml of *A. baumanni* in water after 6 h and 14 h of incubation at 37^{o}C, respectively. The RT-PCR developed in this study enabled specific detection of *A. baumannii*, and may be useful in the rapid detection of the pathogen from environmental and clinical samples.

1. INTRODUCTION

Acinetobacter baumannii is a Gram-negative, aerobic, coccobacillus belonging to the family Moraxellaceae in the order Gammaproteobacteria (Peleg et al., 2008; Perez et al., 2007). *Acinetobacter* species are ubiquitously present in soil and water, which are considered to be their natural habitats (Baumann,et al., 1968). *A. baumannii* has also been detected in water systems from multiple parts of the world (Ferreira et al., 2011; Zhang et al., 2013). In addition, *A. baumannii* can persist in biofilms on a variety of abiotic surfaces for prolonged periods of time in the hospital environment, thereby serving as a continuous source of infection. *A. baumannii* is the most common bacterium associated with infections linked to intensive care units (Giamarellou, 2010, Lambert et al., 2011), and it can cause a variety of nosocomial infections in humans, including meningitis, pneumonia, urinary tract infections and bacteremia, with a high mortality rate (Seifert et al., 1995; Dijkshoorn et al., 2007; Perez et al., 2007; Wisplinghoff et al., 2004; Peleg et al., 2008; Pereira et al., 2013). During the last decade, *A. baumannii* has emerged as an important human pathogen due to increasing reports of infections associated with the organism and rapid emergence of multidrug-resistant (MDR), extensively-drug resistant (XDR), and pandrug-resistant (PDR) strains (Peleg et al, 2008; Durante-Mangoni and Zarrilli, 2011). The Infectious Diseases Society of America has ranked *A. baumannii* as one of the highest priority, antibiotic-resistant pathogens to control due to its rapid propensity to develop drug resistance, and the availability of a restricted choice of antibiotics to treat infections caused by the bacterium (Talbot et al., 2006).

Bacteriological detection of *A. baumannii* by traditional cultural methods requires atleast 48 to 72 h for obtaining accurate results (Levi et al., 2003; Jamal et al., 2014). Furthermore, the four *Acinetobacter* species, namely *A. calcoaceticus* (formerly *Acinetobacter* genomospecies 1), *A.*

baumannii (formerly *Acinetobacter* genomospecies 2), *Acinetobacter pitti* (formerly *Acinetobacter* genomospecies 3) and *Acinetobacter nosocomalis* (formerly *Acinetobacter* genomospecies 13TU) are phenotypically and genotypically closely related, and are difficult to be distinguish from each other. Therefore, they are grouped together and referred to as *A. baumannii -A. calcoaceticus* complex (ABC) (Gerner-Smidt et al., 1991; Gerner-Smidt, 1992). Although several *Acinetobacter* species show hemolysis on sheep blood agar at 37^{o}C, this property, which is sometimes used for species identification is not exhibited by the members of ABC complex (Peleg, 2008). In Gram staining procedure, *Acinetobacter* spp. is sometimes difficult to de-stain, and depending on its stage of growth, can exhibit either Gram negative or Gram positive characteristics (Howard et al., 2012). Moreover, species identification using biochemical tests cannot differentiate *A. calcoaceticus* from *A. baumannii*, and specific detection needs to be complemented by further tests, which could be time consuming in clinical diagnosis. Thus, there is a need for accurate assays for specific detection of *A.baumannii* in clinical and environmental samples.

Polymerase chain reaction (PCR) is a widely used method for rapid, specific and sensitive detection of microorganisms (Garibyan and Avashia, 2013; Law et al., 2015).Real-time Taqman PCR assays are faster and more sensitive than conventional PCR, and allow the quantification of target DNA (Livak et al., 1995). The objective of this study was to develop and optimize a real time-PCR for specific detection of *A. baumannii* from water and blood by targeting *adeT*, a RND (resistance-nodulation-division) type efflux pump gene in *A. baumannii* not reported to be present in any other bacteria.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Growth Conditions

The various bacterial strains used in the study are listed in Table 1. A total of seventeen clinical and environmental isolates of *A. baumannii* were used to study the specificity of the PCR. The negative control isolates included important water- and blood-borne pathogens as well as other *Acinetobacter* species from the ABC complex- *A. calcoaceticus*, *A. nosocomalis* and *A. pitti*. Each strain of *A. baumannii* was individually grown on MDR *Acinetobacter* and Leeds *Acinetobacter* agar plates (Hardy Diagnostics, Santa Maria, CA, USA), and a single colony from these media was sub-cultured at least three times in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) for 24 h at 37ºC. The bacteria used as negative controls were cultured in TSB (Difco).

2.2. Preparation of Genomic DNA

The genomic DNA from Gram-negative and Gram-positive bacteria was extracted,as previously described by Mohan Nair and Venkitanarayanan (2006). For testing the sensitivity of PCR, *A. baumannii* DNA was extracted from one milliliter aliquots of sterile deionized water or defibrinated sheep blood (Quad Five, Ryegate, MT, USA) containing 10^6, 10^5, 10^4, 10^3, 10^2, or 10^1 CFU using InstaGene Matrix (Bio-Rad, Hercules, CA, USA) or BiOstic bacteremia DNA isolation kit (Mo Bio, Carlsbad, CA), respectively. Concurrently, the sensitivity of PCR was also tested with serial dilutions of DNA ranging from 100 ng to 0.1 ng/µl.

2.3. Detection of A. baumannii in water and blood on pre-enrichment

In order to increase the sensitivity of the PCR, an enrichment of the sample was done in TSB (Mohan Nair and Venkitanarayanan, 2006; Afendy and Son, 2015), where one milliliter aliquots of water or blood containing 10^3, 10^2 or 10^1*A. baumannii* were separately transferred to

9 ml of TSB, and incubated at 37^{o}C for 6 or 14 h. Following incubation, *A. baumannii* genomic DNA was extracted and subjected to real-time PCR analysis.

2.4. Primers for A. baumannii specific PCR

Using Basic Local Alignment Search Tool (BLAST), a primer pair targeting *A. baumannii* RND efflux pump gene *adeT* was designed. The forward primer (5'-CGGAATATCCGCTTTCATTT-3') and reverse primer (5'-GACAAATTAAAAGCTGGTTT-3') target a highly conserved 102 bp region of the gene. The probe sequence was (5' CGCGGTAACTGACCAGCCACC–3') with FAM- labeled on the 5' end and TAMRA on the 3' end. All oligonucleotides were synthesized, labeled and purified by Integrated DNA Technologies (Coralville, IA, USA).

2.5. Real-Time TaqMan PCR Assay

Real-time TaqMan PCR assay was performed using StepOnePlus™ Real-time PCR detection system (Applied Biosystems, Foster city, CA, USA). Thirty µl reactions containing 15 µl 2x Taqman universal PCR master mix (Applied Biosystems), PCR primers at a concentration of 300 nM and the probe at 100 nM, and 6 µlof DNA were prepared. The PCR parameters were 50^{o}C for 2 min, 95^{o}C for 10 min and then 40 cycles at 95^{o}C for 15s and 60^{o}C for 1 min. The software in the instrument calculates and plots the changes in the fluorescence signalfrom the probe (Δ Rn) versus the cycle number during the PCR reaction.Δ Rn is calculated using the formula Δ Rn = (Rn+) – (Rn-), where Rn+ is the fluorescence signal of the PCR product at a given time and Rn- is the fluorescence signal of the baseline emission during the initial amplification cycles, where no signal was detected. To determine the efficiency of the PCR, a standard curve was plotted using the Ct number (cycle number at which the Δ Rn crosses the threshold) and log CFU/ml of *A. baumannii*. The primer-probe set was subsequently tested using standard curve

dilutions of the *A. baumannii* or *A. baumannii* DNA. Regression analyses of the data wasdone to determine the correlation coefficient.

4. RESULTS AND DISCUSSION

Clinically, the rapid detection of *A. baumannii* is important for reducing the time before patients receive appropriate treatment, which is especially critical in light of its multi-drug resistance, and ability to cause a wide spectrum of infections with high mortality rates. Moreover, accurate detection of *A. baumannii* in water is crucial as its ability to survive in water may result in fatal infections in all age groups (Kempf et al., 2012). Therefore, this study was undertaken to develop a real-time PCR for rapid and specific detection of *A. baumannii* in blood and water. A literature search revealed that a few PCRs have been developed for the specific detection of *A. baumannii* or multiplex detection for *Acinetobacter* spp. For example, McConnell and group (2012) reported a real-time PCR based on the conserved sequence of *ompA* for detecting *A. baumannii*. Although these researchers reported a high sensitivity for the PCR, they failed to include *A. calcoaceticus*, *A. nosocomalis* and *A. pitti* from the ABC complex as negative controls in the assay. Similarly, Higgins et al. (2007) developed a conventional PCR based on *gyrB* for differentiating between *A. baumannii* and *A. nosocomalis*; however, they did not screen the PCR with highly related *A. calcoaceticus* and *A. pitti* from the ABC complex. Recently, Chen and coworkers reported a multiplex PCR based on 16S-23S ribosomal RNA intergenic region, *gyrB*, and *recA* genes for the identification of *A. baumannii*, *A. nosocomialis* and *A. pitti*. Although a large number of isolates were screened in the PCR, the authors did report 16 false positives and 10 false negatives in their results (Chen et al., 2014).

The PCR developed in this study is based on a highly conserved 102-bp DNA sequence of *adeT*, a RND type efflux pump gene reportedly present only in *A. baumannii*. The specificity

of this DNA sequence was confirmed by BLAST analysis with all published prokaryotic gene sequences, including related *Acinetobacter* sequences available in the NCBI GenBank database. The specificity of the primers was further tested using *In silico* PCR (Bikandi et al., 2004), a computational tool to detect the efficacy of primer-probe set to amplify the target DNA sequence. The primer pair was used to amplify the target genomic DNA sequence from 17 strains of *A. baumannii* and 28 strains of other important bacteria,including members from the ABC complex, and important water-borne bacteria such as *Escherichia coli*, *Salmonella*, *Vibrio cholera*, *Shigella* and *Pseudomonas*, and other major pathogens such as *Klebsiella pneumoniae*, *Listeria monocytogenes* and *Yersinia pseudotuberculosis*.All the isolates of *A. baumannii* showed the amplification of a 102-bp DNA sequence within the targeted 40 cycles, whereas none of the negative isolates, including the other members of the ABC complex yielded any amplification (Fig. 1 and 2), thereby indicating that the PCR developed was able to specifically detect *A. baumannii*.

The sensitivity of PCR was tested with DNA extracted from serial dilutions of *A. baumannii* counts (ranging from 1 $\log_{10}$ to 6 $\log_{10}$ CFU/ml) present in water and blood. After 40 cycles of amplification, the PCR could detect as low as 3 $\log_{10}$ CFU/ml of *A. baumannii* in both water (Fig 3A) and blood (Fig 4A). A standard curve showing the relation between the threshold cycle and *A. baumannii* counts was plotted for detection in water (Fig 3B), where each point represents *A. baumannii* count corresponding to the CT value. The regression analysis performed with bacterial count as the independent variable and CT value as the dependent variable revealed a correlation coefficient (R^2) of 0.94. Similarly, the relationship between threshold cycle and *A. baumannii* counts in blood was found to have a correlation coefficient of 0.99 (Fig 4B). The PCR sensitivity was also tested with serial dilutions of *A. baumannii* DNA, where the lowest limit of

detection was found to be 0.1ng/μl of DNA (Fig 5A), with a correlation coefficient of 0.98 (Fig 5B).

Since the lowest detection limit of the PCR was 3 $\log_{10}$CFU/ml of *A. baumannii* both in water and blood, a pre-PCR enrichment of the samples was done to improve the sensitivity of PCR detection. The results revealed that enrichment for 6 h at 37^{o}C improved the sensitivity of detection to 2 $\log_{10}$ CFU/ml (Fig. 6A), whereas the PCR could detect as low as 1 $\log_{10}$ CFU/ml of *A. baumannii* in water after 14 h of enrichment (Fig. 6B). However, the enrichment step failed to improve the sensitivity of detection in blood samples, which remained at 3 $\log_{10}$ CFU/ml even after enrichment (data not shown). This could be attributed to the presence of potential PCR inhibitors such as heme (Akane et al., 1994), and hemoglobin and lactoferrin (Al-Soud et al., 2001) present in blood.

In summary, the PCR reported in the manuscript was able to detect *A. baumannii* specifically, without any false positives, especially from other species in the ABC complex. The PCR could potentially be used for rapid detection of the pathogen from environmental and clinical samples; however, further optimization for improving its sensitivity of detection in clinical samples is warranted.

References

Afendy, M.A.T., Son, R., 2015. Pre-enrichment effect on PCR detection of Salmonella Enteritidis in artificially contaminated raw chicken meat. International food research journal 22, 2576-2576.

Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., Kimura, K.,1994. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. Journal of forensic sciences 39, 362-362.

Al-Soud, W.A., Radstrom, P.,2001. Purification and characterization of PCR-inhibitory components in blood cells. Journal of clinical microbiology 39, 485-493.

Baumann, P., Doudoroff, M., Stanier, R.Y.,1968. A study of the Moraxella group. II. Oxidative-negative species (genus *Acinetobacter*). Journal of Bacteriology 95, 1520-1541.

Bikandi, J., San Millan, R., Rementeria, A., Garaizar, J.,2004. In silico analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. Bioinformatics (Oxford, England) 20, 798-799.

Chen, T.L., Lee, Y.T., Kuo, S.C., Yang, S.P., Fung, C.P., Lee, S.D., 2014. Rapid identification of Acinetobacter baumannii, Acinetobacter nosocomialis and Acinetobacter pittii with a multiplex PCR assay. Journal of medical microbiology 63, 1154-1159.

Dijkshoorn, L., Nemec, A., Seifert, H.,2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter* baumannii. Nature Reviews Microbiology 5, 939-951.

Durante-Mangoni, E., Zarrilli, R.,2011. Global spread of drug-resistant *Acinetobacter* baumannii: molecular epidemiology and management of antimicrobial resistance. Future microbiology 6, 407-422.

Ferreira, A.E., Marchetti, D.P., De Oliveira, L.M., Gusatti, C.S., Fuentefria, D.B., Corção, G.,2011. Presence of OXA-23-producing isolates of *Acinetobacter* baumannii in wastewater from hospitals in southern Brazil. Microbial Drug Resistance 17, 221-227.

Garibyan, L., Avashia, N.,2013. Polymerase chain reaction. The Journal of investigative dermatology 133, e6.

Gerner-Smidt, P.,1992. Ribotyping of the *Acinetobacter* calcoaceticus-*Acinetobacter* baumannii complex. Journal of clinical microbiology 30, 2680-2685.

Gerner-Smidt, P., Tjernberg, I., Ursing, J.,1991. Reliability of phenotypic tests for identification of *Acinetobacter* species. Journal of clinical microbiology 29, 277-282.

Giamarellou, H.,2010. Multidrug-resistant Gram-negative bacteria: how to treat and for how long. International journal of antimicrobial agents 36, S50-S54.

Higgins, P., Wisplinghoff, H., Krut, O., Seifert, H.,2007. A PCR-based method to differentiate between *Acinetobacter* baumannii and *Acinetobacter* genomic species 13TU. Clinical Microbiology and Infection 13, 1199-1201.

Howard, A., O'Donoghue, M., Feeney, A., Sleator, R.D.,2012. *Acinetobacter* baumannii: an emerging opportunistic pathogen. Virulence 3, 243-250.

Jamal, W., Al Roomi, E., AbdulAziz, L.R., Rotimi, V.O.,2014. Evaluation of Curetis Unyvero, a multiplex PCR-based testing system, for rapid detection of bacteria and antibiotic resistance and impact of the assay on management of severe nosocomial pneumonia. Journal of clinical microbiology 52, 2487-2492.

Kempf, M., Rolain, J.M., Diatta, G., Azza, S., Samb, B., Mediannikov, O., Gassama Sow, A., Diene, S.M., Fenollar, F., Raoult, D.,2012. Carbapenem resistance and *Acinetobacter* baumannii in Senegal: the paradigm of a common phenomenon in natural reservoirs. PloS one 7, e39495.

Lambert, M., Suetens, C., Savey, A., Palomar, M., Hiesmayr, M., Morales, I., Agodi, A., Frank, U., Mertens, K., Schumacher, M.,2011. Clinical outcomes of health-care-associated infections and antimicrobial resistance in patients admitted to European intensive-care units: a cohort study. The Lancet infectious diseases 11, 30-38.

Law, J.W., Ab Mutalib, N.S., Chan, K.G., Lee, L.H.,2015. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. Frontiers in microbiology 5, 770.

Levi, K., Bailey, C., Bennett, A., Marsh, P., Cardy, D.L., Towner, K.J.,2003. Evaluation of an isothermal signal amplification method for rapid detection of methicillin-resistant Staphylococcus aureus from patient-screening swabs. Journal of clinical microbiology 41, 3187-3191.

Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W., Deetz, K.,1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR methods and applications 4, 357-362.

Mohan Nair, M.K., Venkitanarayanan, K.S.,2006. Cloning and sequencing of the ompA gene of Enterobacter sakazakii and development of an ompA-targeted PCR for rapid detection of Enterobacter sakazakii in infant formula. Applied and Environmental Microbiology 72, 2539-2546.

Peleg, A.Y., Seifert, H., Paterson, D.L.,2008. *Acinetobacter* baumannii: emergence of a successful pathogen. Clinical microbiology reviews 21, 538-582.

Pereira, C., Marra, A.R., Camargo, L., Pignatari, A.C.C., Sukiennik, T., Behar, P.R.P., Medeiros, E.A.S., Ribeiro, J., Girão, E., Correa, L.,2013. Nosocomial bloodstream infections in Brazilian pediatric patients: microbiology, epidemiology, and clinical features. PloS one 8, e68144.

Perez, F., Hujer, A.M., Hujer, K.M., Decker, B.K., Rather, P.N., Bonomo, R.A.,2007. Global challenge of multidrug-resistant *Acinetobacter* baumannii. Antimicrobial Agents and Chemotherapy 51, 3471-3484.

Seifert, H., Strate, A., Pulverer, G.,1995. Nosocomial bacteremia due to *Acinetobacter* baumannii: clinical features, epidemiology, and predictors of mortality. Medicine 74, 340-349.

Talbot, G.H., Bradley, J., Edwards, J.E.,Jr, Gilbert, D., Scheld, M., Bartlett, J.G., Antimicrobial Availability Task Force of the Infectious Diseases Society of America,2006. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 42, 657-668.

Wisplinghoff, H., Bischoff, T., Tallent, S.M., Seifert, H., Wenzel, R.P., Edmond, M.B.,2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 39, 309-317.

Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z.,2013. Higher isolation of NDM-1 producing *Acinetobacter* baumannii from the sewage of the hospitals in Beijing.

Table 1**:** List of bacterial strains used in detecting specificity of PCR

No.	Species	Strain
1	*A. baumannii*	189632[a]
2	*A. baumannii*	260177 [a]
3	*A. baumannii*	474030 [a]
4	*A. baumannii*	190451 [a]
5	*A. baumannii*	251847 [a]
6	*A. baumannii*	251382 [a]
7	*A. baumannii*	249958 [a]
8	*A. baumannii*	252128 [a]
9	*A. baumannii*	313099 [a]
10	*A. baumannii*	195652 [a]
11	*A. baumannii*	173795 [a]
12	*A. baumannii*	163731 [a]
13	*A. baumannii*	320452 [a]
14	*A. baumannii*	134882 [a]
15	*A. baumannii*	Naval 17[b]
16	*A. baumannii*	OIFC 109 [b]
17	*A. baumannii*	ATCC 17978
18	*Acinetobactercalcoaceticus*	ATCC BAA-346
19	*Acinetobacter* genomospecies 3	ATCC 17922
20	*Acinetobacter* genomospecies 13 TU	ATCC 17903
21	*Burkholderia cepacia*	ATCC 25608
22	*Serratia marcescens*	361[c]
23	*Escherichia coli*K88	842[d]
24	*Yersinia pseudotuberculosis*	399 [c]
25	*Edwardsiella tarda*	296 [c]
26	*Proteus mirabilis*	366 [c]
27	*Proteus vulgaris*	365 [c]
28	*Aeromonas hydrophilia*	191 [c]
29	*Shigella flexneri*	387 [c]
30	*Edwardsiella ictaluri*	92/132 [d]
31	*Erwinia carotovora*	351[c]
32	*Bacillus cereus*	F3802A/84 [d]
33	*Klebsiella pneumoniae*	344 [c]
34	*Pseudomonas fragi*	110 [c]
35	*Salmonella Enteritidis*	SE 90 [d]
36	*Salmonella enterica serovar typhi*	372[c]

37	*Listeria monocytogenes*	Scott A[e]
38	*Staphylococcus aureus*	ATCC 35556
39	*Enterobacter aerogenes*	NRRL B-115[f]
40	*Providencia alcalifaciens*	368 [c]
41	*Citrobacter fruendii*	239 [c]
42	*Chromobacterium violaceum*	294 [c]
43	*Pseudomonas fluorescence*	105 [c]
45	*Vibrio parahaemolyticus*	ATCC 17802
46	*Vibrio cholerae*	ATCC 51394

[a] International Health Management Associates (IHMA), Schaumburg, IL, USA
[b] Bei resources, Manassas, VA
[c] Presque Isle cultures, Presque Isle, PA
[d] Culture collection, Food Microbiology Laboratory, University of Connecticut, Storrs, CT
[e] M.P. Doyle, Center for Food Safety, University of Georgia, Griffin, GA
[f] A. P. Rooney, Microbial Genomics and Bioprocessing Research Unit, Agricultural research Service Culture Collection, USDA, Peoria, IL

Fig 1: Specificity of the primer-probe set in differentiating *A. baumannii* from other members of ABC complex

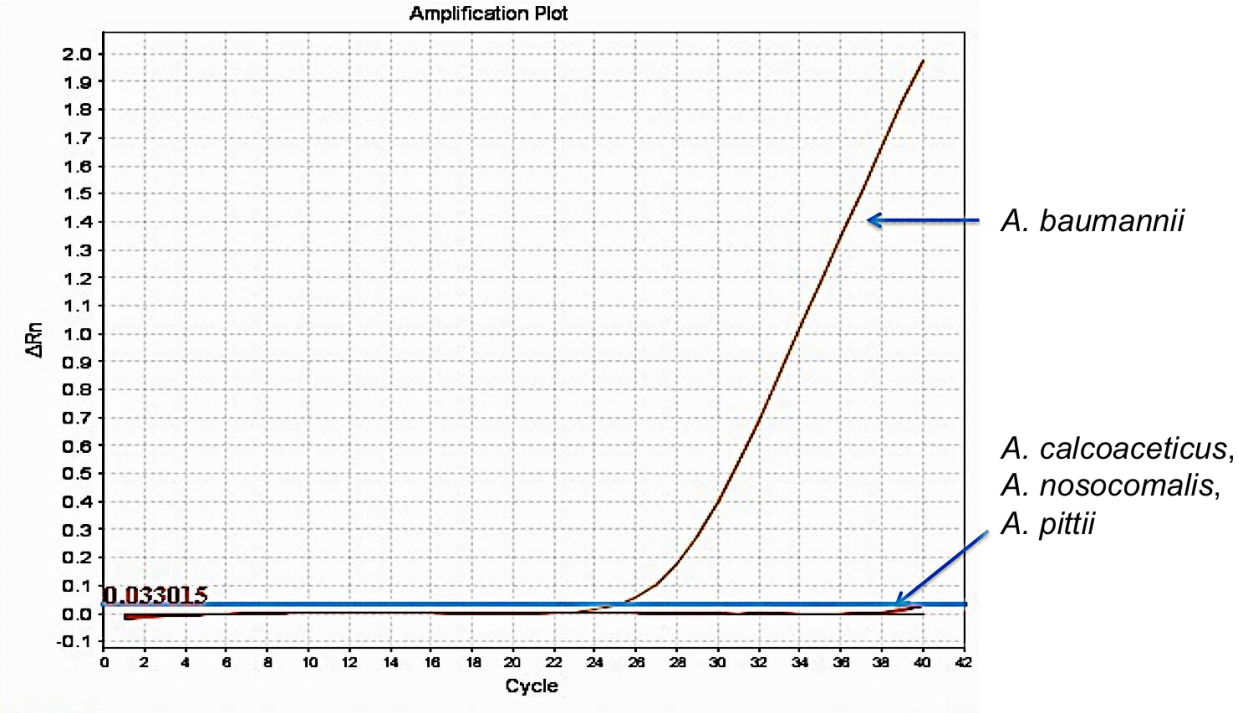


Fig 2: Specificity of the primer-probe set in differentiating *A. baumannii* from other important Gram-negative and Gram-positive pathogens used as negative controls

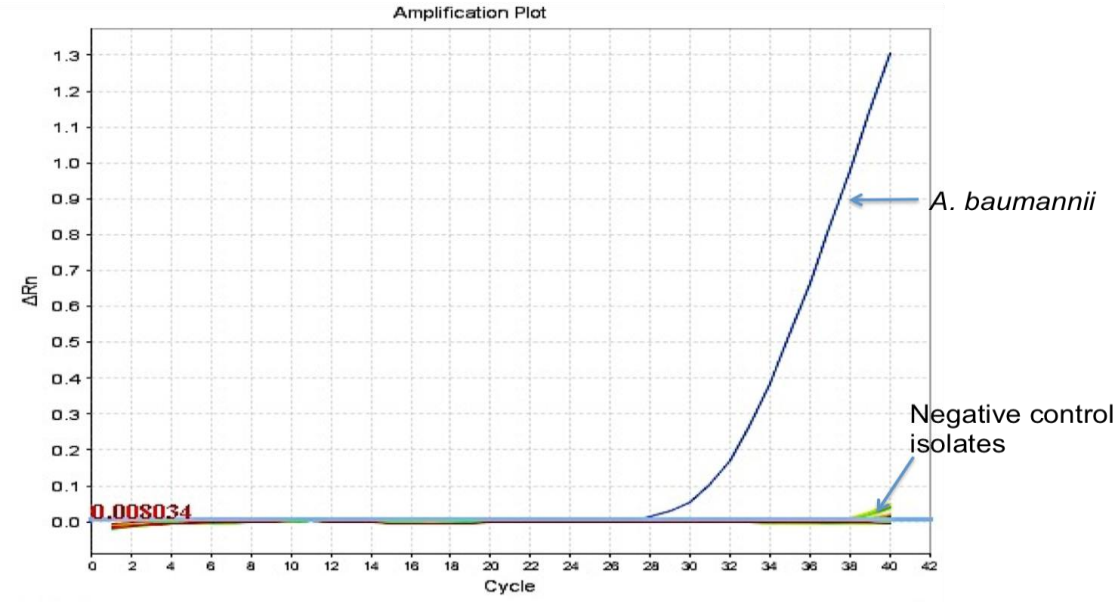
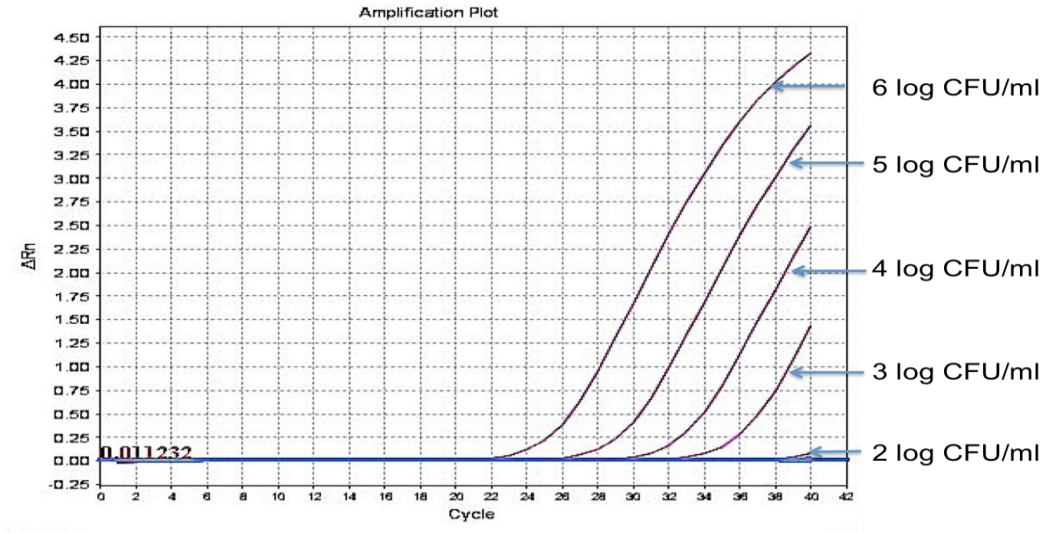
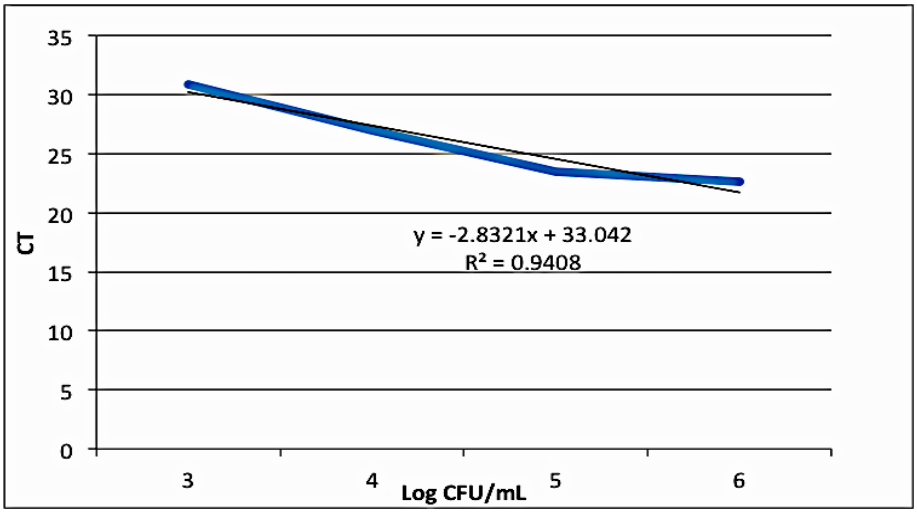


Fig 3: (A) Sensitivity of the primer-probe set in detecting *A. baumannii* in water

(B) Standard curve generated from the amplification plot (Fig 3A).R^2 was calculated from plots of measured CT (y axis) versus counts of *A. baumannii* log CFU/mlin water (x axis).



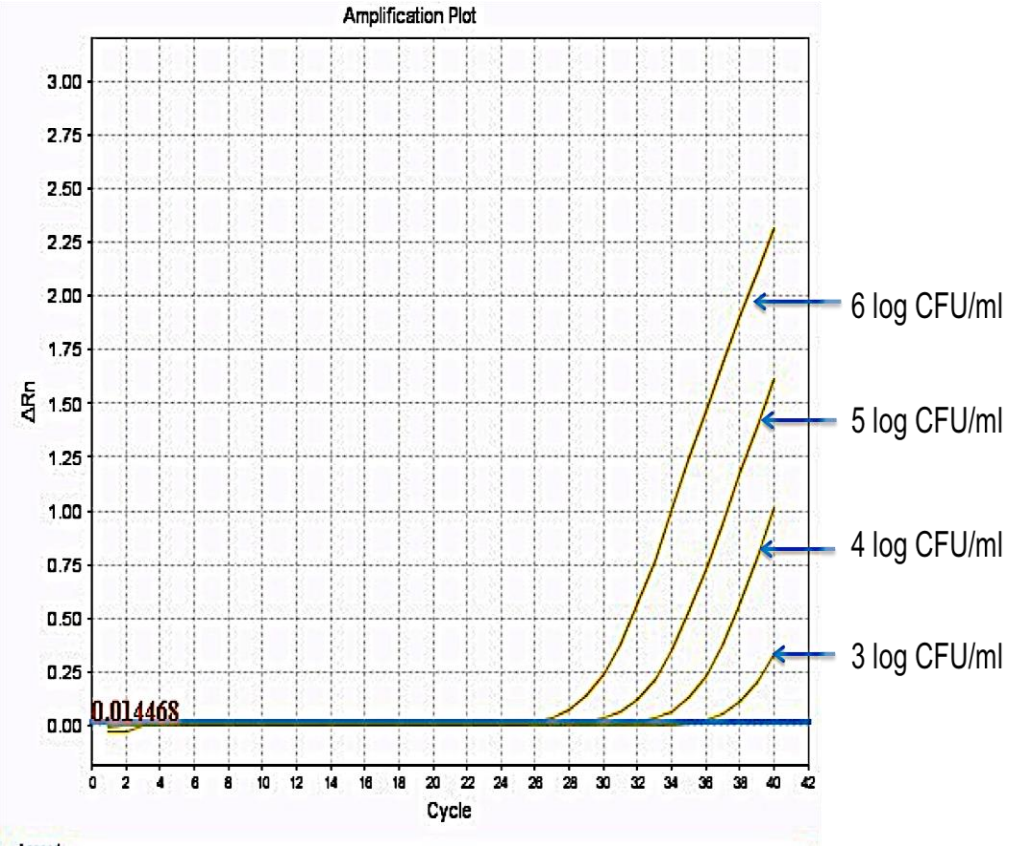
(A)



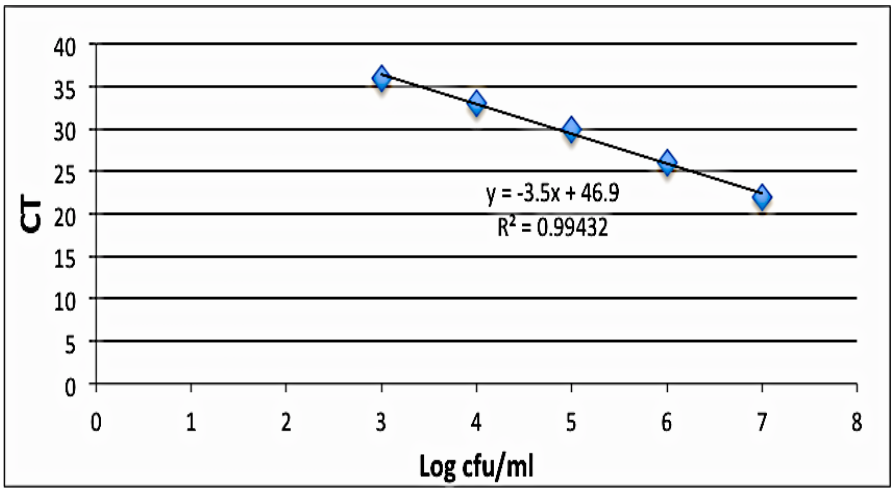
(B)

Fig 4: (A) Sensitivity of the primer-probe set in detecting *A. baumannii* in blood

(B) Standard curve generated from the amplification plot (Fig 4A). R^2 was calculated from plots of measured CT (y axis) versus counts of *A. baumannii* log CFU/ml in blood (x axis).



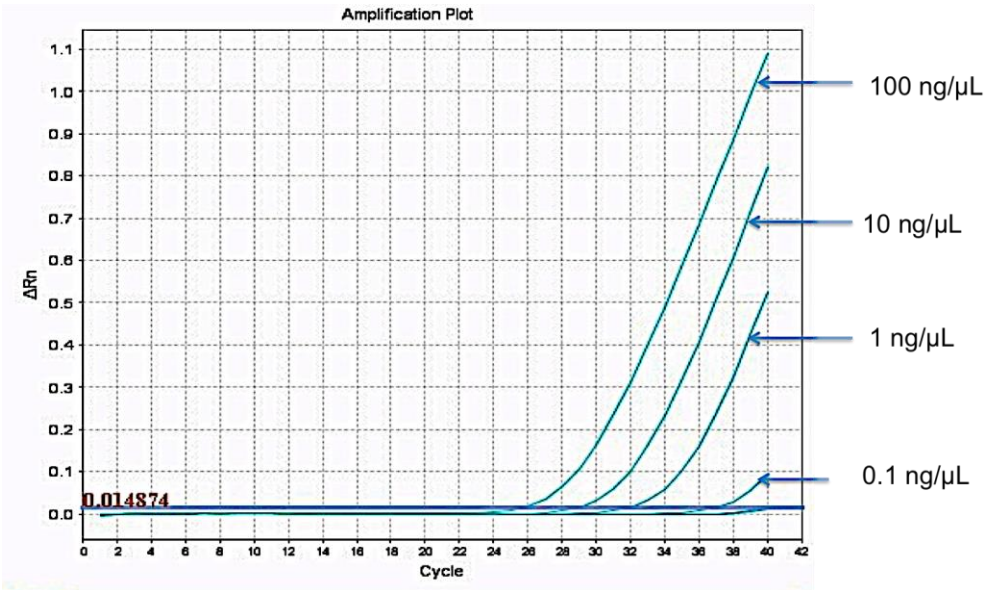
(A)



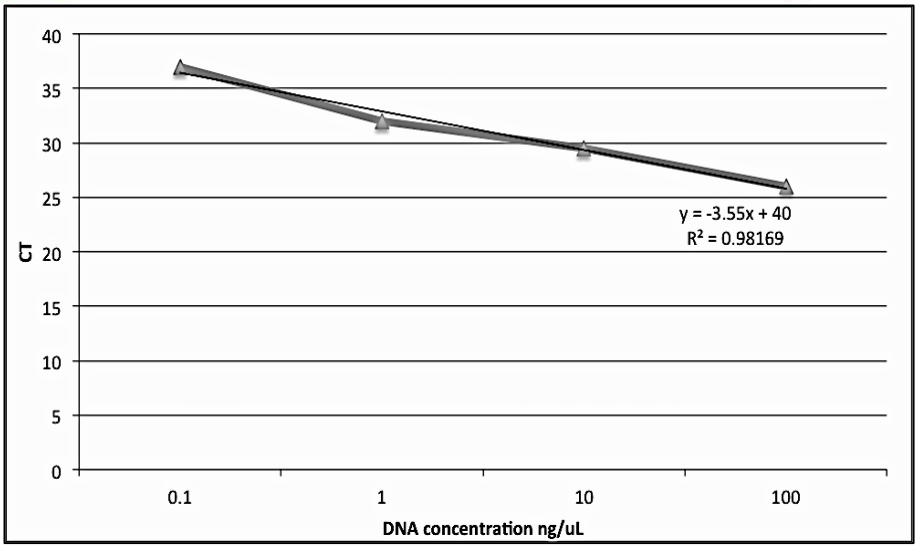
(B)

Fig 5: (A) Sensitivity of the primer-probe set in detecting *A. baumannii* DNA

(B) Standard curve generated from the amplification plot (Fig 5A). R^2 was calculated from plots of measured CT (y axis) versus *A. baumannii*DNA (ng/µl) (x axis).



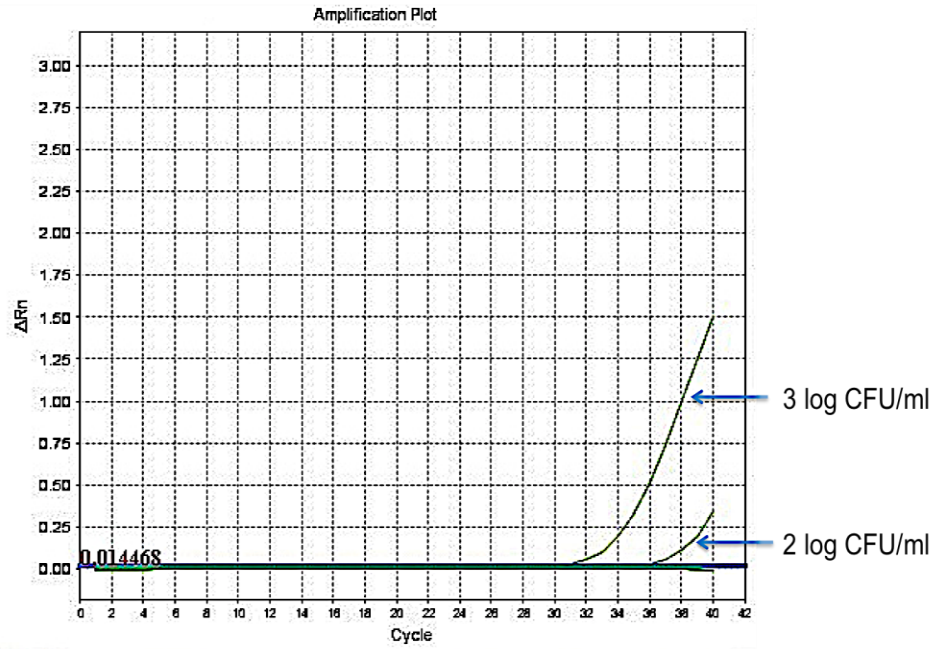
(A)



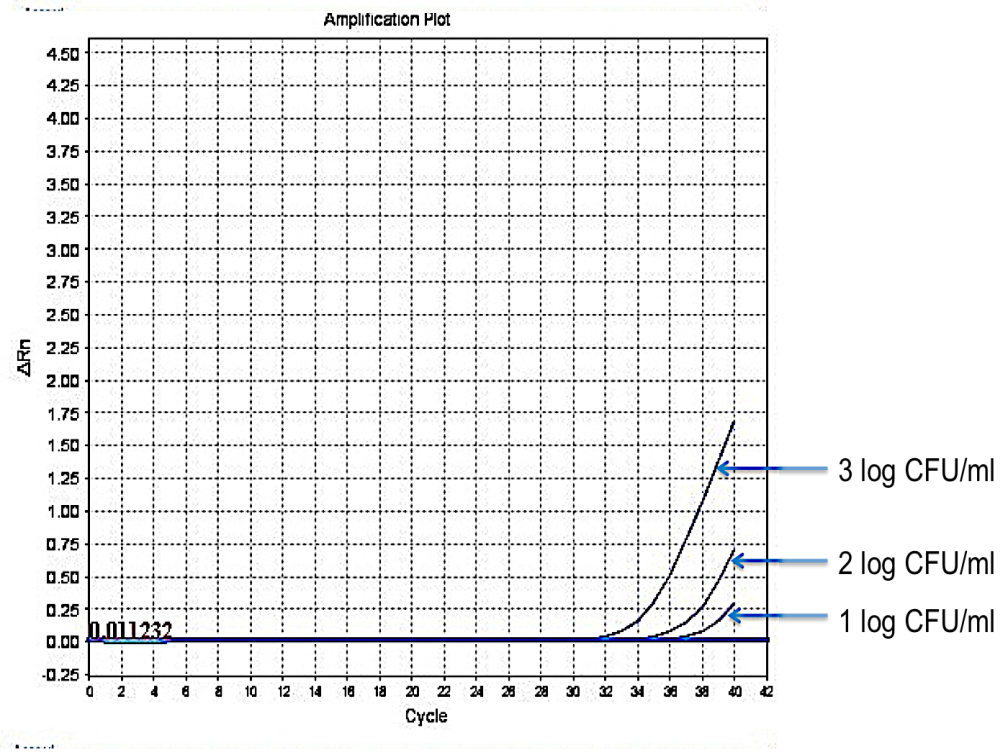
(B)

Fig 6: Sensitivity of the primer-probe set in detecting *A. baumannii* in water after enrichment

A. Following 6 h of incubation at 37^{O}C (B) Following 14 h of incubation at 37^{O}C



(A)



(B)

Chapter VI

Effect of Trans-cinnamaldehyde and Eugenol on decreasing *A. baumannii* resistance to β-lactam antibiotics.

ABSTRACT

Multidrug resistant (MDR) *Acinetobacter baumannii* is a major nosocomial pathogen causing a wide range of clinical conditions with significant mortality rates. *A.baumannii* strains are equipped with a multitude of antibiotic resistance mechanisms, rendering them resistant to most of the currently available antibiotics. Thus, there is a critical need to explore novel strategies for controlling antibiotic resistance in *A.baumannii*. This study investigated the efficacy of two food-grade, plant-derived antimicrobials (PDAs), namely trans-cinnamaldehyde(TC) and eugenol (EG) in decreasing *A.baumannii*'s resistance to seven β-lactam antibiotics, including ampicillin, methicillin, meropenem, penicillin, aztreonam, amoxicillin and piperacillin. Two MDR *A.baumannii* isolates (ATCC 17978 and AB251847) were separately cultured in tryptic soy broth (~6logCFU/ml) containing the m inimum inhibitory concentration (MIC) of TC or EG with or without the MIC of each antibiotic at 37^{o}C for 18 h. *A.baumannii* strains not exposed to the PDAs or antibiotics served as controls. Following incubation, *A.baumannii* counts were determined by broth dilution assay. In addition, the effect of PDAs on the permeability of outer membrane and efflux pumps in *A. baumannii* was measured. Further, the effect of TC and EG on the expression of *A.baumannii* genes encoding resistance to β-lactam antibiotics (*blaP*), efflux pumps (*adeABC*) and multi-drug resistant protein (*mdrp*) was studied using real-time quantitative PCR (RT-qPCR). The experiment was replicated three times with duplicate samples of each treatment and control. The results from broth dilution assay indicated that both TC and EG in combination with antibiotics increased the sensitivity of *A.baumannii* to all the tested antibiotics ($P<0.05$). The two PDAs inhibited the function of *A. baumannii* efflux pump, (AdeABC), but did not increase the permeability of its outer membrane. Moreover, RT-qPCR data revealed that TC and EG down-regulated the expression of majority of the genes associated

with β-lactam antibiotic resistance, especially *blaP*and *adeABC* ($P<0.05$).The results suggest that TC and EG could potentially be used along with β-lactam antibiotics for controlling MDR *A.baumannii* infections; however, their clinical significance needs to be determined using *in vivo* studies.

1. INTRODUCTION

Acinetobacter baumannii is a multi-drug resistant (MDR) Gram-negative, aerobic bacillus that has emerged as a major cause of nosocomial infections with mortality rates ranging from 34 to 61% (Wiexaorek et al., 2008; Karageorgopoulos and Falagas, 2008; Esterly et al., 2011). In humans, MDR *A. baumannii* causes a wide-spectrum of clinical conditions, including pneumonia (Leung et al., 2006), blood-stream infections (Wisplingoff et al., 2004; CDC, 2014), meningitis (Metan et al., 2007), urinary tract infections (Sunenshine et al., 2007), and wound infections (Scott et al., 2007). In addition, reports of other manifestations such as endocarditis, peritonitis, and osteomyelitis associated with *A. baumannii* have been reported (Olut and Erkek, 2005; Menon et al., 2006; Wieczorek et al., 2008). *A. baumannii* is ranked as one of the most common bacteria associated with intensive care units (Garnacho-Montero and Amaya-Villar, 2010; Ulu-Kilic et al., 2013), and is difficult to treat due to its resistance to most of the currently available antibiotics (Maragakis and Perl., 2008; Doi et al., 2009; Neonakis et al., 2011; Al Mobarak et al., 2014; Ellis et al., 2015). For example, carbapenems, which were once the antimicrobials of choice for treating *A. baumannii*, are no longer completely effective due to resistance development by the bacterium (Falagas et al., 2006; Bassetti et al., 2008; Abbott et al., 2013; Fonseca et al., 2013). Similarly, although polymyxins have been successfully used to treat *A. baumannii* infections, strains resistant to these drugs have appeared (Hernan et al., 2009; Lean et al., 2014; Pogue et al., 2015). In light of these reports, the Infectious Diseases Society of America ranked *A. baumannii* as one of the top priority, antibiotic-resistant pathogens to target due to its rapid propensity to develop drug resistance, and a limited choice of antibiotics available to treat infections caused by this bacterium (Talbot et al., 2006; Shales et al., 2013).

A. baumannii is considered to be multidrug resistant, if it exhibits resistance to more than three classes of antibiotics (Falagas et al., 2006). The resistance of *A. baumannii* to antibiotics has been attributed to multiple mechanisms, including reduced permeability of its outer membrane to antibiotics, constitutive expression of drug efflux pumps, and its ability to acquire and incorporate genetic elements such as plasmids, transposons, and integrons (Giamarellou et al., 2008; Cai et al., 2012). In addition, *A. baumannii* has a significantability to produce biofilms on various surfaces (Espinal et al., 2012; Longo et al., 2014), which not only increases the potential of *A. baumannii* for nosocomial spread, but also contributes to its resistance to antibiotics and virulence (Lee et al., 2008; Rao et al., 2008). Thus, there is a critical need to explore novel strategies for treating *A. baumannii* infections.

Traditionally, plants have served as a source of novel drugs for treating a variety of diseases in humans (Cowan, 1999; FAO, 2013). A variety of plant-derived compounds possessing antimicrobial properties against a wide range of microorganisms have been documented (Kon and Rai, 2012; Upadhyay et al., 2014). Wang et al. (2010) reported the antimicrobial effects of four components of ginger against MDR strains of the bacterium. Similarly, the essential oil from coriander was found to exert either synergistic or additive effects with antibiotics such as tetracycline, chloroamphenicol, ciprofloxacin, gentamicin, piperacillin and cefoperazone against *A. baumannii* (Duarte et al., 2012).Recently, Saghi et al. (2015) showed that the essential oil from *Origanum vulgare* possessed potent antimicrobial activity against MDR *A. baumannii*. In addition, previous research from our laboratory indicated that several plant-derived antimicrobials (PDAs), including trans-cinnamaldehyde, an ingredient in cinnamon, eugenol (present in clove) and carvacrol and thymol obtained from oregano oil and oil of thyme, respectively decreased antibiotic resistance in MDR *S.* Typhimurium DT 104

(Kollanoor-Johny et al., 2010). These investigators observed that trans-cinnamaldehyde reduced DT 104's resistance to 5 antibiotics, where thymol and carvacrol decreased resistance to 3 antibiotics.

The β-lactam group of antibiotics are the most commonly prescribed antibiotics for the treatment of bacterial infections worldwide (Pitout et al., 1997; Thakuria and Lahon, 2013). *A. baumannii* is capable of producing β-lactamases that can hydrolyze the β-lactam ring of penicillins, cephalosporins, and carbapenems, thereby conferring resistance to these antibiotics. Therefore, this study investigated the efficacy of trans-cinnamaldehyde(TC) and eugenol (EG) in increasing the sensitivity of *A. baumannii* to seven β-lactam antibiotics. In addition, the effect of these PDAs on genes conferring resistance to β-lactam antibiotics in *A. baumannii* was determined.

2. MATERIALS AND METHODS

2.1. A. baumannii Cultures and Growth Conditions

Two clinical isolates of *A. baumannii*, namely 251847 (International Health Management Associates, IL), and 17978 (ATCC; fatal meningitis isolate) were used in the study.All bacteriological media used in the study, except Leeds MDR *Acinetobacter* agar, were purchased from Difco (Becton Dickinson, Sparks, MD, USA). Leeds MDR agar was procured from Hardy Diagnostics (Santa Maria, CA, USA). The bacterial isolates were cultured separately overnight in 10 ml tryptic soy broth (TSB), followed by streaking on Leeds MDR *Acinetobacter* agar plates and incubation at 37^{o}C for 24 h. An individual colonyfrom Leeds MDR *Acinetobacter* agar was sub-cultured twice in 10 ml of TSB at 37^{o}C for 24 h with agitation to reach ~ 8 $\log_{10}$ CFU/ml. The cultures were sedimented by centrifugation (3700 x g, 15 min, 4^{o}C) and the pellet was washed twice and re-suspended in sterile phosphate buffered saline (PBS; pH 7.2). The cultures

were then diluted appropriately in PBS to obtain 5 to 6 $\log_{10}$ CFU/ml to be used as the inoculum. The bacterial populationin the inoculum was confirmed by plating on tryptic soy agar (TSA) with incubation at 37^{o}C for 24 h.

2.2. Plant-Derived Antimicrobials (PDAs) and chemicals

Trans-cinnamaldehyde (≥98%; TC, trans-3-Phenyl-2-propenal), Eugenol (≥98%; EG,4-Allyl-2-methoxyphenol), 1- N-phenylnaphthylamine (NPN), EDTA, ethidium bromide (EtBr), carbonyl cyanide m-chlorophenylhydroazone (CCCP) and pyronin Y were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Determination of sub-inhibitory concentration (SIC) and minimum inhibitory concentration (MIC) of PDAs against A. baumannii

The SIC and MIC of TC and EG against *A. baumannii* were determined as previously reported (Johny et al., 2010; Amalaradjou and Venkitanarayanan, 2011). Tryptic soy broth (10 ml) tubes containing 1 to 10 µlof TC or EG (Sigma-Aldrich) in increments of 0.5 µlwere inoculated separately with *A. baumannii* at ~ 6 $\log_{10}$CFU/ml, and incubated at 37^{o}C for 24 h. Tubes without any added PDAs served as controls. After incubation, the samples were serially diluted (1:10) in PBS, plated on TSA, and incubated at 37^{o}C for 24 h before counting the colonies. The highest concentration of TC or EG that did not inhibit *A. baumannii* growth after 24 h of incubation was selected as the SIC, while the lowest concentration of the antimicrobial that inhibited visible growth of the bacteria after 24 h incubation was taken as the MIC of that treatment. The experiment was done in duplicates and repeated three times.

2.4. Effect of PDAs on antibiotic resistance in A. baumannii

The β-lactam antibiotics tested in the current study included Ampicillin, Meropenem, Methicillin, Penicillin, Aztreonam, Amoxicillin and Piperacillin (Sigma-Aldrich). Previously

published MIC of each aforementioned antibiotic against *A. baumannii*was used in the study (Table 2) (Vashist et al., 2011; Morfin-Otero and Dowzicky et al., 2012; Malone and Kwon, 2013).

To determine the effect of combination of PDAs and antibiotics on *A. baumannii*, the MIC of each antibiotic and that of TC/EG were added to 10 ml TSB containing *A. baumannii*(~ 6 $\log_{10}$ CFU/ml), and incubated at 37ºC for 24 h (Kollanoor-Johny et al., 2010). The bacterial counts were determined after broth dilution assay and surface plating of appropriate dilutions on TSA. The treatments included only *A. baumannii* (positive control), *A. baumannii* + antibiotic, *A. baumannii*+ PDA and *A. baumannii*+ antibiotic + PDA. In addition, suitable controls, including *A. baumannii* + diluent (ethanol) and *A. baumannii* + ethanol + antibiotic were also included. Duplicate samples were included for each treatment, and the experiment was replicated three times.

2.5. Efflux pump inhibition assay

To study the effect of TC and EG on inhibiting the action of efflux pumps in *A. baumannii*, ethidium bromide (EtBr) and pyronin Y efflux assays were performed according to a published protocol (Chusri et al., 2009). Overnight cultures of *A. baumannii* were washed twice and resuspended in PBS containing 0.4 % glucose to an OD_{600} of ~ 0.5. The bacterial suspension was added with the MIC of TC/EG or CCP (positive control, 100 μM), and incubated at 37ºC for 5 h. *A. baumannii* suspension in PBS + 0.4 % glucose served as control. After incubation, 200 μl of the treatments/control was separately transferred to a 96-well microtiter plate, followed by addition of EtBr (Sigma) to a final concentration of 4 mg/l, and the fluorescence was measured at excitation 530 nm and emission 645 nm. The assay was repeated with pyronin Y (Sigma) at a

final concentration of 5 mg/l at 530 nm and emission 645 nm. The experiment was repeated three times with duplicates for *A. baumannii* 17978 and *A. baumannii* 251847.

2.6. Outer membrane permeabilization assay

To study the effect of TC and EG on the outer membrane of *A. baumannii*, NPN uptake assay was performed using a published protocol (Chusri et al., 2009). Overnight *A. baumannii* 17978 and *A. baumannii* 251847 cultures were separately washed and resuspended in 5 mM HEPES buffer to an OD_{600} ~ 0.5. Aliquots of 100 μl of *A. baumannii* suspension were transferred to a microtiter plate along with the MIC of TC/EG or EDTA 1 mM/HEPES buffer. This was followed by addition of 40 μM of NPN to make the total volume to 200 μl, the fluorescence was measured within 3 min at excitation 355 nm and emission 460 nm, and continuously recorded for 3 h every 10 min. The experiment was repeated three times with duplicates in each treatment.

2.7. Effect of PDAs on antibiotic resistance gene expression in A. baumannii

2.7.1. RNA extraction and cDNA synthesis

To study the effect of TC and EG on genes associated with resistance to β-lactam antibiotics in *A. baumannii*, bacterial cultures were grown separately with or without the SIC of TC/EG at 37^{o}C in TSB to mid-log phase. *A. baumannii* grown witheither antibiotic alone or PDA alone were also included as controls. After incubation, the cultures were subjected to centrifugation (12000 x g, 15 min, 4^{o}C) and the resultant pellet was added with 0.5 ml of RNAase free, sterile water and 1 ml of RNA protect reagent (Qiagen, Valencia, CA, USA). The total RNA from each sample was extracted using the RNeasy mini kit (Qiagen), and the manufacturer's instructions were followed in estimating the total RNAusing Nanodrop (ThermoFisher Scientific, Waltham, MA, USA). Super-script II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis, and the resultant cDNA was

used as a template for RT-qPCR. The amplified product was detected using SYBR Green reagents.

2.7.2.Real-Time Quantitative PCR (RT-qPCR)

The antibiotic resistance genes of *A. baumannii*assayedfor expression analysis included efflux pump genes, namely *adeA, adeB, adeC*; β-lactam resistance gene,*blaP*; and the multidrug resistance protein gene, *mdrp*. Primer Express software® (Applied Biosystems, Foster city, CA, USA) was used for designing the primers specific for the genes and for the endogenous control (16S rRNA). The primers were designed from*A. baumannii* AB0057 genome (CP001182.1) published in the NCBI database (Adams et al., 2008), and their sequences are provided in Table 1. The custom synthesized primers were obtained from Integrated DNA Technologies (Foster City, CA, USA). RT-qPCR was performed with StepOnePlus™ Real Time PCR system (Applied Biosystems) using the SYBR green assay (Applied Biosystems) under custom thermal cycling conditions with the normalized RNA as the template (Bookout and Mangelsdorf, 2003). Duplicate samples were analyzed and standardized against 16S rRNA gene expression. The relative changes in mRNA expression levels were determined using comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$) between the control and the treatments.

2.8. Statistical analysis

A completely randomized design with a factorial treatment structure was used. The factors included two *A. baumannii* strains, seven antibiotics, and two PDAs. The data were analyzed using the PROC GENMOD procedure of Statistical Analysis Software (SAS ver. 9.4; SAS Institute Inc., Cary, NC). Least square means were considered significant at $P< 0.05$. The data comparisons for gene expression study were made using Students t-test. The difference was considered significant at $P<0.05$.

3. RESULTS

The SICs of TC and EG against *A. baumannii* were found to be 1.1 mM (0.015 %) and 1.8 mM (0.03 %), respectively, while the MIC was 4 mM (0.05% TC and 0.065 % EG) for both PDAs. Although a previously reported MIC of each antibiotic against *A. baumannii* was used in the study, both *A. baumannii* isolates grew approximately by 2.0 $\log_{10}$CFU/ml after 24 h (antibiotic control), as observed in Fig 1-4. In the presence of the MIC of TC, the bacterial count after 24 h did not change significantly from the inoculation of level of 6.0 $\log_{10}$CFU/ml (Fig. 1 and 2) as expected. However, when *A. baumannii* was grown in the presence of each antibiotic and TC, its growth after 24 h was significantly decreased in comparison to that in the positive control, antibiotic control and TC control ($P < 0.05$). In both *A. baumannii* isolates, the greatest sensitivity was observed to methicillin and lowest sensitivity was seen against ampicillin and meropenem (Fig. 1 and 2). Similarly, the sensitivity of both *A. baumannii* isolates to all seven antibiotics was significantly increased in the presence of EG, as indicated by the reduced growth after 24 h ($P < 0.05$) (Fig. 3 and 4). In *A. baumannii* 17978, the greatest sensitivity was observed against ampicillin (Fig. 3), whereas *A. baumannii* 251847 was maximally sensitive to amoxicillin (Fig. 4).

The results of the efflux pump inhibition assay using EtBr and pyronin Y in *A. baumannii* ATCC 17978 and *A. baumannii* 251847 are presented in Fig. 5-8. The MIC of TC and EG resulted in an increased accumulation of EtBr inside bacterial cells, as indicated by an increase in fluorescence compared to the PBS control ($P<0.05$) (Fig. 5-6).CCCP, an efflux pump inhibitor used as a positive control (Magnet et al., 2001) also resulted in an increase in fluorescene indicatingsupression of efflux pump in *A. baumannii*. However, a similar increase in the fluorescence was not observed for pyronin Y compared to the PBS control (Fig. 7 and 8).

The results of NPN uptake assay in *A. baumannii* ATCC 17978 and *A. baumannii* 251847 are presented in Fig. 9 and Fig. 10, respectively. Neither TC nor EG increased NPN uptake, while the EDTA control did show an increase in fluorescence. Further, the samples treated with TC or EG demonstrated a decrease in fluorescence over time (data not shown).

The effect of seven antibiotics and two PDAs and their combination on the expression of various antibiotic resistance genes in *A. baumannii*17978 is depicted in Fig. 11A-G. It was observed that compared to control, the expression of all tested genes was up-regulated ($P < 0.05$) following *A. baumannii* growth in the presence of the antibiotics. However, TC significantly down-regulated the expression of major genes conferring resistance to β-lactam antibiotics compared to control ($P< 0.05$). The genes encoding efflux pump *adeA* and *adeB* were down regulated by ~ 6- and 9-fold, respectively. Moreover, the expression of genes encoding β-lactamase (*blaP*) and the multiple drug resistance protein (*mdrp*) was decreased by ~3-folds($P< 0.05$)Similar to the results observed with TC, all antibiotic resistance genes were down-regulated on exposure to EG compared to control ($P< 0.05$). The expression of genes encoding efflux pumps, *adeA* and *adeB* were down-expressed by 3- and 14- fold, respectively. Similarly, *blaP* and *mdrp* were also down-regulated in both strains ($P< 0.05$). The combination of TC or EG with the antibiotics also resulted in a down-regulation of majority of the tested genes ($P< 0.05$). Among the combinations of TC or EG with the seven antibiotics, the combination containingaztreonam resulted in a significant down-regulation of all the tested genes compared to control (Fig. 11D). The combination of EG with piperacillin (Fig. 11C) or penicillin (Fig. 11F) also significantly reduced the expression of all the antibiotic resistance genes ($P < 0.05$).

4. DISCUSSION

Rapid emergence of antibiotic resistance in pathogenic microorganisms, especially to multiple antibiotics has ignited research efforts to discover novel antibiotics and develop effective derivatives of currently available antibiotics. However, few promising antibiotics are under development, and the rapidity and complexity of resistance development in pathogens have further exacerbated the situation. In light of this, a potential viable approach was explored to reduce bacterial antibiotic resistance in the development of inhibitors of resistance mechanisms in bacteria (Renau et al., 1999; Walsh and Fanning, 2008). This strategy involves the co-administration of an antibiotic with an "inhibitor", which counteracts bacterial resistance mechanism(s), thereby rendering the resistant pathogen sensitive to the drug. The advantage of this approach is that it makes it possible to continue the use of current antibiotics, for which in-depth pharmacological and toxicological data are already available. In this regard, PDAs represent a potential natural group of "inhibitors" of bacterial antibiotic resistance mechanisms.

Abundant literature exists on the antimicrobial properties of a variety of plant compounds against a wide range of microorganisms (Upadhyay et al., 2014; Burt 2004), however, only a handful of studies have addressed their effect on bacterial antibiotic resistance, especially in Gram negative bacteria (Galluci et al., 2006; Chursi et al., 2009; Kollanoor-Johny et al., 2010; Ilic et al., 2014). In the current study, both TC and EG enhanced the sensitivity of *A. baumannii* to all seven β-lactam antibiotics tested (Fig. 1-4). This is evident from the significant reductions in bacterial counts observed in the samples containing PDA and antibiotics as compared to that in the treatments containing each PDA or antibiotic alone.

A. baumannii has been reported to exhibit resistance to β-lactam antibiotics through several mechanisms, including the production of β-lactamases, changes in penicillin-binding

proteins (PBPs), altering the structure and number of porin proteins, decreased membrane permeability, and by use of efflux pumps that exit antibiotics out of the bacterial cell (Vila et al., 2007; Manchanda et al., 2010; Bonin et al., 2013; Tang et al., 2014). Moreover, the presence of efflux pumps and MDR proteins in *A. baumannii* contribute significantly to both intrinsic and acquired resistance to antibiotics (Lomovskaya and Bostian, 2006). The *A. baumannii* genome encodes a wide array of multidrug efflux systems, including AdeABC, a resistance-nodulation-division (RND) family-type pump (Damier-Piolle et al., 2008;Wieczorek et al., 2008; Sun et al., 2014; Yoon et al., 2013). The substrates for this pump include beta-lactams, aminoglycosides, erythromycin, chloramphenicol, tetracycline, fluoroquinolone, trimethoprim, and ethidium bromide (Magnet et al., 2001., Higgins et al., 2004; Heritier et al., 2005; Peleg et al., 2007). The three component structures of AdeABC include the inner membrane fusion protein (AdeA), transmembrane component (AdeB) and an outer membrane protein (AdeC), with the inactivation of *adeB* resulting in the loss of pump function and multidrug resistance (Magnet et al., 2001).

In order to determine if TC or EG exerted an inhibitory effect on the aforementioned efflux pumps in *A. baumannii*, an efflux pump inhibition assay was performed with EtBr and pyronin Y along withCCCP, a documented efflux pump inhibitor(Magnet et al., 2001; Chusri et al., 2009). Ethidium bromide and Pyronin Y are known substrates for AdeABC and AdeIJK efflux pumps, respectively (Magnet et al., 2001; Chusri et al., 2009; Damier-Piolle et al., 2008; Xing et al., 2014). Both TC and EG resulted in the inhibition of AdeABC efflux pump in the two tested *A. baumannii* strains, as evident from the increase in fluorescence in the treated samples compared to PBS control (Fig. 5 and 6). However, the PDAs did not exert any inhibitory effect on the action of AdeIJK efflux pump in *A. baumannii*, as evident from the lack of difference in fluorescence units between PDA-treated and PBS control samples (Fig. 7 and 8). Similarly, the

results from the NPN uptake assay revealed that TC and EG did not increase *A. baumannii*'s outer membrane permeability, as seen from Fig. 9 and 10, where no increase in fluorescence was observed in PDA-treated *A. baumannii* as against the samples treated with EDTA, a known outer membrane permeabilizer in Gram-negative bacteria (Alakomi et al., 2006; Helander and Sandholm, 2008). These results indicate that TC and EG increased the sensitivity of *A. baumannii* to the tested antibiotics at least in part by inhibiting the efflux pump, AdeABC.

For ascertaining if TC or EG exerted an inhibitory effect on any of the antibiotic resistance genes conferring resistance to β-lactam antibiotics in *A. baumannii*, we performed a RT-qPCR on mRNA extracted from *A. baumannii* following growth in the presence and absence of the PDAs. The results from the RT-qPCR revealed that TC and EG in combination with or without each antibiotic significantly down-regulated the expression of the majority of genes that confer resistance to β-lactam antibiotics (Fig. 11A-G). Among the various genes screened, those encoding efflux pumps, *adeA* and *adeB* were maximally down-regulated by both PDAs. These results concur with the results from the EtBr efflux pump inhibition assay, and suggest that TC and EG enhanced the efficacy of the seven β-lactam antibiotics against *A. baumannii* by thwarting the various resistance mechanisms, especially those involving the efflux pumps.

In conclusion, the results of this study suggest the potential use of TC and EG in conjunction with the currently available β-lactam antibiotics for the treatment for MDR *A. baumannii* infections. However, efficacy studies in suitable animal models are warranted before recommending their clinical usage.

References

Abbott, I., Cerqueira, G.M., Bhuiyan, S., Peleg, A.Y.,2013. Carbapenem resistance in Acinetobacter baumannii: laboratory challenges, mechanistic insights and therapeutic strategies.

Adams, M.D., Goglin, K., Molyneaux, N., Hujer, K.M., Lavender, H., Jamison, J.J., MacDonald, I.J., Martin, K.M., Russo, T., Campagnari, A.A., Hujer, A.M., Bonomo, R.A., Gill, S.R.,2008. Comparative genome sequence analysis of multidrug-resistant Acinetobacter baumannii. Journal of Bacteriology 190, 8053-8064.

Al Mobarak, M.F., Matbuli, R.M., Meir, H., Al Gehani, N., ElToukhy, A.A.M., Al Qureshey, K.F., Mutwalli, A.H.,2014. Antimicrobial Resistance Patterns Among Acinetobacter baumannii Isolated From King Abdulaziz Hospital, Jeddah, Saudi Arabia, Four-Year Surveillance Study (2010–2013). Egyptian Journal of Medical Microbiology 23

Alakomi, H.L., Paananen, A., Suihko, M.L., Helander, I.M., Saarela, M.,2006. Weakening effect of cell permeabilizers on gram-negative bacteria causing biodeterioration. Applied and Environmental Microbiology 72, 4695-4703.

Amalaradjou, M.A.R., Venkitanarayanan, K.,2011. Effect of trans-cinnamaldehyde on reducing resistance to environmental stresses in Cronobacter sakazakii. Foodborne pathogens and disease 8, 403-409.

Bassetti, M., Righi, E., Esposito, S., Petrosillo, N., Nicolini, L.,2008. Drug treatment for multidrug-resistant Acinetobacter baumannii infections.

Bonnin, R.A., Nordmann, P., Poirel, L.,2013. Screening and deciphering antibiotic resistance in Acinetobacter baumannii: a state of the art. Expert review of anti-infective therapy 11, 571-583.

Bookout, A.L., Mangelsdorf, D.J.,2003. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nuclear receptor signaling 1, e012.

Burt, S.,2004. Essential oils: their antibacterial properties and potential applications in foods—a review. International journal of food microbiology 94, 223-253.

Cai, Y., Chai, D., Wang, R., Liang, B., Bai, N.,2012. Colistin resistance of Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. The Journal of antimicrobial chemotherapy 67, 1607-1615.

Center of Disease Control, 2014. Acinetobacter baumannii infections among patients at military medical facilities creating injured US service members 2002-2004, MMWR 53 (45); 1063-1066.

Chusri, S., Villanueva, I., Voravuthikunchai, S.P., Davies, J.,2009. Enhancing antibiotic activity: a strategy to control Acinetobacter infections. The Journal of antimicrobial chemotherapy 64, 1203-1211.

Cowan, M.M.,1999. Plant products as antimicrobial agents. Clinical microbiology reviews 12, 564-582.

Damier-Piolle, L., Magnet, S., Bremont, S., Lambert, T., Courvalin, P.,2008. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy 52, 557-562.

Doi, Y., Husain, S., Potoski, B.A., McCurry, K.R., Paterson, D.L.,2009. Extensively drug-resistant Acinetobacter baumannii. Emerging infectious diseases 15, 980-982.

Duarte, A., Ferreira, S., Silva, F., Domingues, F.C.,2012. Synergistic activity of coriander oil and conventional antibiotics against Acinetobacter baumannii. Phytomedicine : International Journal of Phytotherapy and Phytopharmacology 19, 236-238.

Ellis, D., Cohen, B., Liu, J., Larson, E.,2015. Risk factors for hospital-acquired antimicrobial-resistant infection caused by Acinetobacter baumannii. Antimicrobial resistance and infection control 4, 1-5.

Espinal, P., Marti, S., Vila, J.,2012. Effect of biofilm formation on the survival of Acinetobacter baumannii on dry surfaces. Journal of Hospital Infection 80, 56-60.

Esterly, J.S., Griffith, M., Qi, C., Malczynski, M., Postelnick, M.J., Scheetz, M.H.,2011. Impact of carbapenem resistance and receipt of active antimicrobial therapy on clinical outcomes of Acinetobacter baumannii bloodstream infections. Antimicrobial Agents and Chemotherapy 55, 4844-4849.

Falagas, M.E., Koletsi, P.K., Bliziotis, I.A.,2006. The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) Acinetobacter baumannii and Pseudomonas aeruginosa. Journal of medical microbiology 55, 1619-1629.

Fonseca, E.L., Scheidegger, E., Freitas, F.S., Cipriano, R., Vicente, A.C.,2013. Carbapenem-resistant Acinetobacter baumannii from Brazil: role of carO alleles expression and blaOXA-23 gene. BMC microbiology 13, 245-2180-13-245.

Gallucci, N., Casero, C., Oliva, M., Zygadlo, J., Demo, M.,2006. Interaction between terpenes and penicillin on bacterial strains resistant to beta-lactam antibiotics. Mol Med Chem 10, 30-32.

Garnacho-Montero, J., Amaya-Villar, R., 2010. Multiresistant Acinetobacter baumannii infections: epidemiology and management. Current opinion in infectious diseases 23, 332-339.

Gaynes, R., Edwards, J.R., National Nosocomial Infections Surveillance System,2005. Overview of nosocomial infections caused by gram-negative bacilli. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 41, 848-854.

Giamarellou, H.,2010. Multidrug-resistant Gram-negative bacteria: how to treat and for how long. International journal of antimicrobial agents 36, S50-S54.

Giamarellou, H., Antoniadou, A., Kanellakopoulou, K.,2008. Acinetobacter baumannii: a universal threat to public health? International journal of antimicrobial agents 32, 106-119.

Helander, I.M., Mattila-Sandholm, T.,2000. Fluorometric assessment of gram-negative bacterial permeabilization. Journal of applied microbiology 88, 213-219.

Heritier, C., Poirel, L., Lambert, T., Nordmann, P.,2005. Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy 49, 3198-3202.

Hernan, R.C., Karina, B., Gabriela, G., Marcela, N., Carlos, V., Angela, F.,2009. Selection of colistin-resistant Acinetobacter baumannii isolates in postneurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. Diagnostic microbiology and infectious disease 65, 188-191.

Higgins, P.G., Wisplinghoff, H., Stefanik, D., Seifert, H.,2004. Selection of topoisomerase mutations and overexpression of adeB mRNA transcripts during an outbreak of Acinetobacter baumannii. The Journal of antimicrobial chemotherapy 54, 821-823.

Ilić,B.S., Kocić,B.D., Ćirić,V.M., Cvetković,O.G and Miladinović, D.L, 2014 An In Vitro Synergistic Interaction of Combinations ofThymus glabrescens Essential Oil and Its Main Constituents with Chloramphenicol**.** The Scientific World Journal Volume 2014, Article ID 826219, 12 pages http://dx.doi.org/10.1155/2014/826219

Johny, A.K., Hoagland, T., Venkitanarayanan, K.,2010. Effect of subinhibitory concentrations of plant-derived molecules in increasing the sensitivity of multidrug-resistant Salmonella enterica serovar Typhimurium DT104 to antibiotics. Foodborne pathogens and disease 7, 1165-1170.

Karageorgopoulos, D.E., Falagas, M.E.,2008. Current control and treatment of multidrug-resistant Acinetobacter baumannii infections. The Lancet.Infectious diseases 8, 751-762.

Kollanoor-Johny, A., Mattson, T., Baskaran, S.A., Amalaradjou, M.A., Babapoor, S., March, B., Valipe, S., Darre, M., Hoagland, T., Schreiber, D., Khan, M.I., Donoghue, A., Donoghue, D., Venkitanarayanan, K.,2012. Reduction of Salmonella enterica serovar enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds trans-cinnamaldehyde and eugenol. Applied and Environmental Microbiology 78, 2981-2987.

Kon, K.V., Rai, M.K.,2012. Plant essential oils and their constituents in coping with multidrug-resistant bacteria. Expert review of anti-infective therapy 10, 775-790.

Lean, S., Suhaili, Z., Ismail, S., Rahman, N.I.A., Othman, N., Abdullah, F.H., Jusoh, Z., Yeo, C.C., Thong, K.,2014. Prevalence and genetic characterization of carbapenem-and polymyxin-

resistant Acinetobacter baumannii Isolated from a tertiary hospital in Terengganu, Malaysia. ISRN microbiology 2014

Lee, H., Koh, Y., Kim, J., Lee, J., Lee, Y., Seol, S., Cho, D.,2008. Capacity of multidrug- resistant clinical isolates of Acinetobacter baumannii to form biofilm and adhere to epithelial cell surfaces. Clinical microbiology and infection 14, 49-54.

Leung, W.S., Chu, C.M., Tsang, K.Y., Lo, F.H., Lo, K.F., Ho, P.L.,2006. Fulminant community-acquired Acinetobacter baumannii pneumonia as a distinct clinical syndrome. Chest 129, 102-109.

Lomovskaya, O., Bostian, K.A.,2006. Practical applications and feasibility of efflux pump inhibitors in the clinic--a vision for applied use. Biochemical pharmacology 71, 910-918.

Longo, F., Vuotto, C., Donelli, G.,2014. Biofilm formation in Acinetobacter baumannii. New Microbiol 37, 119-127.

Magnet, S., Courvalin, P., Lambert, T.,2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454. Antimicrobial Agents and Chemotherapy 45, 3375-3380.

Malone, L., Kwon, D.H., 2013. Carbapenem-associated multidrug-resistant Acinetobacter baumannii are sensitised by aztreonam in combination with polyamines. International journal of antimicrobial agents 41, 70-74.

Manchanda, V., Sanchaita, S., Singh, N.,2010. Multidrug resistant acinetobacter. Journal of global infectious diseases 2, 291-304.

Maragakis, L.L., Perl, T.M.,2008. Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 46, 1254-1263.

Menon, T., Shanmugasundaram, S., Nandhakumar, B., Nalina, K., Balasubramaniam,2006. Infective endocarditis due to Acinetobacter baumannii complex--a case report. Indian journal of pathology & microbiology 49, 576-578.

Metan, G., Alp, E., Aygen, B., Sumerkan, B.,2007. Acinetobacter baumannii meningitis in post-neurosurgical patients: clinical outcome and impact of carbapenem resistance. The Journal of antimicrobial chemotherapy 60, 197-199.

Morfin-Otero, R., Dowzicky, M.J.,2012. Changes in MIC within a global collection of Acinetobacter baumannii collected as part of the Tigecycline Evaluation and Surveillance Trial, 2004 to 2009. Clinical therapeutics 34, 101-112.

Neonakis, I.K., Spandidos, D.A., Petinaki, E.,2011. Confronting multidrug-resistant Acinetobacter baumannii: a review. International journal of antimicrobial agents 37, 102-109.

Olut, A.I., Erkek, E.,2005. Early prosthetic valve endocarditis due to Acinetobacter baumannii: a case report and brief review of the literature. Scandinavian Journal of Infectious Diseases 37, 919-921.

Peleg, A.Y., Adams, J., Paterson, D.L.,2007. Tigecycline Efflux as a Mechanism for Nonsusceptibility in Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy 51, 2065-2069.

Peleg, A.Y., Seifert, H., Paterson, D.L.,2008. Acinetobacter baumannii: emergence of a successful pathogen. Clinical microbiology reviews 21, 538-582.

Pitout, J.D., Sanders, C.C., Sanders, W.E.,1997. Antimicrobial resistance with focus on β-lactam resistance in gram-negative bacilli. The American Journal of Medicine 103, 51-59.

Pogue, J., Kaye, K., Cohen, D., Marchaim, D.,2015. Appropriate antimicrobial therapy in the era of multidrug-resistant human pathogens. Clinical Microbiology and Infection 21, 302-312.

Rao, R.S., Karthika, R.U., Singh, S.P., Shashikala, P., Kanungo, R., Jayachandran, S., Prashanth, K.,2008. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of Acinetobacter baumannii. Indian journal of medical microbiology 26, 333-337.

Renau, T.E., Leger, R., Flamme, E.M., Sangalang, J., She, M.W., Yen, R., Gannon, C.L., Griffith, D., Chamberland, S., Lomovskaya, O., Hecker, S.J., Lee, V.J., Ohta, T., Nakayama, K.,1999. Inhibitors of efflux pumps in Pseudomonas aeruginosa potentiate the activity of the fluoroquinolone antibacterial levofloxacin. Journal of medicinal chemistry 42, 4928-4931.

Saghi, H., Bahador, A., Khaledi, A., Kachoei, R.A., Dastjerdi, F.A., Esmaeili, D.,2015. Antibacterial Effects of Origanum vulgareEssence Against Multidrug-Resistant.

Scott, P., Deye, G., Srinivasan, A., Murray, C., Moran, K., Hulten, E., Fishbain, J., Craft, D., Riddell, S., Lindler, L., Mancuso, J., Milstrey, E., Bautista, C.T., Patel, J., Ewell, A., Hamilton, T., Gaddy, C., Tenney, M., Christopher, G., Petersen, K., Endy, T., Petruccelli, B.,2007. An outbreak of multidrug-resistant Acinetobacter baumannii-calcoaceticus complex infection in the US military health care system associated with military operations in Iraq. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 44, 1577-1584.

Shlaes, D.M., Sahm, D., Opiela, C., Spellberg, B.,2013. The FDA reboot of antibiotic development. Antimicrobial Agents and Chemotherapy 57, 4605-4607.

Sun, J., Deng, Z., Yan, A.,2014. Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. Biochemical and biophysical research communications 453, 254-267.

Sunenshine, R.H., Wright, M.O., Maragakis, L.L., Harris, A.D., Song, X., Hebden, J., Cosgrove, S.E., Anderson, A., Carnell, J., Jernigan, D.B., Kleinbaum, D.G., Perl, T.M., Standiford, H.C.,

Srinivasan, A.,2007. Multidrug-resistant Acinetobacter infection mortality rate and length of hospitalization. Emerging infectious diseases 13, 97-103.

Talbot, G.H., Bradley, J., Edwards, J.E.,Jr, Gilbert, D., Scheld, M., Bartlett, J.G., Antimicrobial Availability Task Force of the Infectious Diseases Society of America,2006. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 42, 657-668.

Tang, S.S., Apisarnthanarak, A., Hsu, L.Y.,2014. Mechanisms of β-lactam antimicrobial resistance and epidemiology of major community- and healthcare-associated multidrug-resistant bacteria. Advanced Drug Delivery Reviews 78, 3-13.

Thakuria, B., Lahon, K.,2013. The Beta Lactam Antibiotics as an Empirical Therapy in a Developing Country: An Update on Their Current Status and Recommendations to Counter the Resistance against Them. Journal of clinical and diagnostic research : JCDR 7, 1207-1214.

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., Venkitanarayanan, K.,2014. Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis. BioMed research international 2014

Ulu-Kilic A, Ahmed SS, Alp E, Doğanay M. Challenge of intensive care unit-acquired infections and Acinetobacter baumannii in developing countries. OA Critical Care 2013 Mar 01;1(1):2.

Vashist, J., Tiwari, V., Das, R., Kapil, A., Rajeswari, M.R.,2011. Analysis of penicillin-binding proteins (PBPs) in carbapenem resistant Acinetobacter baumannii. The Indian journal of medical research 133, 332-338.

Vila, J., Martí, S., Sánchez-Céspedes, J.,2007. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. Journal of antimicrobial chemotherapy 59, 1210-1215.

Walsh, C., Fanning, S.,2008. Antimicrobial Resistance in Foodborne Pathogens-A Cause for Concern? Current Drug Targets 9, 808-815.

Wang, H., Chen, C., Chen, H., Huang, W., Lin, W., Chen, T., Lin, C., Chien, H., Lu, P., Lin, C.,2010. Zingiber officinale (ginger) compounds have tetracycline- resistance modifying effects against clinical extensively drug- resistant Acinetobacter baumannii. Phytotherapy Research 24, 1825-1830.

Wieczorek, P., Sacha, P., Hauschild, T., Zorawski, M., Krawczyk, M., Tryniszewska, E.,2008. Multidrug resistant Acinetobacter baumannii--the role of AdeABC (RND family) efflux pump in resistance to antibiotics. Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society 46, 257-267.

Wiexaorek et al., 2008 . Wieczorek P, Sacha P, Hauschild T, Zorawski M, Krawczyk M, Tryniszewska E. Multidrug resistant Acinetobacter baumannii —the role of AdeABC (RND family) efflux pump in resistance to antibiotics. Folia Histochem Cytobiol 2008;46:257-67.

Wisplinghoff, H., Bischoff, T., Tallent, S.M., Seifert, H., Wenzel, R.P., Edmond, M.B.,2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 39, 309-317.

Xing, L., Barnie, P., Su, Z., Xu, H.,2014. Development of Efflux Pumps and Inhibitors (EPIs) in A. baumanii. Clin Microbial 3, 135.

Yoon, E.J., Courvalin, P., Grillot-Courvalin, C.,2013. RND-type efflux pumps in multidrug-resistant clinical isolates of Acinetobacter baumannii: major role for AdeABC overexpression and AdeRS mutations. Antimicrobial Agents and Chemotherapy 57, 2989-2995

Table 1: List of primers used in detecting *A. baumannii* antibiotic resistance genes

Gene	Sequence (5'→3')	Function
adeA (F)	TGACCGACCAATGCACCTT	Efflux pump
(R)	GCAACAGTTCGAGCGCCTA	
adeB (F)	CCGATGACGTATCGAAGTT	Efflux pump
(R)	CCGATGACGTATCGAAGTT	
adeC (F)	ACGGCCCCAGAAGTCTAGT	Efflux pump
(R)	CGATTAACCCCAATAACCC	
blaP (F)	ACACTAGGAGAAGCCATGA	Beta-lactam resistance
(R)	GCATGAGATCAAGACCGAT	
mdrp (F)	GTACGGCTTCTAGACCCAC	Multiple drug resistance
(R)	ACAAAGAGCCGTGCACAGT	
rRNA-16S(F)	TCGCTAGTAATCGCGGATC	Endogenous control
(R)	GACGGGCGGTGTGTACAAG	

Note: (F), forward; (R), reverse.

Table 2: MIC of antibiotics used in testing against *A. baumannii*

Antibiotics	MIC (µg/ml)
Ampicillin	64
Meropenem	32
Methicillin	256
Penicillin	256
Aztreonam	256
Amoxicillin	64
Piperacillin	128

Fig 1. Effect of TC in combination with β-lactam antibiotics in *A. baumannii* 17978

Bars with different superscripts differ from each other within a cluster ($P < 0.05$). *A. baumannii* 17978 was grown with each β-lactam antibiotic either alone or in combination with TC. Bacteria not exposed to any treatments (PC) and bacteria exposed to only TC (TC Ctrl) served as controls for the experiment.

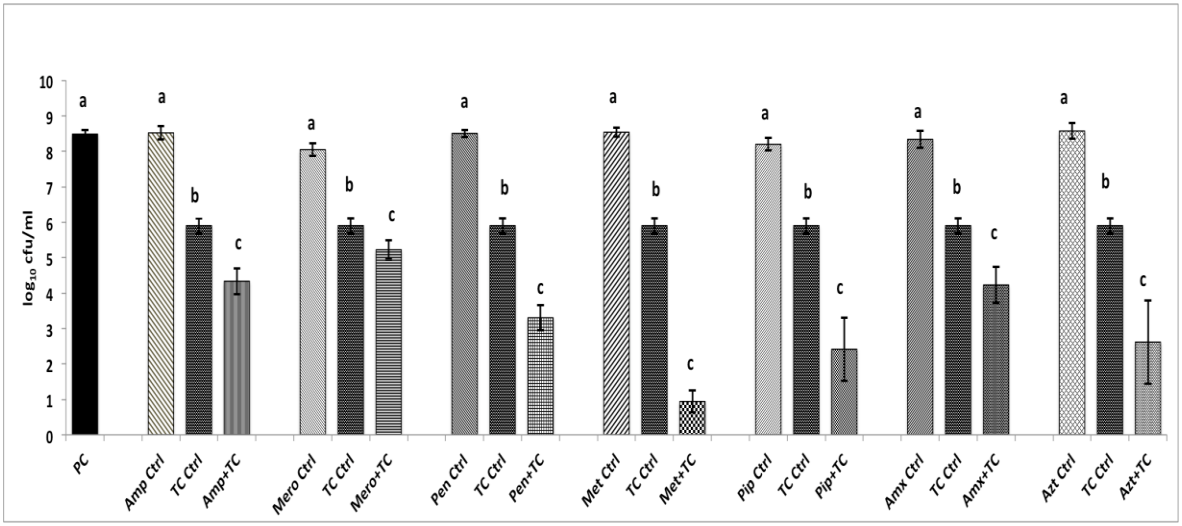


Fig 2. Effect of TC in combination with β-lactam antibiotics in *A. baumannii* 251847

Bars with different superscripts differ from each other within a cluster ($P < 0.05$). *A. baumannii* 251847 was grown with each β-lactam antibiotic either alone or in combination with TC. Bacteria not exposed to any treatments (PC) and bacteria exposed to only TC (TC Ctrl) served as controls for the experiment.

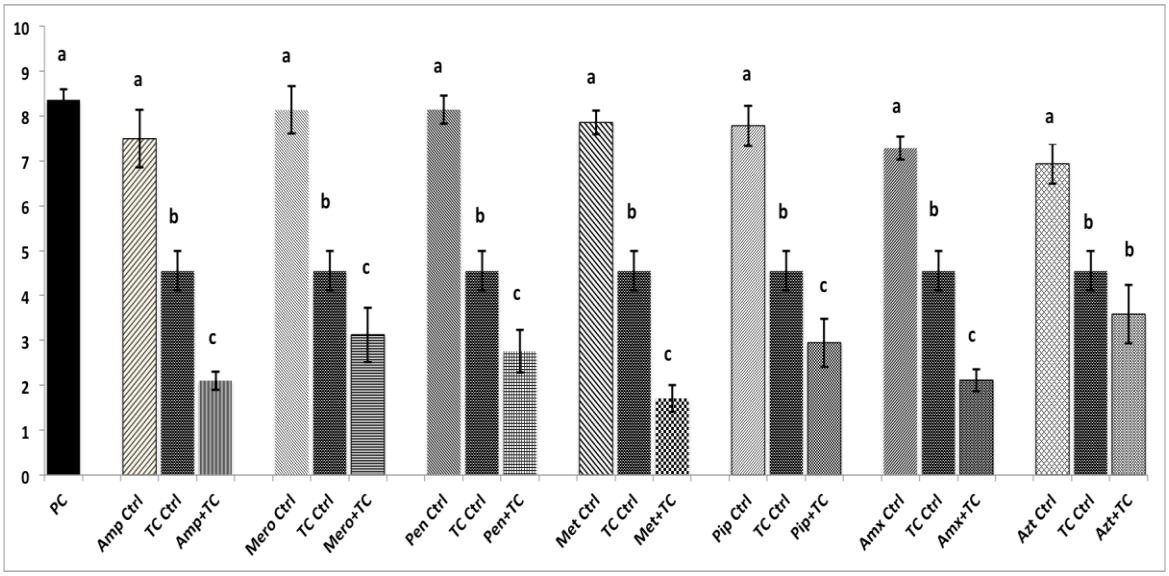


Fig 3. Effect of EG in combination with β-lactam antibiotics in *A. baumannii* 17978

Bars with different superscripts differ from each other within a cluster ($P < 0.05$). *A. baumannii* 17978 was grown with each β-lactam antibiotic either alone or in combination with EG. Bacteria not exposed to any treatments (PC) and bacteria exposed to only EG (EG Ctrl) served as controls for the experiment.

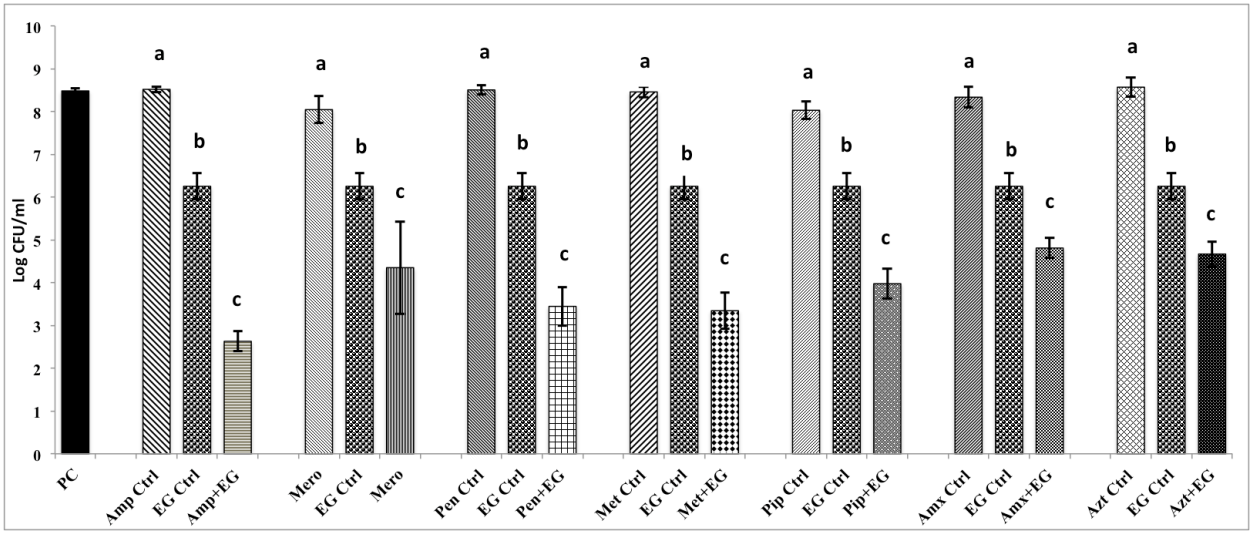


Fig 4. Effect of EG in combination with β-lactam antibiotics in *A. baumannii* 251847

Bars with different superscripts differ from each other within a cluster ($P < 0.05$). *A. baumannii* 251847 was grown with each β-lactam antibiotic either alone or in combination with EG. Bacteria not exposed to any treatments (PC) and bacteria exposed to only EG (EG Ctrl) served as controls for the experiment.

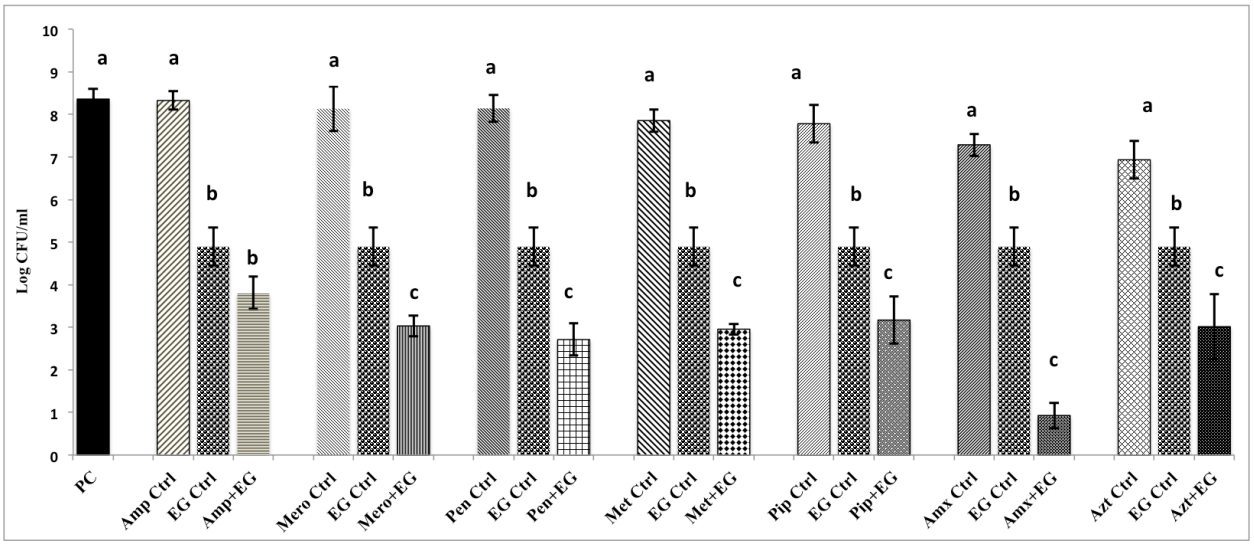


Fig 5. Intracellular accumulation of ethidium bromide (EtBr) in *A. baumannii* ATCC 17978 after treatment with MIC of TC and EG, as measured by fluorescence intensity

A. baumannii ATCC 17978 was added with MIC of TC/EG or CCCP (100 μM) and incubated for 5 h at 37ºC. Fluorescence (excitation 530 nm and emission 645 nm) was measured after addition of EtBr (4 mg/l). Treatments with * are significantly different from PBS (control) ($P < 0.05$)

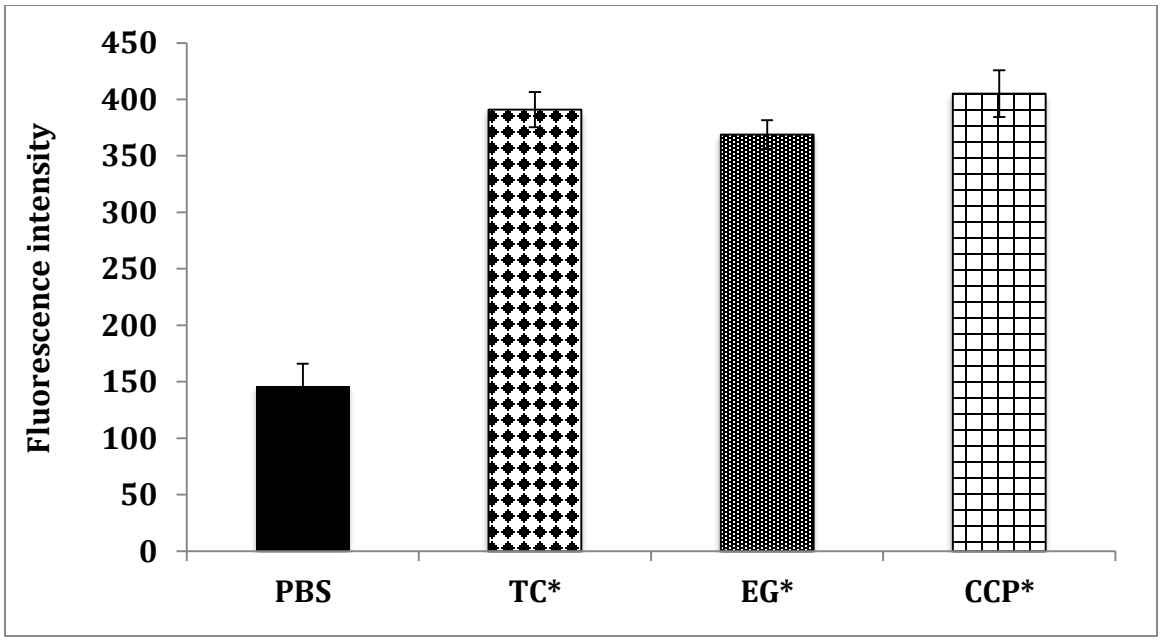


Fig 6. Intracellular accumulation of ethidium bromide (EtBr) in *A. baumannii* 251847 after treatment with MIC of TC and EG, as measured by fluorescence intensity.

A. baumannii 251847 was added with MIC of TC/EG or CCCP (100 μM) and incubated for 5 h at 37ºC. Fluorescence (excitation 530 nm and emission 645 nm) was measured after addition of EtBr (4 mg/l). Treatments with * are significantly different from PBS (control) (P <0.05)

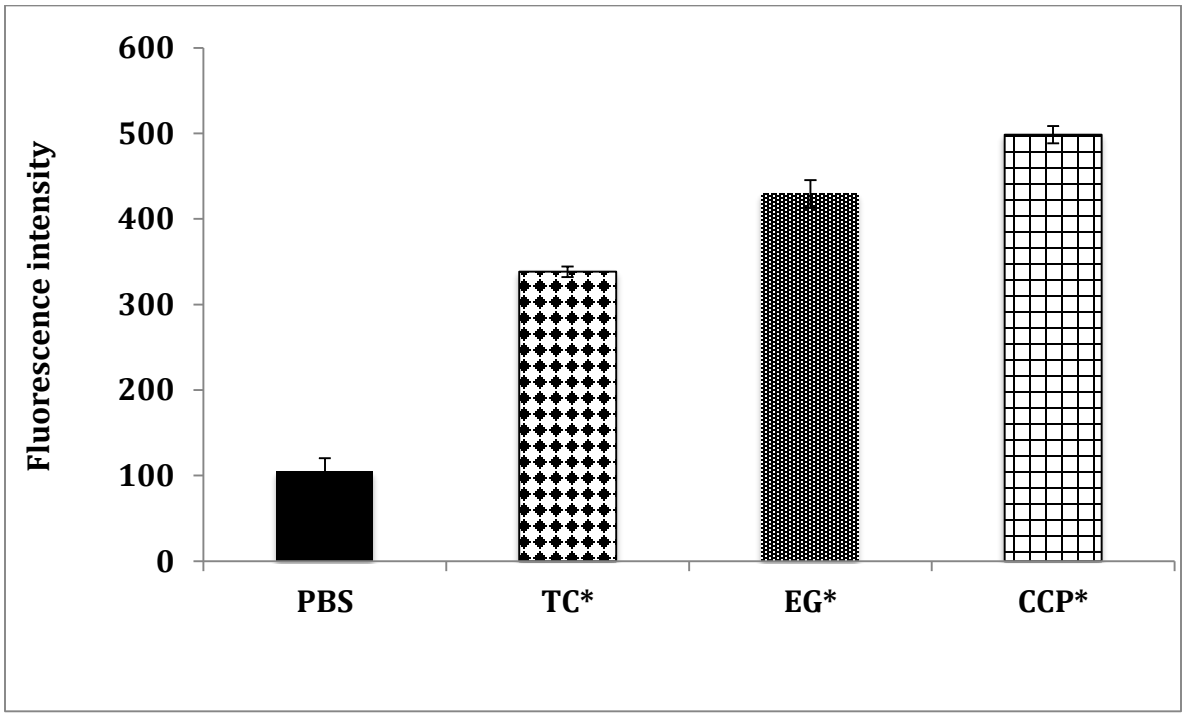


Fig 7. Intracellular accumulation of pyronin Y in *A. baumannii* ATCC 17978 after treatment with MIC of TC and EG, as measured by fluorescence intensity.

A. baumannii ATCC 17978 was added with MIC of TC/EG and incubated for 5 h at 37ºC. Fluorescence (excitation 530 nm and emission 645 nm) was measured after addition of pyronin Y (5 mg/l). Treatments with * are significantly different from PBS (control) ($P < 0.05$).

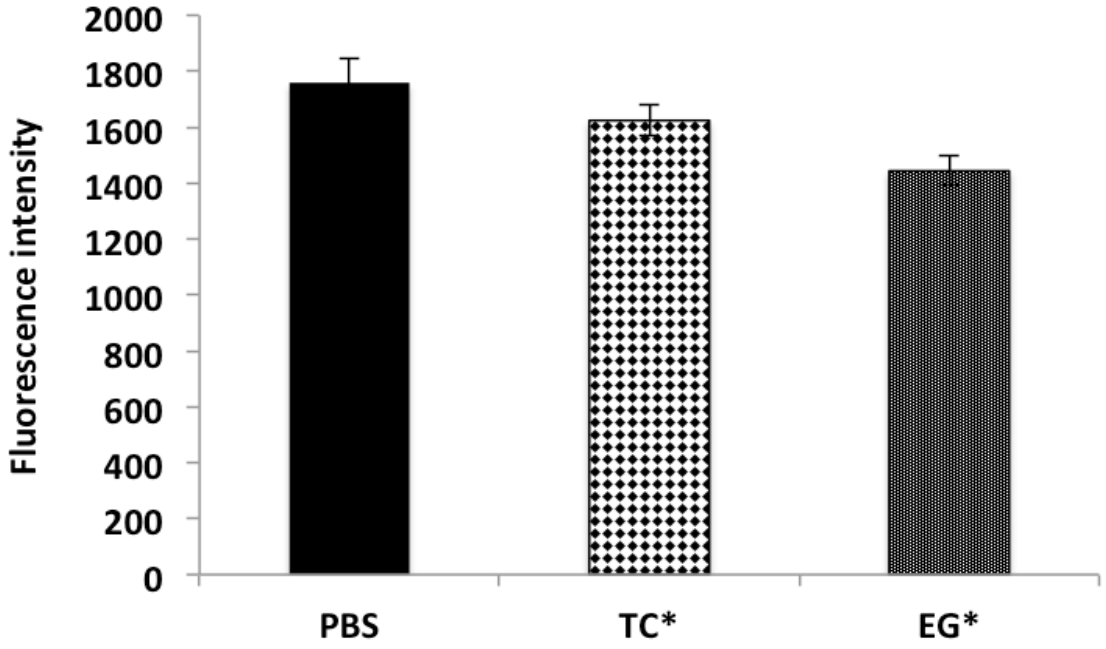


Fig 8. Intracellular accumulation of pyronin Y in *A. baumannii* 251847 after treatment with MIC of TC and EG, as measured by fluorescence intensity.

A. baumannii 251847 was added with MIC of TC/EG and incubated for 5 h at 37ºC. Fluorescence (excitation 530 nm and emission 645 nm) was measured after addition of pyronin Y (5 mg/l). Treatments with * are significantly different from PBS (control) ($P < 0.05$)

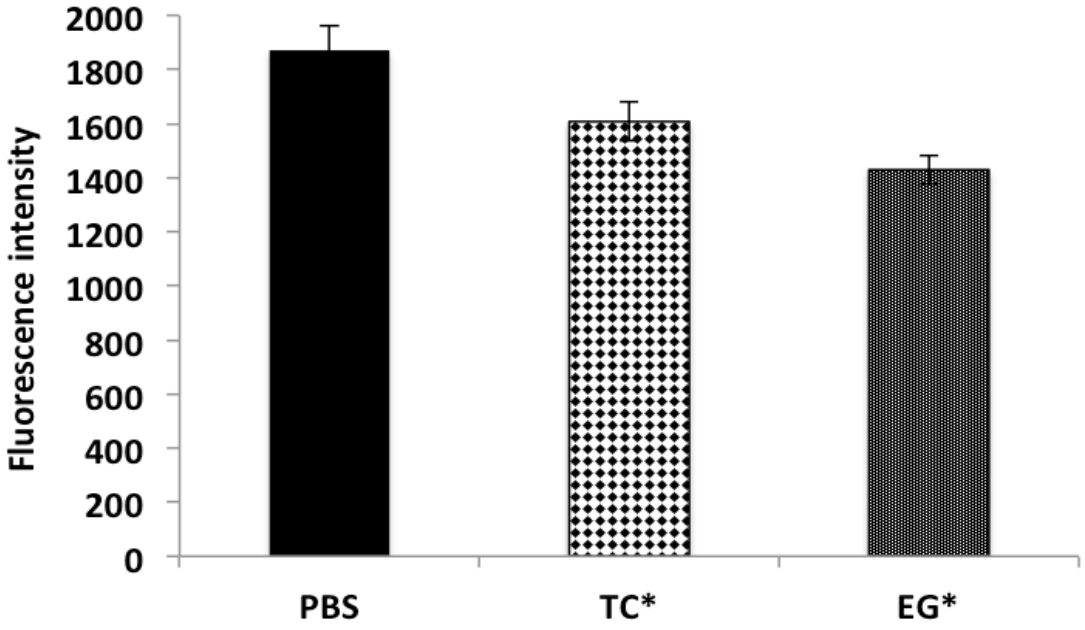


Fig 9. Effect of TC and EG on *A. baumannii* 17978 outer membrane, as measured by fluorescence intensity. HEPES buffer (5.0 mM) was used as a control.

A. baumannii ATCC 17978 was added with MIC of TC/EG or EDTA (1 mM) and incubated for 5 h at 37^{o}C. Fluorescence (excitation 530 nm and emission 645 nm) was measured after addition of NPN (40 μM). Treatments with * are significantly different from HEPES (control) ($P < 0.05$).

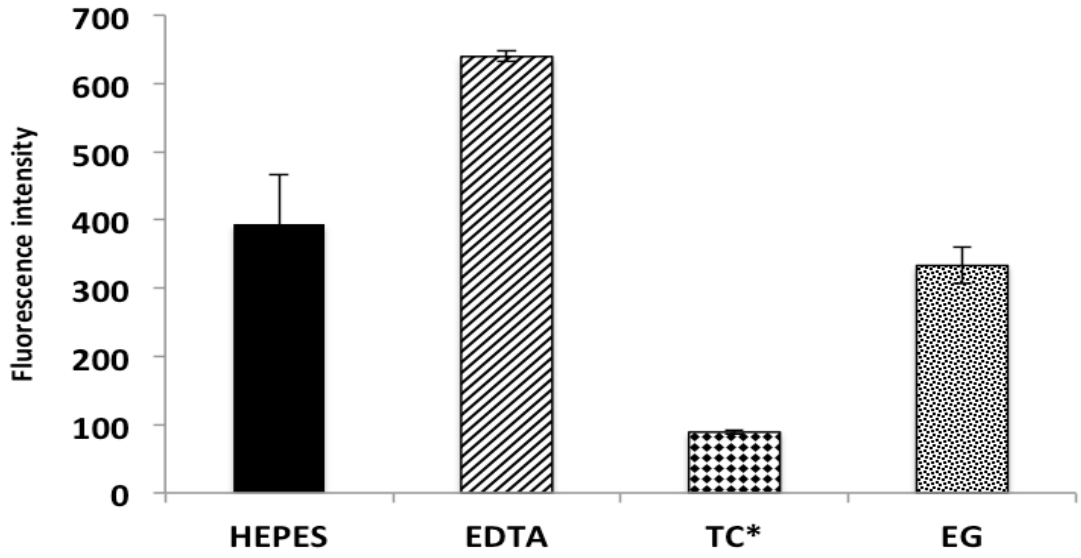


Fig 10. Effect of TC and EG on *A. baumannii* 251847 outer membrane, as measured by fluorescence intensity. HEPES buffer (5.0 mM) was used as a control.

A. baumannii 251847 was added with MIC of TC/EG or EDTA (1 mM) and incubated for 5 h at 37^{o}C. Fluorescence (excitation 530 nm and emission 645 nm) was measured after addition of NPN (40 μM). Treatments with * are significantly different from HEPES (control) ($P < 0.05$).

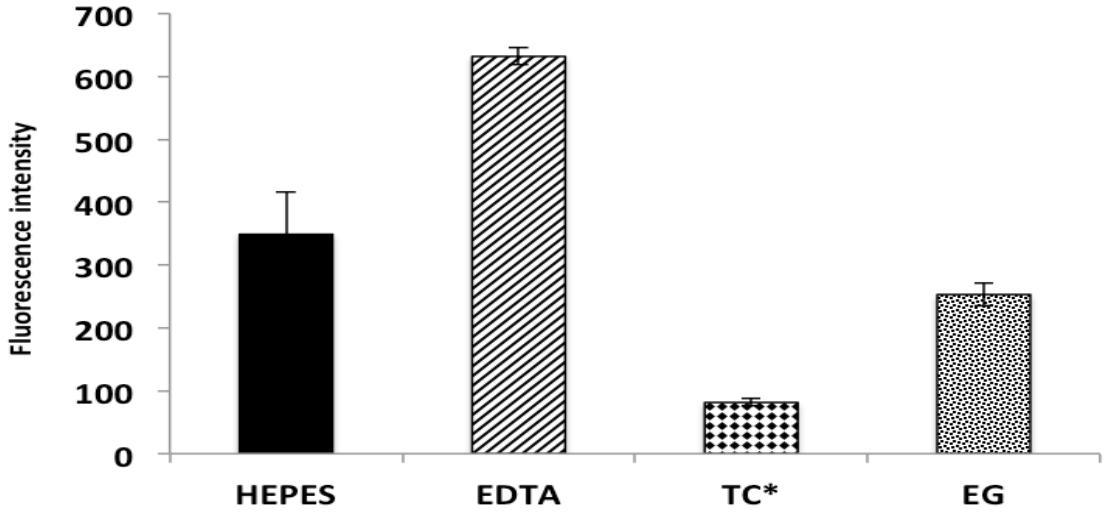
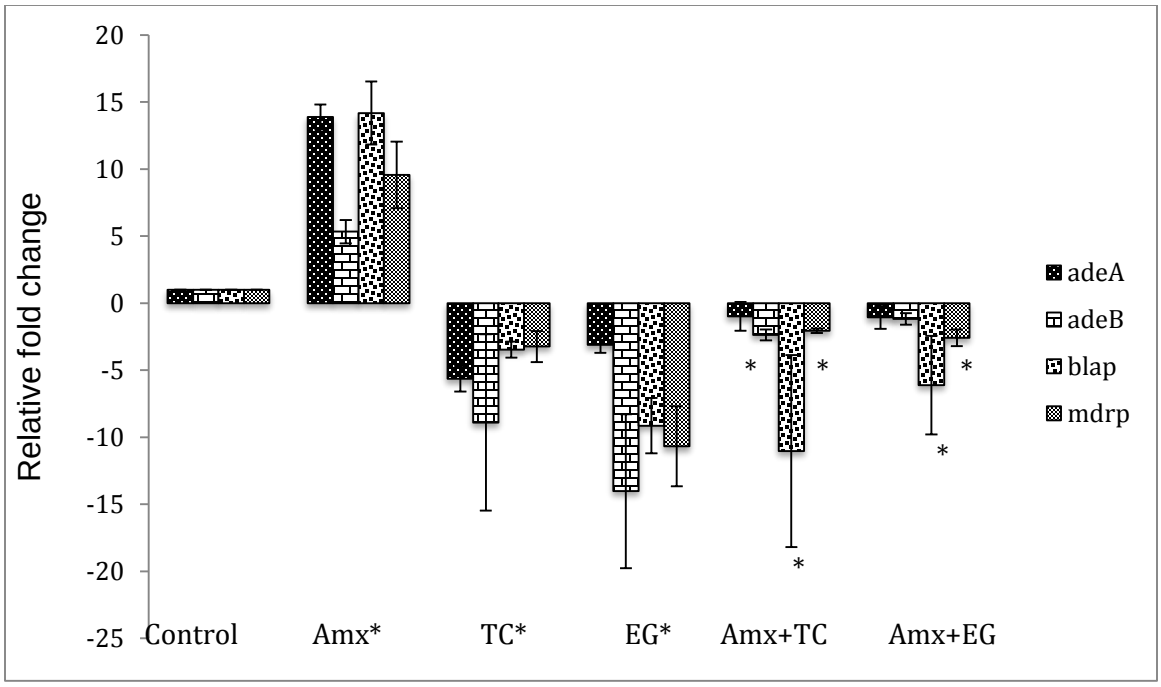
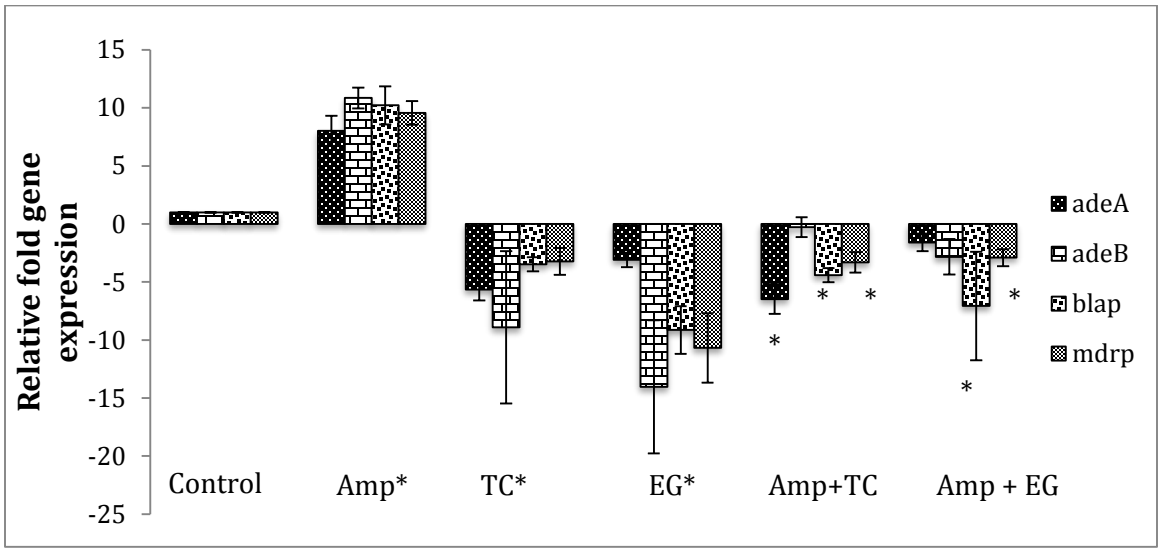


Fig 11. Effect of TC and EG on antibiotic resistance genes in *A. baumannii* 17978.

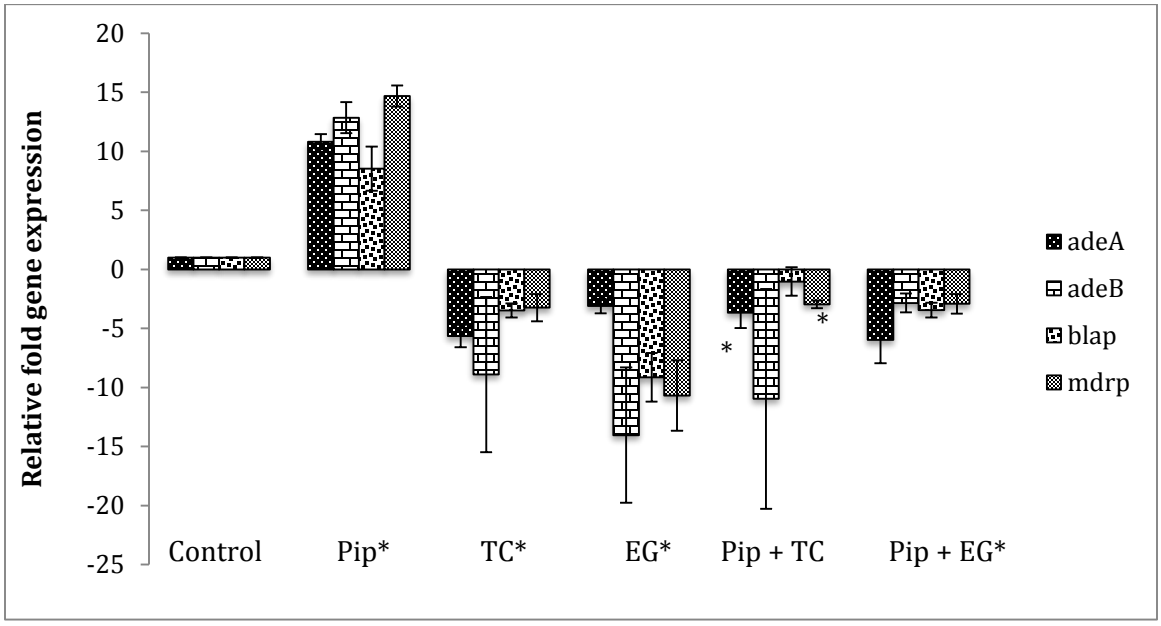
(A) *A. baumannii* 17978 was grown withthe SIC of TC/EG either alone or in combination with amoxicillin, and RT-qPCR was done to test the effect of the treatments on major antibiotic genes in *A. baumannii*. Bacteria exposed to amoxicilllin alone and bacteria not exposed to any treatments served as controls. Bars with * are significantly different from control (P <0.05).



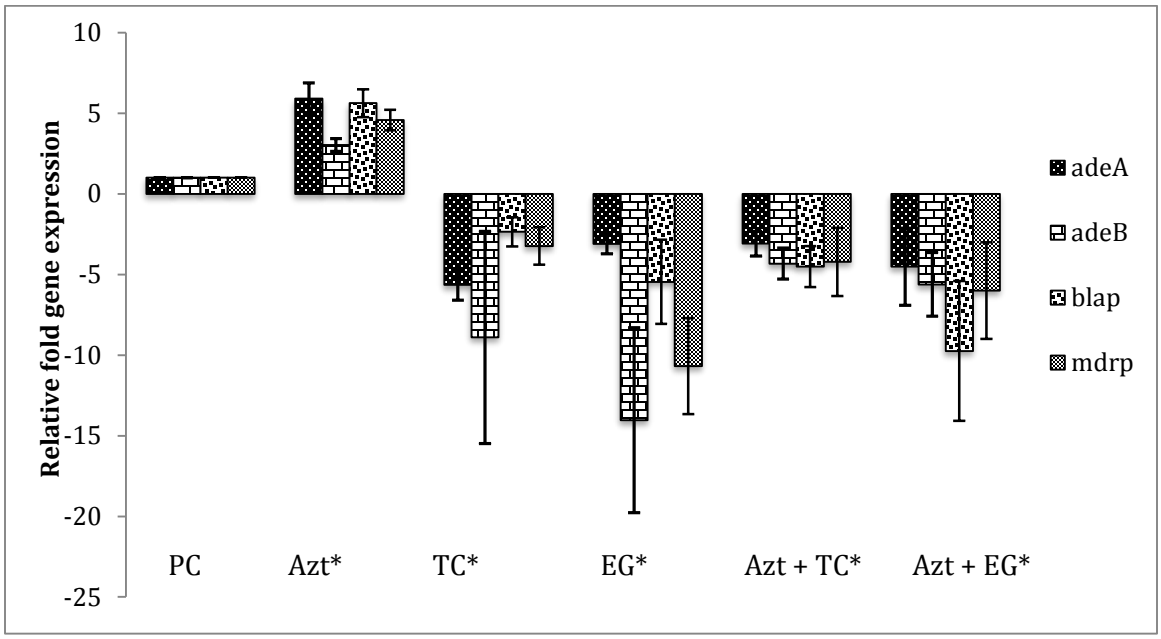
(B) *A. baumannii* 17978 was treated with SICs of TC/EG either alone or in combination with ampicillin and RT-qPCR was done to test the effect of the treatments on major antibiotic genes in *A. baumannii*. Bacteria exposed to ampicillin alone and bacteria not exposed to any treatments served as controls. Bars with * are significantly different from control (P <0.05).



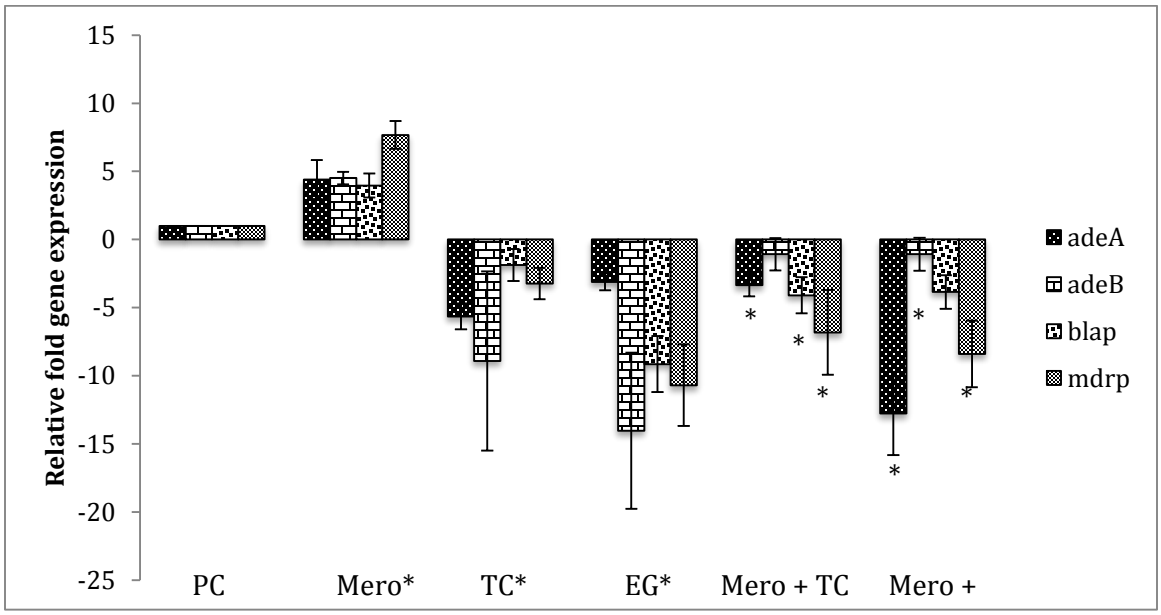
(C) *A. baumannii* 17978 was treated with SICs of TC/EG either alone or in combination with piperacillin and RT-qPCR was done to test the effect of the treatments on major antibiotic genes in *A. baumannii.* Bacteria exposed to piperacillin alone and bacteria not exposed to any treatments served as controls. Bars with * are significantly different from control (P <0.05).



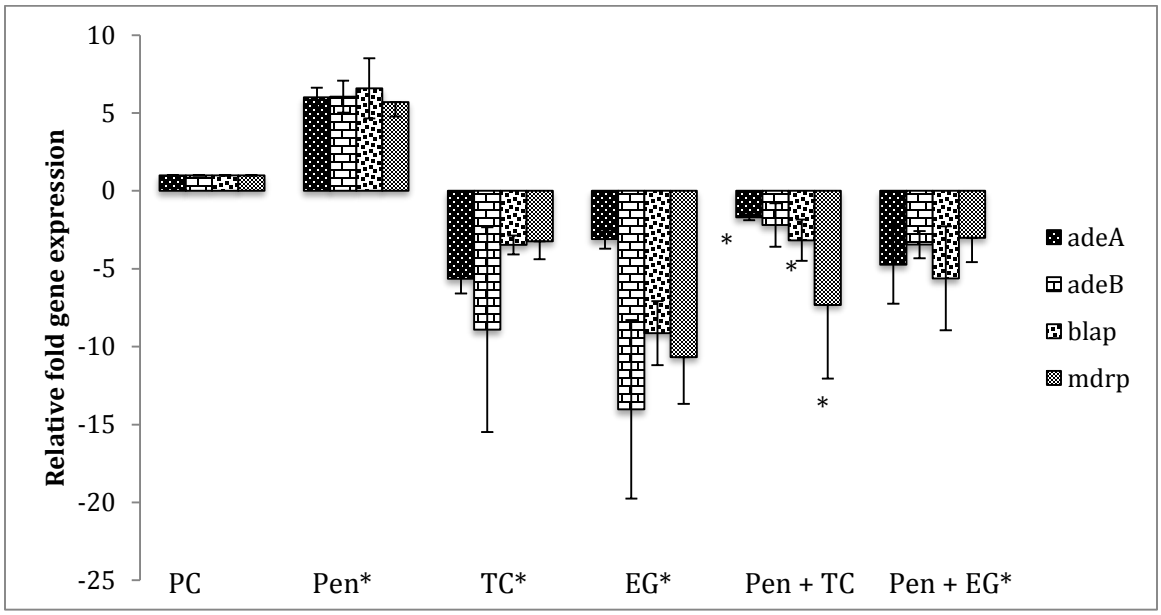
(D) *A. baumannii* 17978 was treated with SICs of TC/EG either alone or in combination with aztreonam and RT-qPCR was done to test the effect of the treatments on major antibiotic genes in *A. baumannii*. Bacteria exposed to aztreonam alone and bacteria not exposed to any treatments served as controls. Bars with * are significantly different from control (P <0.05).



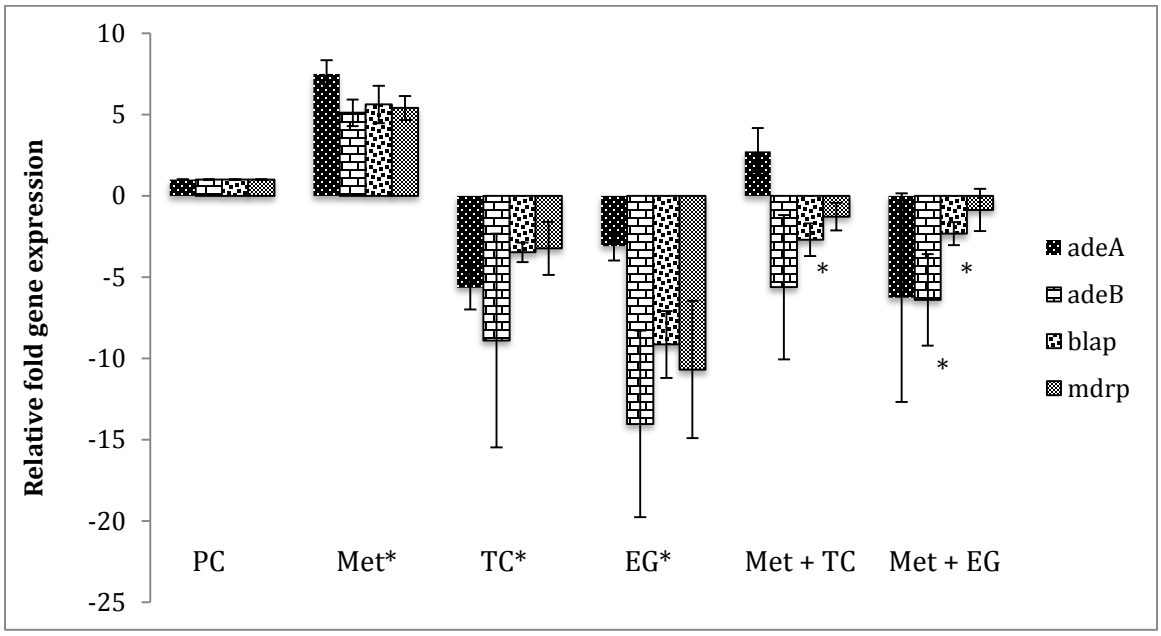
(E) *A. baumannii* 17978 was treated with SICs of TC/EG either alone or in combination with meropenem and RT-qPCR was done to test the effect of the treatments on major antibiotic genes in *A. baumannii*. Bacteria exposed to meropenem alone and bacteria not exposed to any treatments served as controls. Bars with * are significantly different from control (P <0.05).



(F) *A. baumannii* 17978 was treated with SICs of TC/EG either alone or in combination with penicillin and RT-qPCR was done to test the effect of the treatments on major antibiotic genes in *A. baumannii.* Bacteria exposed to penicillin alone and bacteria not exposed to any treatments served as controls. Bars with * are significantly different from control (P <0.05).



(G) *A. baumannii* 17978 was treated with SICs of TC/EG either alone or in combination with methicillin and RT-qPCR was done to test the effect of the treatments on major antibiotic genes in *A. baumannii*. Bacteria exposed to methicillin alone and bacteria not exposed to any treatments served as controls. Bars with * are significantly different from control (P <0.05).



Chapter VII

Efficacy of trans-cinnamaldehyde and eugenol in reducing *A. baumannii* adhesion to and invasion of human keratinocytes and controlling wound infections *in vitro*

ABSTRACT

Acinetobacter baumannii is a multi-drug resistant, nosocomial pathogen causing a variety of disease conditions in humans. After *A. baumannii* outbreaks in military combat personnel in Iraq and Afghanistan, reports of *A. baumannii* wound infections are increasingly recognized. In addition, *A. baumannii*'s ability to form biofilms and colonize epithelial cells potentially increases the invasiveness of this pathogen. Thus, in light of the multidrug resistance and biofilm producing capacity, new strategies for controlling *A. baumannii* wound infections are necessary. This study investigated the efficacy of two natural, plant-derived antimicrobials (PDAs), namely trans-cinnamaldehyde (TC) and eugenol (EG) for decreasing *A. baumannii* adhesion to and invasion of normal human keratinocytes (HEK001). The efficacy of two PDAs for inhibiting *A. baumannii* biofilm formation was determined using an *in vitro* collagen matrix wound model. In addition, the effect of TC and EG on *A. baumannii* biofilm architecture was visualized using confocal scanning microscopy. Further the effect of both PDAs on *A. baumannii* genes critical for biofilm synthesis was determined using real-time quantitative PCR (RT-qPCR). Both TC and EG significantly reduced *A. baumannii* adhesion to HEK001 by ~2 to 2.5 $\log_{10}$ CFU/ml ($P < 0.05$), and invasion by ~2 to 3 log CFU/ml, compared to the controls ($P < 0.05$). Further, after 24 and 48 h, TC inhibited biofilm formation by ~1.5 and ~2 to 3.5 $\log_{10}$ CFU/ml, while EG decreased biofilm-associated bacteria by ~2 and ~3.5 $\log_{10}$ CFU/ml, respectively, compared to controls ($P < 0.05$). Confocal microscopy revealed that TC and EG resulted in the death of biofilm-associated *A. baumannii*, and disrupted the biofilm architecture. RT-qPCR results indicated that the two phytochemicals significantly down-regulated the transcription of genes associated with *A. baumannii* biofilm production. The results suggest that both TC and EG could

potentially treat *A. baumannii* wound infections; however, their efficacy in *in vivo* models needs to be validated.

1. INTRODUCTION

Acinetobacter baumannii has emerged into a significant human pathogen, especially linked to infections acquired from hospital settings, with high case fatality rates (Karageorgopoulos and Falagas, 2008; Wieczaorek et al., 2008; Esterly et al., 2011). In addition, *A. baumannii* has the ability to form biofilms on a variety of biotic and abiotic surfaces (Gaddy et al., 2009). The ability of *A. baumannii*to persist in the nosocomial environment is the most important factor aiding in the transmission of infection in nursing homes and nosocomial settings (Simor et al., 2002; Denton et al., 2005; Rastogi et al., 2007). During the last few years, *A. baumannii* has become resistant to the majority of currently available antibiotics, and the emergence of multidrug-resistant (MDR), extensively-drug resistant (XDR), and pandrug-resistant (PDR) strains have been documented (Peleg et al, 2008; Durante-Mangoni and Zarrilli, 2011). This has generated concern among health-care professionals due to the limited choice of drugs available for controlling *A. baumannii* infections, especially in light of its ability to cause a wide range of infections, including pneumonia, bacteremia, meningitis and urinary tract infections (Bergogne-Bérézin and Towner, 1996; Peleg et al., 2008). In addition to these conditions, MDR *A. baumannii* has been increasingly linked to skin and soft-tissue infections (Ali et al., 2014). Further, *A. baumannii* is an important pathogen causing persistent wound infections in burn victims, leading to loss of skin grafts and delayed wound healing (Bergogne-Berezin, 1995; Santucci et al., 2003; Sharma, 2007). During the last decade, the US military health care system has recorded a significant increase in the incidence of MDR *A. baumannii* in combat-related wound infections (Scott et al., 2007; Murray, et al., 2008).

Traditionally, the management of wound infections consists of using gauze and cotton wool to protect the wound from outside contamination (Jones et al., 2006), coupled with creams

or powders incorporated with topical bioactive agents that either act as antimicrobial drugs or aacelerate the wound healing process (Katti et al., 2004; Dai et al., 2010). Antibiotics such as minocycline, vancomycin, neomycin and streptomycin incorporated in novel delivery systems such as chitosan, polyox and carrageenan have been found effective in the treatment of wounds (Aoyagi et al., 2007; Labovitiadi et al., 2012; Boateng et al., 2013; Pawar and Rhee., 2014). However, the multi drug resistance of *A. baumannii* combined with the inefficacy of systemic antibiotics in reducing pathogen loads in granulation wounds has necessitated alternate strategies for treating and controlling wound infections caused by the pathogen (Selcuk et al., 2012; Thompson et al, 2014).

Several plant-derived compounds have been investigated for their potential would healing properties. Aloe vera was reported to inhibit bacterial growth and enhance wound healing by stimulating macrophage activity (Djeraba and Quere, 2000; Ni, 2004). Plant compounds such as curcumin haveaccelerated healing of irradiated wounds in mice (Jagetia and Rajanikant, 2012). The treated mice had an increased collagen deposition, fibroblast and vascular densities compared to untreated mice. Similarly, essential oils from citrus, thyme, and cinnamon have exhibited wound-healing properties *in vivo* and/or *in vitro* (Dursun et al., 2003; Woollard et al., 2007; Ranasinghe et al., 2013). Liakos et al. (2014) reported the efficacy of essential oils such as cinnamon, elicriso italic, chamomile blue or lemon incorporated at 66% (dry weight percentage) in sodium alginate films against *E. coli* and *C. albicans*, and proposed their application as disposable wound dressings. Chitosan films incorporated with thyme oil were also effectively applied as antibacterial and permeable films for wound healing (Altoik et al., 2010). Farahpour and Habibi (2012) reported that extracts of cinnamon improved the healing of excised wounds in rats by increasing epithelialization. Similarly, Kamath et al. (2003) observed that oral

supplementation of ethanolic extracts of cinnamon bark increased the rate of wound contraction and granulation tissue weight in Wistar rats. Additional studies revealed that phytochemicals such as trans-cinnamaldehyde and eugenol did not produce any detrimental effect on keratinocyte cell growth and proliferation (Daker et al., 2013; Kalmes and Blomeke et al., 2012).

The objective of this study was to investigate the efficacy of trans-cinnamaldehyde and eugenol in reducing the adhesion and invasion of *A. baumannii* in human keratinocytes, and to inhibit *A. baumannii*biofilm formation in an *in vitro* wound model.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Three clinical isolates of *A. baumannii* isolated from wound infections (Navel-17, OIFC-109 and WC-487) (BEI resources, Manassas, VA, USA) were used in the study. Each strain was cultured separately in 10 ml of Tryptic soy broth (TSB, Difco, Becton Dickinson, Sparks, MD, USA) at 37^{o}C for 24 h to reach an optical density at 600 nm of ≥ 0.7, yielding ~ 8 logCFU/ml. The cultures were sedimented by centrifugation (3,600 X g, 15 min at 4^{o}C), the pellet was washed twice, re-suspended in sterile PBS, and used as the inoculum. The *A. baumannii* population in the inoculum was determined by serial dilution and plating on Tryptic soy agar (TSA, Difco) with incubation at 37^{o}C for 24 h.

2.2. Plant-Derived Antimicrobials (PDAs)

Trans-cinnamaldehyde (≥98%; trans-3-Phenyl-2-propenal, TC) and eugenol (≥98%; 4-Allyl-2-methoxyphenol, EG) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Determination of Sub-inhibitory concentrations (SIC) and Minimum inhibitory concentration (MIC) of PDAs

The SIC and MIC of TC and EG against *A. baumannii* were determined as previously reported (Amalaradjou and Venkitanarayanan, 2011; Kollanoor-Johny et al., 2010). Tryptic soy broth (10 ml) tubes containing 1 to 10 µl of TC or EG (Sigma-Aldrich) in increments of 0.5 µlwere inoculated separately with *A. baumannii* at ~6 $\log_{10}$CFU/ml, and incubated at 37^{o}C for 24 h. Tubes without any added PDAs served as controls. After incubation, the samples were serially diluted (1:10) in PBS, plated onTSA, and incubated at 37^{o}C for 24 h before counting the colonies. The highest concentration of TC or EG that did not inhibit *A. baumannii* growth after 24 h of incubation was selected as the SIC, whereasthe lowest concentration of eachantimicrobial that inhibited bacteria growth after 24 h incubation was selected as the MIC of that treatment. The experiment was done in duplicates and repeated three times.

2.4. Keratinocyte cell culture

Human skin keratinocyte, HEK001 (ATCC CRL-2404) was obtained from the American Type Culture Collection (Manassas). HEK001 cells were grown in a 25 cm^2 cell culture flask containingkeratinocyte serum free medium (K-SFM) supplemented with human recombinant epidermal growth factor (Invitrogen, Carlsbad, CA, USA) at 37^{o}C for 24-48 hin an aerobic incubator containing 5% CO_2.

2.5. Adhesion and invasion assay

The effect of MIC of TC and EG on *A. baumannii* adhesion to and invasion of HEK001 keratinocyte cellswas determined, aspreviously described (Muthaiyan et al., 2012). Twenty four-well tissue culture plates (BD, Franklin Lakes, NJ, USA) were seeded with ~10^5 cells/well, and incubated at 37^{o}C for 24 h in 5% CO_2 incubatorto form a monolayer. *A. baumannii* was grown to mid-log phase at 37^{o}C, washed and re-suspended in K-SFM with MIC of TC or EG. Bacteria suspended in K-SFM alone or with ethanol (diluent) were used as controls. Aliquots of 100 µl of

the bacterial suspension containing approximately 6 log_{10}CFU/well (MOI 1:10) was inoculated in duplicates into the HEK001 monolayer, and incubated at 37ºC in 5% CO_2 incubator for 2 h. For the adhesion assay, the infected monolayers after incubation was washed three times with PBS, and the cells were lysed using 0.1 % Triton X-100 (Invitrogen). The number of viable adhered bacteria was enumerated by serial dilution and culturing on TSA plates. For the invasion assay, the HEK001 monolayer was washed three times with PBS, followed by incubation for 2 h in K-SFM containing gentamicin (100 µg/ml) (Invitrogen) in order to kill the extracellular bacteria. Subsequently, the wells were washed three times with PBS and the cells were lysed using 0.1 % Triton X-100 to release the intracellular bacteria. The number of invaded bacteria was enumerated by serial dilution in PBS and culturing on TSA plates. Both adhesion and invasion assays were done in duplicates and the experiment was repeated three times.

2.6. Quantification of biofilm in invitro

In order to replicate the conditions of a chronic wound, an *in vitro* system that accommodates bacterial aggregates in a simulated wound fluid and collagen matrix model, as described by Werthen et al., (2010) was used. Briefly 24-well cell culture plates were coated with collagen solution (rat tail collagen type 1, BD Biosciences, San Jose, CA). A 10 ml collagen solution (2 mg/ml) contained 1 ml of 0.1% acetic acid, 2 ml collagen stock solution (10 mg/ml) and 6 ml cold simulated wound fluid (SWF, 50% fetal calf serum and 50% physiological NaCl in 0.1% peptone) and 1 ml of 0.1 M NaOH. One ml of the above solution was added to each 24-well plate, which wasincubated at 37ºC for 1 h. After complete polymerization, *A. baumannii* (5 log_{10} CFU/ml) was suspended in SWF with or without the MIC of PDAs to each well in duplicates. The plates were incubated for 48 h, and the polymerized collagen was subsequently dissolved using collagenase solution (500 µl/ml in PBS), followed by incubation for 60 min at

37ºC. *A. baumannii* counts were determined using serial dilution and plating on TSA. The experiment was repeated three times.

2.7. Confocal microscopy

The three-dimensional structure of *A. baumannii* biofilm treated with or without TC or EG was visualized *in situ*confocal laser scanning microscopy using a Leica true confocal scanner SP2 microscope with a water immersion lens (Loehfelm et al., 2008). *A. baumannii* exposed to the MIC of TC or EG in TSB was allowed to form biofilms on a Lab-Tech eight-chamber no.1 borosilicate glass coverslip system (Lab-tek, Nalge Nunc International, Rochester, NY, USA) by incubating it at 37ºC for 48 h, followed by gently washing with PBS three times. The live and dead bacteria in the biofilm were imaged after staining with 2.5 μM Syto9 (Molecular Probes, Eugene, Oregon, USA) and 5 μM propidium iodide (Molecular Probes) for 5 min prior to imaging on a Nikon AIR laser scanning confocal. Syto9 is a live cell permanent DNA stain, while propidium iodide is a nucleic acid stain that can only enter dead or dying cells. Syto staining was imaged with 488 nm excitation and a 525/50 emission filter, whereas propidium iodide staining was imaged with 560 nm excitation and a 600/50 emission filter. For each treatment, four randomly selected fields were analyzed. Optical sections of approximately 0.2 μm in height were collected starting from the bottom and moving upward through the entire biofilm.

2.8.Effect of TC and EG on biofilm genes in A. baumannii

2.8.1. RNA extraction and cDNA synthesis

To study the effect of TC and EGon genes critical for biofilm formation in *A. baumannii*, bacterial cultures (Navel-17, OIFC-109) were grown separately in TSB with or without the SIC of TC or EG at 37ºC to mid-log phase. Control without any PDA was also included. After

incubation, the cultures were subjected to centrifugation (12000 x g, 15 min, 4^{o}C), and the resultant pellet was added with 0.5 ml of RNAase free, sterile water and 1 ml of RNA protect reagent (Qiagen, Valencia, CA, USA). The total RNA from each sample was extracted using the RNeasy mini kit (Qiagen), and the manufacturer's instructions were followed in estimating the total RNAusing Nanodrop (ThermoFisher Scientific, Waltham, MA, USA). Super-script II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis, and the resultant cDNA was used as the template for RT-qPCR. The amplified product was detected using SYBR Green reagents.

2.8.2. Real-Time Quantitative PCR (RT-qPCR)

The biofilm genes of *A. baumannii* analysed for the gene expression study included *csuE,* that codes for putative tip adhesin critical forinitiation of biofilm formation (Gaddy and Actis, 2009); *bfmS*, a component of the regulatory system that controls *csuE* (Liou et al., 2014)*;* and *ompA*, a critical gene for attachment to eukaryotic cells for biofilm formation (Liou et al., 2014). Previously published primers were used for *csuE* and *bfmS*, while the primer pair for *ompA* was designed using Basic Logic Alignment Search Tool (BLAST) (Table 1). The custom synthesized primers were obtained from Integrated DNA Technologies (Foster City, CA, USA). RT-qPCR was performed with StepOnePlus^{TM} Real Time PCR system (Applied Biosystems, Foster city, CA, USA) using the SYBR green assay (Applied Biosystems) under custom thermal cycling conditions with the normalized RNA as the template (Bookout and Mangelsdorf, 2003). Duplicate samples were analyzed and standardized against 16S rRNA gene expression. The relative changes in mRNA expression levels were determined using comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$) between the control and the treatments.

2.9. Statistical analysis

A completely randomized design was used for the study. All the experiments had duplicate samples and were repeated three times. The data for each treatment and control were pooled and analyzed using the PROC GENMOD procedure of Statistical Analysis Software (SAS ver. 9.4; Institute, Inc., Cary, NC, USA). A Pvalue of < 0.05 was considered to be statistically significant.

3. RESULTS AND DISCUSSION

Skin is the largest organ in the human body and constitutes an integral part of the innate immune system; it is the first line of defense against any infection by preventing microbial adhesion and invasion (Nizet et al., 2001; Schroder and Harder, 2006). Skin is colonized with microbial populations consisting of resident flora and transient microflora (Price, 1938; Mackowiak, 1982). However, when a disruption occurs to the anatomical structure of the skin, an onset of infection can happen, if the homeostatic physiological conditions are not rapidly restored (Boateng and Catanzano, 2015). While studying the distribution of *Acinetobacter* species on human skin, Seifert et al. (1997) reported that *Acinetobacter* spp. colonized in 75% of patients, with the colonization rates increasing with the length of stay in hospitals. *A. baumannii* is considered as one of the most significant bacteria causing persistent wound infections, especially in burn patients (Uygur et al., 2009) and combat-related injuries (Johnson et al., 2007; Scott et al., 2007). Previous studies have reported the formation of biofilms by *A. baumannii* on human skin surfaces (Guerrero et al., 2010; deBreij et al., 2012). Besides its multi-drug resistance, difficulties in treating *A. baumannii* wound infections areattributed to its ability to form biofilms, which resist the action of host immune defenses as well as antimicrobial interventions, thereby delaying the healing process (Dallo and Weitao, 2010). Bacterial biofilms

are also important in chronic wound infections (Martin et al., 2010);60% of the chronic wounds testedin hospitals containbiofilms (James et al., 2008). Although it is not clearly recognized how the wound-associated microflora affect healing (Percival et al., 2012), pathogens are known to produce extracellular adherence proteins which can inhibit action of leucocytes besidesexerting anti-angiogenic and anti-inflammatory effects (Athanaspoulos et al., 2006). Therefore, in this study, we investigated efficacy of TC and EG in reducing *A. baumannii* colonization of human keratinocytes as well as inhibiting its biofilm in an *in vitro* wound model (Werthen et al., 2010).

The SIC of TC and EG against *A. baumannii* were found to be 1.1 mM (0.015 %) and 1.8 mM (0.03 %), respectively, while the MICs of TC and EG were 4.0 mM (0.05% TC and 0.065 % EG (data not shown). The efficacy the MIC of TC and EG in reducing adhesion to and invasion of keratinocytes HEK001 by three clinical strains of *A. baumannii* is presented in Fig 1-3. The results revealed that both TC and EG were effective in reducing *A. baumannii* adhesion to and invasion of HEK001 cells compared to the controls ($P < 0.05$). The MIC of TC reduced *A. baumannii* adhesion and invasion of keratinocytes by ~1.8 to 2 log CFU/ml, while EG reduced adhesion and invasion of cells by ~2 log and ~2.5-3 $\log_{10}$ CFU/ml, respectively compared to controls ($P < 0.05$). No significant difference was observed between TC and EG in reducing *A. baumannii* adhesion to HEK001 cells, however, EG was found to be more effective than TC in reducing invasion in *A. baumannii* Naval-17 ($P < 0.05$). Ethanol, which was used as a diluent for TC and EG, did not exert any significant effect on the adhesion-invasion properties of *A. baumannii* compared to control ($P > 0.05$).

The effect of PDAs on *A. baumannii* biofilm formation in the *in vitro* wound model is presented in Fig. 4-6. The MIC of both TC and EG significantly reduced biofilm-associated *A. baumannii* counts after 24 and 48 h of incubation at 37^{O}C compared to control ($P < 0.05$). After

24 h of incubation, the MIC of TC and EG resulted in ~1.5$_{10}$ log and ~2 log$_{10}$ CFU/ml reduction in *A. baumannii* counts respectively, compared to control ($P < 0.05$). However, after 48 h of incubation, TC and EG decreased *A. baumannii* counts by ~ 3-3.5 log$_{10}$ CFU/ml and ~ 4 log $_{10}$CFU/ml, respectively compared to control ($P < 0.05$). In addition, EG was generally found to be more effective than TC in inhibiting biofilm, particularly in *A. baumannii* Naval-17 ($P < 0.05$).

The effect of TC and EG on *A. baumannii* biofilm structure, as revealed by confocal laser scan microscopy, is depicted in Fig 7. In *A. baumannii* not exposed to TC/EG (control), a thick uniform layer of biofilm embedded with live cells (stained green by SYTO Green dye) was observed (Fig 7A), while *A. baumannii* treated with the MIC of TC and EG revealed breaks in the biofilm with the presence of dead cells (stained by red propidium iodide dye) (Fig 7 B-C). In addition, the thickness of the biofilms in the treatments (<10 μm) was lesser than in controls (> 20 μm).

The effect of PDAs on the expression of critical biofilm genes in *A. baumannii* is depicted in Fig. 8-10. It was observed that in *A. baumannii* Naval -17 when compared to the control, TC significantly down-regulated the expression the expression of *bfms*, *csuE* and *ompA* by ~2.5, ~1.5 and ~2.5 folds, respectively ($P < 0.05$). Similarly, EG was found to decrease the expression of *bfms*, *csuE* and *ompA* by ~ 3.5, 6 and 4 folds, respectively (Fig 8). In *A. baumannii* OIFC 109, TC down-regulated the expression of *bfmS* and *csuE* by ~2.7 and ~16 fold, respectively ($P < 0.05$), while EG decreased the expression of *bfmS*, *csuE* and *ompA* by ~25, ~11 and ~23 folds, respectively ($P <0.05$) (Fig 9). These results support the findings from the *in vitro* wound biofilm assay as well as the confocal microscopy result.

The efficacy of antibiotics for treating *A. baumannii* wound infections is limited owing to its multi-drug resistance (Karageorgopoulos and Falagas, 2008; Giamarellou et al., 2008). Although studies have shown the efficacy of chlorhexidine as an antiseptic for skin disinfection of *A. baumannii* (Evans et al., 2010; de-Breij et al., 2012), it has been implicated to exert toxicity to fibroblasts and prevent wound healing (Lucarotti et al., 1990; Hidalgo and Dominquez, 2001). In addition, *A. baumannii* resistant to 1% chlorhexidine has been isolated from surfaces of soap dispensers (Brooks et al., 2002). The results from this study indicated that TC and EG were effective for reducing *A. baumannii* colonization of skin cells, and inhibiting bacterial biofilm formation in a collagen matrix. Therefore, TC and EG represent potential antimicrobial agents for treating *A. baumannii* wound infections, where they could be used as ingredients in antimicrobial ointments and creams. However, follow up studies on their safety and efficacy in an appropriate animal model are necessary before recommending their clinical application.

REFERENCES

Ali, A., Botha, J., Tiruvoipati, R.,2014. Fatal skin and soft tissue infection of multidrug resistant Acinetobacter baumannii: A case report. International journal of surgery case reports 5, 532-536.

Altiok, D., Altiok, E., Tihminlioglu, F.,2010. Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications. Journal of Materials Science: Materials in Medicine 21, 2227-2236.

Amalaradjou, M.A.R., Venkitanarayanan, K.,2011. Effect of trans-cinnamaldehyde on reducing resistance to environmental stresses in Cronobacter sakazakii. Foodborne pathogens and disease 8, 403-409.

Aoyagi, S., Onishi, H., Machida, Y.,2007. Novel chitosan wound dressing loaded with minocycline for the treatment of severe burn wounds. International journal of pharmaceutics 330, 138-145.

Athanasopoulos, A.N., Economopoulou, M., Orlova, V.V., Sobke, A., Schneider, D., Weber, H., Augustin, H.G., Eming, S.A., Schubert, U., Linn, T., Nawroth, P.P., Hussain, M., Hammes, H.P., Herrmann, M., Preissner, K.T., Chavakis, T.,2006. The extracellular adherence protein (Eap) of Staphylococcus aureus inhibits wound healing by interfering with host defense and repair mechanisms. Blood 107, 2720-2727.

Bergogne-Berezin, E.,1995. The increasing significance of outbreaks of Acinetobacter spp.: the need for control and new agents. journal of Hospital Infection 30, 441-452

Bergogne-Berezin, E., Towner, K.J.,1996. Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clinical microbiology reviews 9, 148-165.

Boateng, J.S., Pawar, H.V., Tetteh, J.,2013. Polyox and carrageenan based composite film dressing containing anti-microbial and anti-inflammatory drugs for effective wound healing. International journal of pharmaceutics 441, 181-191.

Boateng, J., Catanzano, O.,2015. Advanced Therapeutic Dressings for Effective Wound Healing—A Review. Journal of pharmaceutical sciences 104, 3653-3680.

Bookout, A.L., Mangelsdorf, D.J.,2003. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nuclear receptor signaling 1, e012.

Brooks, S.E., Walczak, M.A., Hameed, R., Coonan, P.,2002. Chlorhexidine resistance in antibiotic-resistant bacteria isolated from the surfaces of dispensers of soap containing chlorhexidine. Infection control and hospital epidemiology 23, 692-695.

Dai, T., Huang, Y.Y., Sharma, S.K., Hashmi, J.T., Kurup, D.B., Hamblin, M.R.,2010. Topical antimicrobials for burn wound infections. Recent patents on anti-infective drug discovery 5, 124-151.

Daker, M., Lin, V.Y., Akowuah, G.A., Yam, M.F., Ahmad, M.,2013. Inhibitory effects of Blume stem bark extract and -cinnamaldehyde on nasopharyngeal carcinoma cells; synergism with cisplatin. Experimental and therapeutic medicine 5, 1701-1709.

Dallo, S.F., Weitao, T.,2010. Insights into acinetobacter war-wound infections, biofilms, and control. Advances in Skin & Wound Care 23, 169-174.

de Breij, A., Haisma, E.M., Rietveld, M., El Ghalbzouri, A., van den Broek, P.J., Dijkshoorn, L., Nibbering, P.H.,2012. Three-dimensional human skin equivalent as a tool to study Acinetobacter baumannii colonization. Antimicrobial Agents and Chemotherapy 56, 2459-2464.

Denton, M., Wilcox, M., Parnell, P., Green, D., Keer, V., Hawkey, P., Evans, I., Murphy, P.,2005. Role of environmental cleaning in controlling an outbreak of Acinetobacter baumannii on a neurosurgical intensive care unit. Intensive and Critical Care Nursing 21, 94-98.

Djeraba, A., Quere, P.,2000. In vivo macrophage activation in chickens with Acemannan, a complex carbohydrate extracted from Aloe vera. International journal of immunopharmacology 22, 365-372.

Durante-Mangoni, E., Zarrilli, R.,2011. Global spread of drug-resistant Acinetobacter baumannii: molecular epidemiology and management of antimicrobial resistance. Future microbiology 6, 407-422.

Dursun, N., Liman, N., Özyazgan, I., Günes, I., Saraymen, R.,2003. Role of thymus oil in burn wound healing. Journal of Burn Care & Research 24, 395-399.

Esterly, J.S., Griffith, M., Qi, C., Malczynski, M., Postelnick, M.J., Scheetz, M.H.,2011. Impact of carbapenem resistance and receipt of active antimicrobial therapy on clinical outcomes of Acinetobacter baumannii bloodstream infections. Antimicrobial Agents and Chemotherapy 55, 4844-4849.

Evans, H.L., Dellit, T.H., Chan, J., Nathens, A.B., Maier, R.V., Cuschieri, J.,2010. Effect of chlorhexidine whole-body bathing on hospital-acquired infections among trauma patients. Archives of surgery (Chicago, Ill.: 1960) 145, 240-246.

Farahpour, M., Habibi, M.,2012. Evaluation of the wound healing activity of an ethanolic extract of Ceylon cinnamon in mice. Vet Med 57, 53-57.

Gaddy, J.A., Actis, L.A.,2009. Regulation of Acinetobacter baumannii biofilm formation.

Gaddy, J.A., Tomaras, A.P., Actis, L.A.,2009. The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infection and immunity 77, 3150-3160.

Giamarellou, H., Antoniadou, A., Kanellakopoulou, K.,2008. Acinetobacter baumannii: a universal threat to public health? International journal of antimicrobial agents 32, 106-119

Guerrero, D.M., Perez, F., Conger, N.G., Solomkin, J.S., Adams, M.D., Rather, P.N., Bonomo, R.A.,2010. Acinetobacter baumannii-Associated Skin and Soft Tissue Infections: Recognizing a Broadening Spectrum of Disease*. Surgical infections 11, 49-57.

Hidalgo, E., Dominguez, C.,2001. Mechanisms underlying chlorhexidine-induced cytotoxicity. Toxicology in vitro : an international journal published in association with BIBRA 15, 271-276.

Inouye, S., Takizawa, T., Yamaguchi, H.,2001. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. The Journal of antimicrobial chemotherapy 47, 565-573.

Jagetia, G.C., Rajanikant, G.K.,2012. Acceleration of wound repair by curcumin in the excision wound of mice exposed to different doses of fractionated γ radiation. International wound journal 9, 76-92.

James, G.A., Swogger, E., Wolcott, R., Secor, P., Sestrich, J., Costerton, J.W., Stewart, P.S.,2008. Biofilms in chronic wounds. Wound Repair and regeneration 16, 37-44.

Johnson, E.N., Burns, T.C., Hayda, R.A., Hospenthal, D.R., Murray, C.K.,2007. Infectious complications of open type III tibial fractures among combat casualties. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 45, 409-415.

Johny, A.K., Hoagland, T., Venkitanarayanan, K.,2010. Effect of subinhibitory concentrations of plant-derived molecules in increasing the sensitivity of multidrug-resistant Salmonella enterica serovar Typhimurium DT104 to antibiotics. Foodborne pathogens and disease 7, 1165-1170.

Jones V, Grey JE, Harding KG. 2006. Wound dressings. BMJ 332(7544):777–780. Accessed May 11, 2015, at: http://www.oxforddictionaries.com/.

Kalmes, M., Blomeke, B.,2012. Impact of eugenol and isoeugenol on AhR translocation, target gene expression, and proliferation in human HaCaT keratinocytes. Journal of toxicology and environmental health.Part A 75, 478-491.

Kamath, J.V., Rana, A.C., Chowdhury, A.R.,2003. Pro-healing effect of Cinnamomum zeylanicum bark. Phytotherapy Research : PTR 17, 970-972.

Karageorgopoulos, D.E., Falagas, M.E.,2008. Current control and treatment of multidrug-resistant Acinetobacter baumannii infections. The Lancet.Infectious diseases 8, 751-762.

Katti, D.S., Robinson, K.W., Ko, F.K., Laurencin, C.T.,2004. Bioresorbable nanofiber-based systems for wound healing and drug delivery: optimization of fabrication parameters. Journal of biomedical materials research.Part B, Applied biomaterials 70, 286-296.

Labovitiadi, O., Lamb, A.J., Matthews, K.H.,2012. In vitro efficacy of antimicrobial wafers against methicillin-resistant Staphylococcus aureus. Therapeutic delivery 3, 443-455.

Liakos, I., Rizzello, L., Scurr, D.J., Pompa, P.P., Bayer, I.S., Athanassiou, A.,2014. All-natural composite wound dressing films of essential oils encapsulated in sodium alginate with antimicrobial properties. International journal of pharmaceutics 463, 137-145.

Liou, M., Soo, P., Ling, S., Kuo, H., Tang, C.Y., Chang, K.,2014. The sensor kinase BfmS mediates virulence in Acinetobacter baumannii. Journal of Microbiology, Immunology and Infection 47, 275-281.

Loehfelm, T.W., Luke, N.R., Campagnari, A.A.,2008. Identification and characterization of an Acinetobacter baumannii biofilm-associated protein. Journal of Bacteriology 190, 1036-1044.

Lucarotti, M.E., White, H., Deas, J., Silver, I.A., Leaper, D.J.,1990. Antiseptic toxicity to breast carcinoma in tissue culture: an adjuvant to conservation therapy? Annals of the Royal College of Surgeons of England 72, 388-392.

Mackowiak, P.A.,1982. The normal microbial flora. The New England journal of medicine 307, 83-93.

Martin, J.M., Zenilman, J.M., Lazarus, G.S.,2010. Molecular microbiology: new dimensions for cutaneous biology and wound healing. Journal of Investigative Dermatology 130, 38-48.

Murray, C.K., Hsu, J.R., Solomkin, J.S., Keeling, J.J., Andersen, R.C., Ficke, J.R., Calhoun, J.H.,2008. Prevention and management of infections associated with combat-related extremity injuries. The Journal of trauma 64, S239-51.

Muthaiyan, A., Biswas, D., Crandall, P.G., Wilkinson, B.J., Ricke, S.C.,2012. Application of orange essential oil as an antistaphylococcal agent in a dressing model. BMC complementary and alternative medicine 12, 125-6882-12-125.

Ni Y, Yates KM, Tizard IR. 2004. Aloes: The genus Aloe. In Reynolds T, ed. New York: CRC Press LLC.

Nizet, V., Ohtake, T., Lauth, X., Trowbridge, J., Rudisill, J., Dorschner, R.A., Pestonjamasp, V., Piraino, J., Huttner, K., Gallo, R.L.,2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414, 454-457.

Peleg, A.Y., Seifert, H., Paterson, D.L.,2008. Acinetobacter baumannii: emergence of a successful pathogen. Clinical microbiology reviews 21, 538-582.

Percival, S.L., Emanuel, C., Cutting, K.F., Williams, D.W.,2012. Microbiology of the skin and the role of biofilms in infection. International wound journal 9, 14-32.

Price, P.B.,1938. The bacteriology of normal skin; a new quantitative test applied to a study of the bacterial flora and the disinfectant action of mechanical cleansing. The Journal of infectious diseases , 301-318.

Ranasinghe, P., Pigera, S., Premakumara, G.A., Galappaththy, P., Constantine, G.R., Katulanda, P.,2013. Medicinal properties of 'true' cinnamon (Cinnamomum zeylanicum): a systematic review. BMC complementary and alternative medicine 13, 275-6882-13-275.

Rastogi, V.K., Wallace, L., Smith, L.S.,2007. Disinfection of Acinetobacter baumannii-contaminated surfaces relevant to medical treatment facilities with ultraviolet C light. Military medicine 172, 1166-1169.

Santucci, S., Gobara, S., Santos, C., Fontana, C., Levin, A.,2003. Infections in a burn intensive care unit: experience of seven years. Journal of Hospital Infection 53, 6-13.

Schröder, J., Harder, J.,2006. Antimicrobial skin peptides and proteins. Cellular and Molecular Life Sciences CMLS 63, 469-486.

Scott, P., Deye, G., Srinivasan, A., Murray, C., Moran, K., Hulten, E., Fishbain, J., Craft, D., Riddell, S., Lindler, L., Mancuso, J., Milstrey, E., Bautista, C.T., Patel, J., Ewell, A., Hamilton, T., Gaddy, C., Tenney, M., Christopher, G., Petersen, K., Endy, T., Petruccelli, B.,2007. An outbreak of multidrug-resistant Acinetobacter baumannii-calcoaceticus complex infection in the US military health care system associated with military operations in Iraq. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 44, 1577-1584.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Tjernberg, I., Vaneechoutte, M.,1997. Distribution of Acinetobacter species on human skin: comparison of phenotypic and genotypic identification methods. Journal of clinical microbiology 35, 2819-2825.

Selçuk, C.T., Durgun, M., Özalp, B., Tekin, A., Tekin, R., Akçay, C., Alabalık, U.,2012. Comparison of the antibacterial effect of silver sulfadiazine 1%, mupirocin 2%, Acticoat and octenidine dihydrochloride in a full-thickness rat burn model contaminated with multi drug resistant Acinetobacter baumannii. Burns 38, 1204-1209.

Sharma, B.,2007. Infection in patients with severe burns: causes and prevention thereof. Infectious disease clinics of North America 21, 745-759.

Simor, A.E., Lee, M., Vearncombe, M., Jones-Paul, L., Barry, C., Gomez, M., Fish, J.S., Cartotto, R.C., Palmer, R., Louie, M.,2002. An outbreak due to multiresistant Acinetobacter baumannii in a burn unit: risk factors for acquisition and management. Infection Control 23, 261-267.

Thompson, M.G., Black, C.C., Pavlicek, R.L., Honnold, C.L., Wise, M.C., Alamneh, Y.A., Moon, J.K., Kessler, J.L., Si, Y., Williams, R., Yildirim, S., Kirkup, B.C.,Jr, Green, R.K., Hall, E.R., Palys, T.J., Zurawski, D.V.,2014. Validation of a novel murine wound model of Acinetobacter baumannii infection. Antimicrobial Agents and Chemotherapy 58, 1332-1342.

Uygur, F., Öncül, O., Evinç, R., Diktas, H., Acar, A., Ülkür, E.,2009. Effects of three different topical antibacterial dressings on Acinetobacter baumannii-contaminated full-thickness burns in rats. Burns 35, 270-273.

Werthén, M., Henriksson, L., Jensen, P.Ø., Sternberg, C., Givskov, M., Bjarnsholt, T.,2010. An in vitro model of bacterial infections in wounds and other soft tissues. Apmis 118, 156-164.

Wieczorek, P., Sacha, P., Hauschild, T., Zórawski, M., Krawczyk, M., Tryniszewska, E.,2008. Multidrug resistant Acinetobacter baumannii--the role of AdeABC (RND family) efflux pump in resistance to antibiotics. Folia Histochemica et Cytobiologica 46, 257-256.

Woollard, A., Tatham, K., Barker, S.,2007. The influence of essential oils on the process of wound healing: a review of the current evidence. Journal

Table 1. List of primers used for RT-PCR for targeting biofilm genes in *A. baumannii*

Gene	Sequence (5'→3')	Function
bfmS[a] (F)	TCGGCGGGTATTACCTTATTTAGCT	Biofilm formation response regulator
(R)	GCCTCAATCAAACGCTGAATATGGT	
csuE[b](F)	TACTGGTTTGGCCTATCC	Putative tip adhesion
(R)	CGTAAAGCTACTCATGTC	
ompA(F)	GGTATTGGAGCTGGTTTATCTTCT	Attachment to eukaryotic
(R)	GATTTTCCCGTAGTCTCTGTTGCT	
rRNA-16S(F)	TGGCTCAGATTGAACGCTGGCGGC	Endogenous control
(R)	TACCTTGTTACGACTTCACCCCA	

[a]Liou et al., 2014; [b]Dorsey et al., 2002

Fig 1. Effect of Trans-cinnamaldehyde (TC) and Eugenol (EG) on *A. baumannii* Naval-17 adhesion to and invasion of HEK001 keratinocytes. *A. baumannii* either alone or with TC/EG/ethanol (diluent) was added to HEK001 monolayer. The adhered or invaded bacterial counts were determined by broth dilution.

Bars with different superscripts differ from each other ($P < 0.05$).

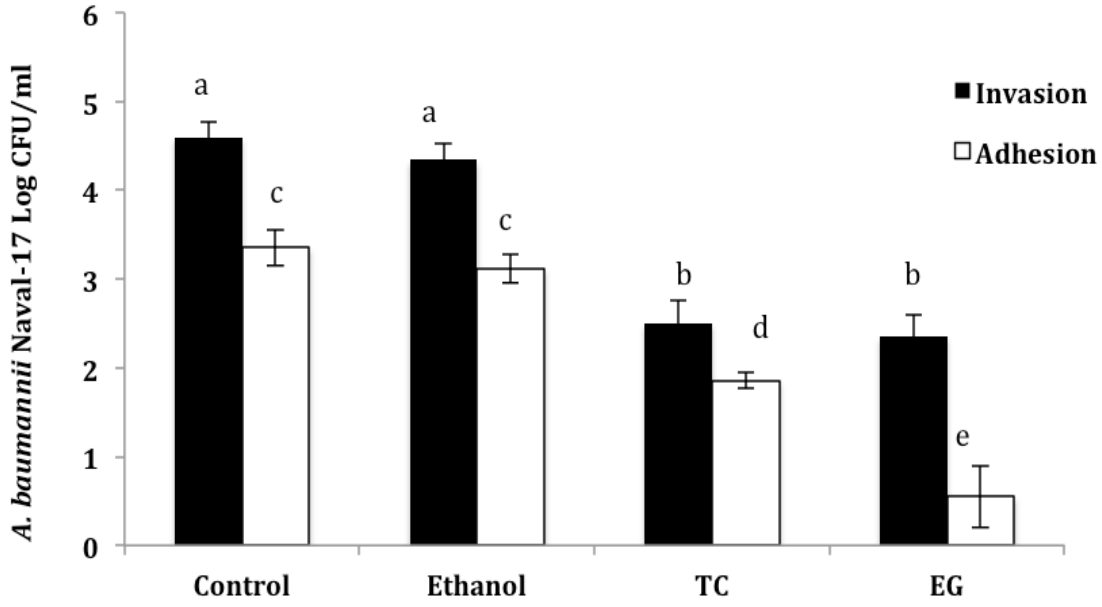


Fig 2. Effect of Trans-cinnamaldehyde (TC) and Eugenol (EG) on *A. baumannii* OIFC 109 adhesion to and invasion of HEK001 keratinocytes.

A. baumannii either alone or with TC/EG/ethanol(diluent) was added to HEK001 monolayer. The adhered or invaded bacterial counts were determined by broth dilution.

Bars with different superscripts differ from each other ($P < 0.05$).

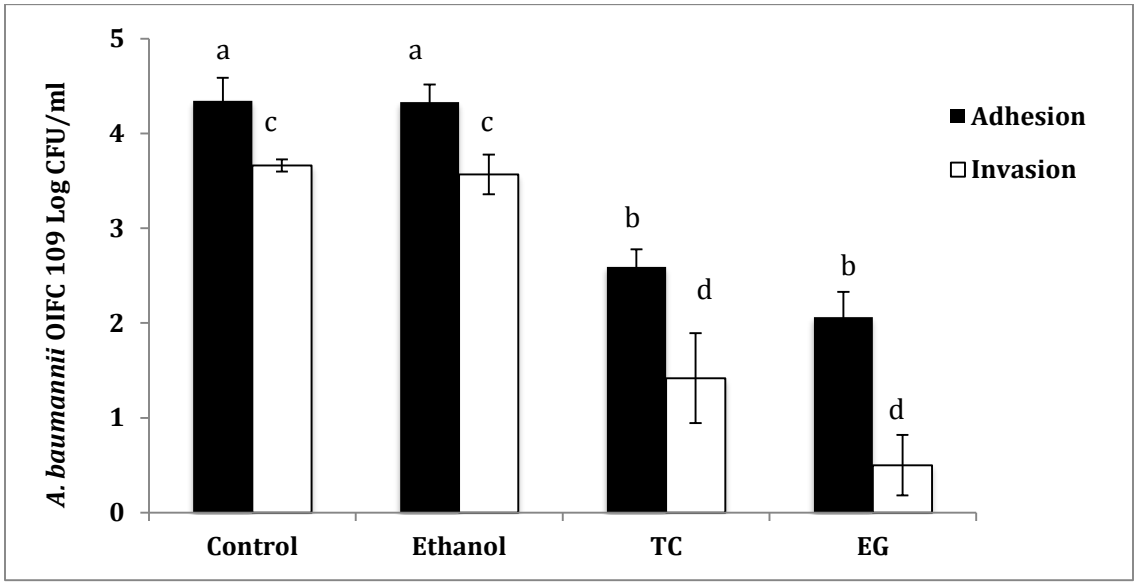


Fig 3. Effect of Trans-cinnamaldehyde (TC) and Eugenol (EG) on *A. baumannii* WC-487 adhesion to and invasion of HEK001 keratinocytes

A. baumannii either alone or with TC/EG/ethanol(diluent) was added to HEK001 monolayer. The adhered or invaded bacterial counts were determined by broth dilution.

Bars with different superscripts differ from each other ($P < 0.05$).

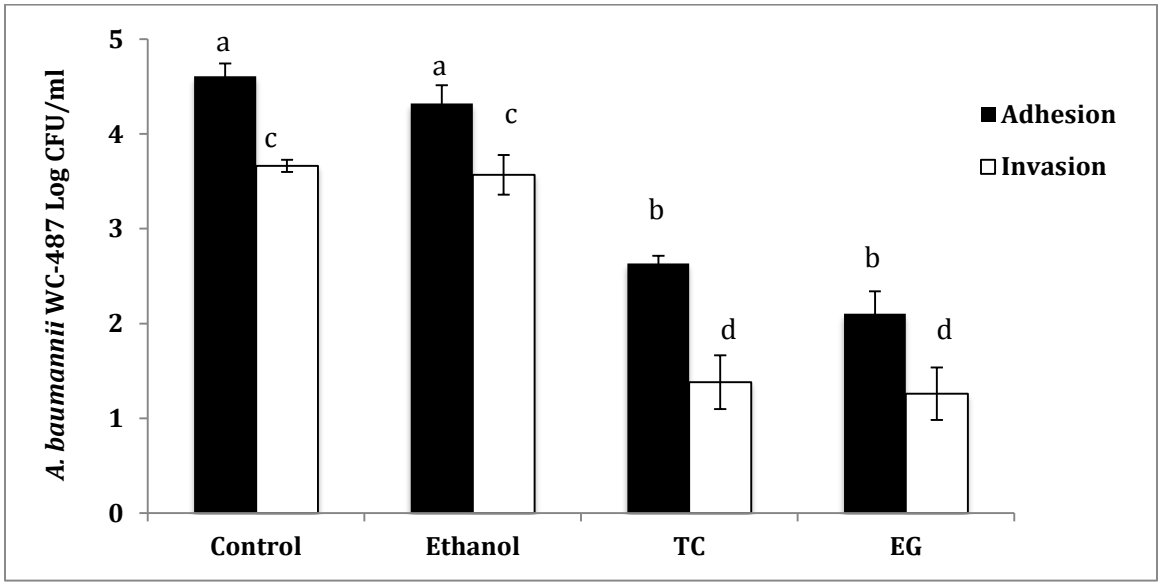


Fig 4. Effect of Trans-cinnamaldehyde (TC) and Eugenol (EG) on *A. baumannii* Naval -17 biofilm in an *in vitro* wound model. *A. baumannii* exposed to MICs of TC/EG were allowed to form a biofilm on a collagen matrix at 37^{O}C. Bacterial counts were enumerated after 24 and 48 h of incubation.Bars with different superscripts differ from each other ($P < 0.05$).

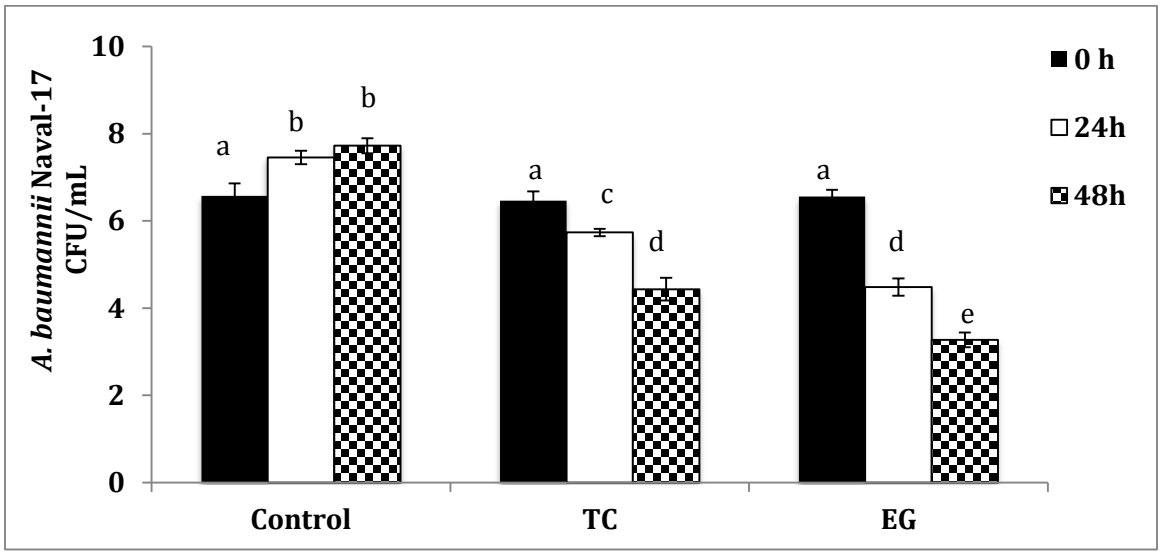


Fig 5. Effect of TC and EG on *A. baumannii* OIFC 109 biofilm in an *in vitro* wound model. *A. baumannii* exposed to MICs of TC/EG were allowed to form a biofilm on a collagen matrix at 37^{O}C. Bacterial counts were enumerated after 24 and 48 h of incubation.Bars with different superscripts differ from each other ($P < 0.05$).

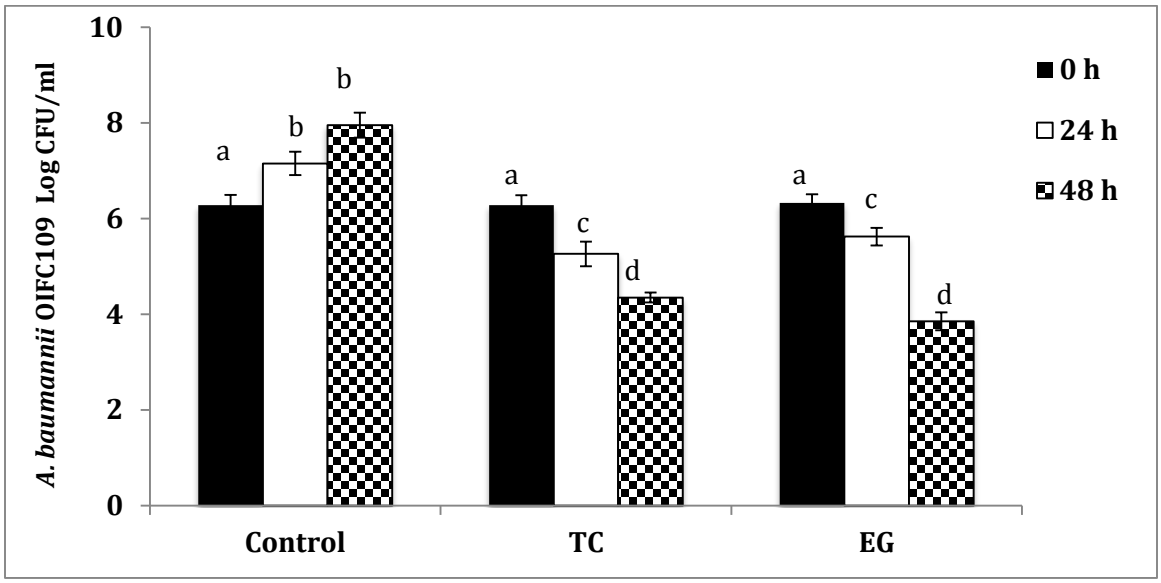


Fig 6. Effect of TC and EG on *A. baumannii* WC-487 biofilm in an *in vitro* wound model. *A. baumannii* exposed to MICs of TC/EG were allowed to form a biofilm on a collagen matrix at 37^{O}C. Bacterial counts were enumerated after 24 and 48 h of incubation.Bars with different superscripts differ from each other ($P < 0.05$).

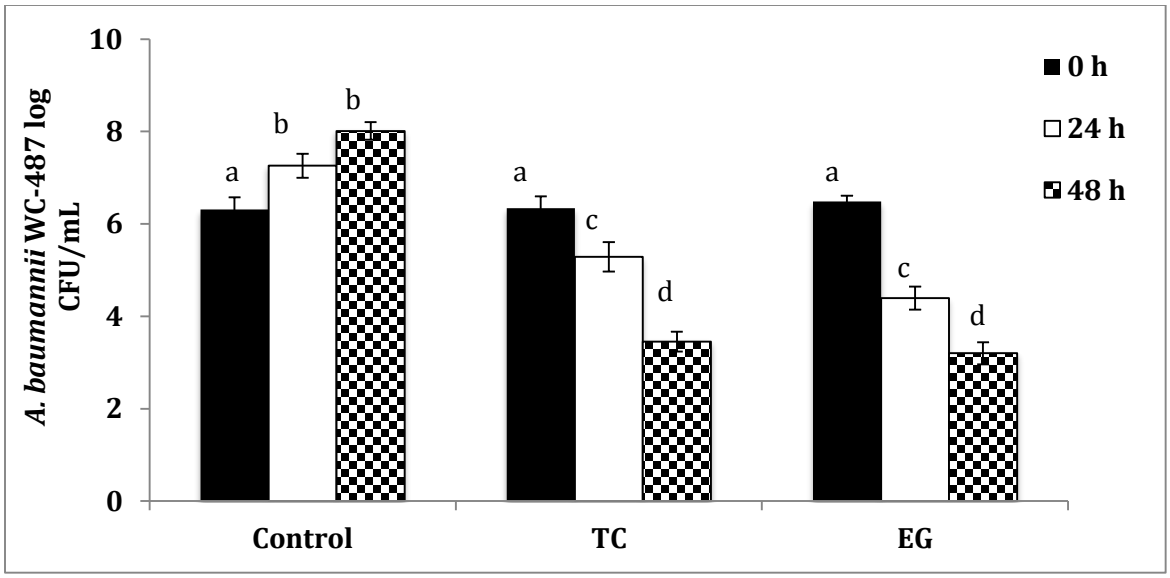


Fig 7. Scanning confocal micrographs of *Acinetobacter baumannii* Naval-17 biofilm

(A) without TC/EG treatment, (B) after treatment with the MIC of trans-cinnamaldehyde (TC; 4.0 mM), (C) after treatment with the MIC of eugenol (EG; 4.0 mM). The bacteria were first treated with TC and EG and allowed to form a biofilm at 37^{O}C for 48 h on borosilicate glass coverslip. Live/Dead staining of the biofilm was done with 2.5 μM Syto9 and 5 μM propidium iodide.

(A) (B) (C)

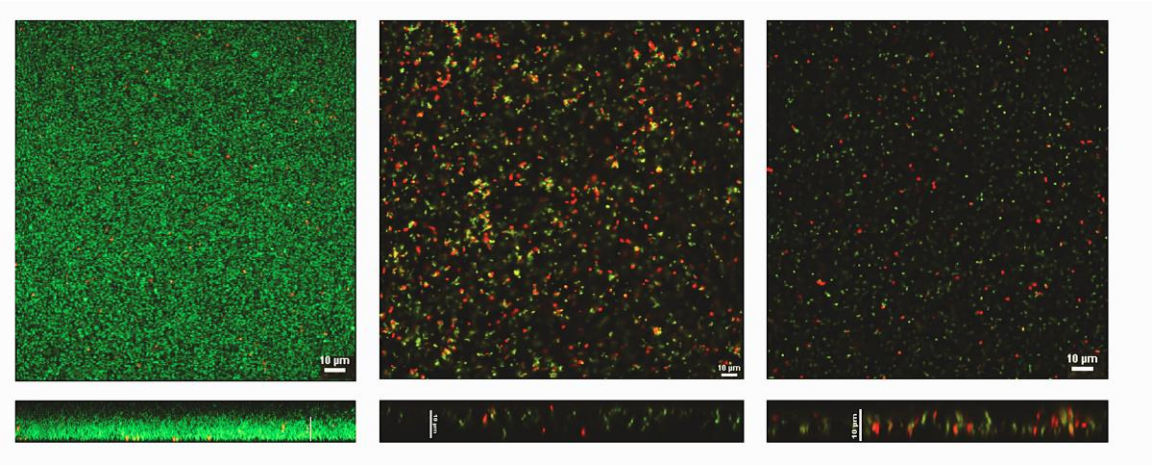


Fig 8. Effect of TC and EG on biofilm genes in *A. baumannii* Naval-17 *(P<0.05)

A. baumannii Naval-17 was exposed to SICs of TC or EG and RT-qPCR was done to test the effect of PDAs on critical biofilm genes in *A. baumannii*. Bacteria not exposed to TC/EG served as control. Bars with * are significantly different from control (P <0.05).

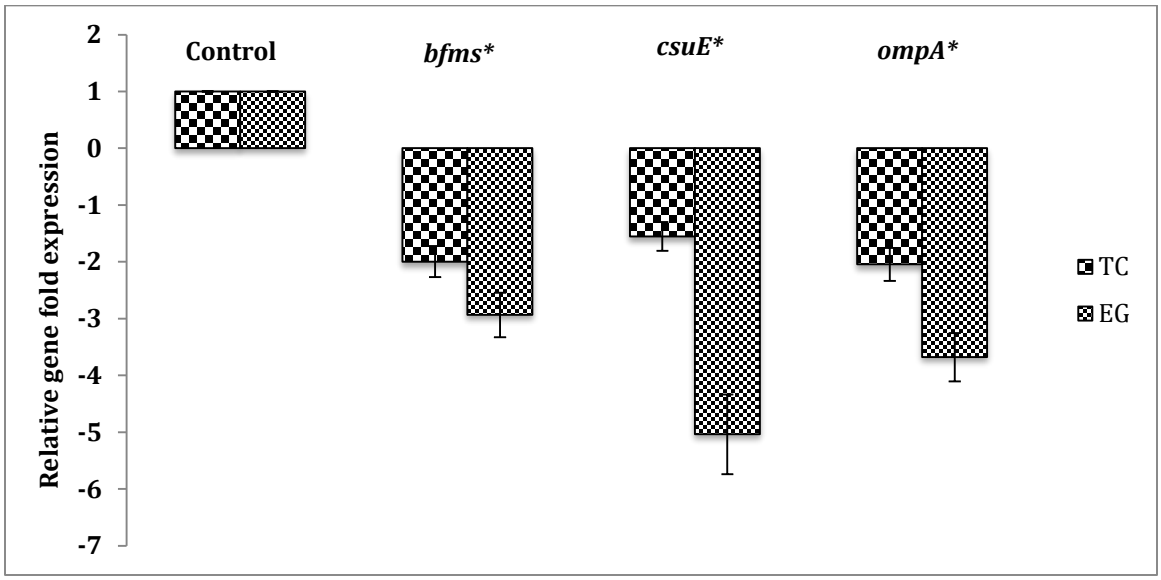
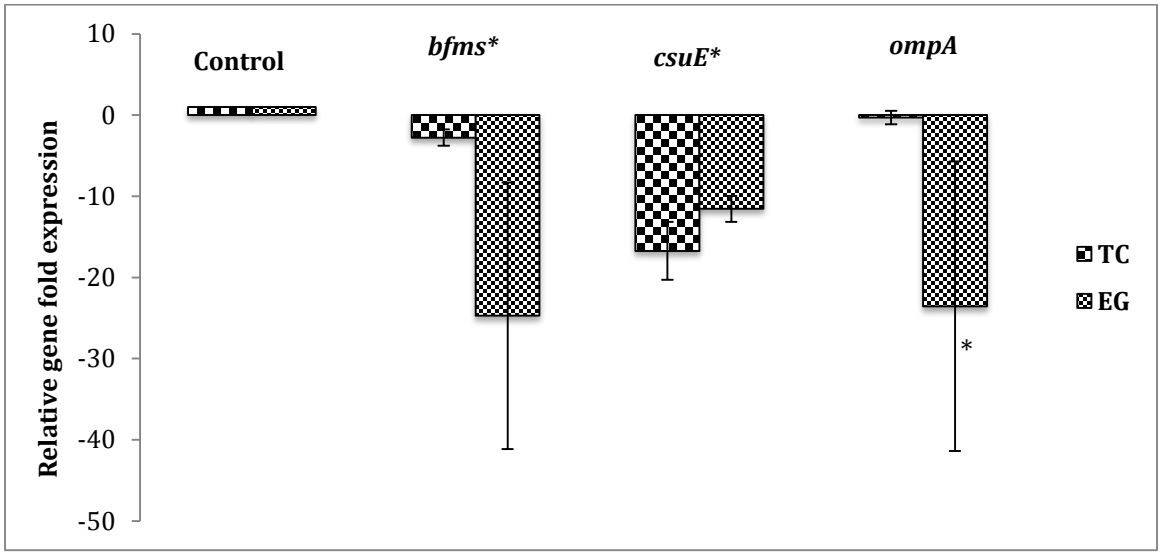


Fig 9. Effect of TC and EG on biofilm genes in *A. baumannii* OIFC 109

A.baumannii OIFC 109 was exposed to SICs of TC or EG and RT-qPCR was done to test the effect of PDAs on critical biofilm genes in *A. baumannii*. Bacteria not exposed to TC/EG served as control. Bars with * are significantly different from control (P <0.05).



Chapter VIII

SUMMARY AND CONCLUSIONS

Acinetobacter baumannii has emerged as a significant, multi-drug resistant (MDR), nosocomial pathogen causing meningitis, bacteremia, urinary tract infections and wound infections in humans. The widespread presence of this pathogen in the environment, including soil and water, potentially leads to the contamination of foods, especially fresh produce In addition, *A. baumannii* is resistant to almost all currently used antibiotics, making the treatment of infections difficult. This underscores the need for alternative strategies for controlling the pathogen.

This study investigated the prevalence of MDR *A. baumannii* on fresh vegetables, and the efficacy of chlorine as a water disinfectant in killing the bacterium. In addition, the study also investigated the potential of two plant-derived antimicrobials (PDAs), *trans*-cinnamaldehyde (TC) and eugenol (EG), for controlling antibiotic resistance and virulence in *A. baumannii*. The first objective of the study investigated the prevalence of MDR *A. baumannii* on vegetables such as carrots, lettuce and potatoes collected from farmers' markets in Connecticut. The results revealed that out of 100 samples of each vegetable tested, *A. baumannii* was recovered from only two samples of potato and one sample of lettuce. Further, no carrot sample was positive for the bacterium. However, besides *A. baumannii*, several other MDR, opportunistic human pathogens such as *Burkholderia cepacia*, *Stenotrophomonas maltophilia* and *Pseudomonas luteola*were recovered from the vegetables. *Burkholderia cepacia* isolates showed a high resistance to ceftriaxone (91%), colistin-sulphate (83%), erythromycin (79%) and streptomycin (70%); *Pseudomonas luteola* to colistin-sulphate (91%) and imipenem (83%); *S. maltophilia* to colistin-sulphate (100%), ceftriaxone (88%), cefepime (66%), erythromycin (66%) and imipenem (55%); and all isolates of *A. baumannii* were resistant to imipenem, ceftriaxone, erythromycin and streptomycin. The results indicated the presence of MDR bacteria, including *A. baumannii* on

fresh produce, thereby highlighting the potential health risk to consumers, especially those who are immune-compromised.

The second objective of this study was to test the efficacy of chlorine as a water disinfectant in killing *A. baumannii*. In addition, RT-qPCR was used to determine the effect of chlorine on the antibiotic resistance genes in *A. baumannii*. Results revealed that all the tested *A. baumannii* strains survived a chlorine concentration up to 4 pm, which typically used for water disinfection. Further, RT-qPCR analysis revealed an up-regulation of all or some of the antibiotic resistance genes in the tested *A. baumannii* isolates, indicating an induction of antibiotic resistance in the presence of chlorine. This is significant as chlorine is widely used as a sanitizer for water and fresh produce.

As routine cultural methods of screening *A. baumannii* from biological materials are cumbersome, the development of a real-time PCR (RT-qPCR) for rapid and specific detection of *A. baumannii* in water and blood was the third objective in the study. The RT-qPCR used a TaqMan primer-probe set targeting a highly conserved 102-bp DNA sequence of an efflux pump gene, *adeT* found in *A. baumannii*. All tested *A. baumannii* isolates yielded a 107-bp PCR product, while none of the negative isolates, including other closely related *Acinetobacter* species produced any amplification. The sensitivity of PCR was 3 $\log_{10}$ CFU/ml *A. baumannii* or 0.1 ng/mL of *A. baumannii* DNA. However, upon enrichment, the PCR detected 2 log and 1 log CFU/ml of *A. baumannii* in water after 6 h and 14 h of incubation at 37^{o}C, respectively.

Beta-lactam antibiotics are the most commonly used antibiotics used in the treatment of human infections. Since *A. baumannii* is resistant to most currently known antibiotics, the fourth objective of the study investigated the efficacy of TC and EG in decreasing *A. baumannii*'s resistance to seven β-lactam antibiotics. The results from broth dilution assay revealed that both

TC and EG increased the sensitivity of *A. baumannii* to all antibiotics tested ($P<0.05$). In addition, RT-qPCR data revealed that TC and EG down-regulated the expression of majority of the genes conferring resistance to beta-lactam antibiotics, especially *blaP* and *adeABC*. The results suggest that TC and EG could potentially be used along with β-lactam antibiotics for controlling MDR *A. baumannii* infections; however, their clinical significance needs to be determined using *in vivo* studies.

The fifth objective of the study was to investigate the efficacy of TC and EG in decreasing *A. baumannii* adhesion to and invasion of normal human keratinocytes, and inhibiting *A. baumannii* biofilm formation in an *in vitro* wound model. Both TC and EG significantly reduced *A. baumannii* adhesion to the skin cells by ~2-2.5 $\log_{10}$ CFU/ml and invasion of the cells by ~2-3 log CFU/m compared to controls ($P<0.05$). Further, both PDAs inhibited biofilm formation by *A. baumannii* in the wound model. These results suggest the potential use of TC and EG for treating *A. baumannii* wound infections.