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The Relationship Between Biofilm Production and Human Respiratory Cell Attachment and Invasion by Acinetobacter Baumannii

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THE RELATIONSHIP BETWEEN BIOFILM PRODUCTION AND HUMAN RESPIRATORY CELL ATTACHMENT AND INVASION BY *ACINETOBACTER BAUMANNII*

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ABSTRACT

Acinetobacter baumannii is an opportunistic gram-negative bacterial pathogen that causes many nosocomial infections in immunocompromised individuals. Common infections include catheter-associated urinary tract infections, ventilator-associated pneumonia, skin and soft tissue infections, and bloodstream infections that often lead to septicemia. Increasing multidrug resistance (MDR) in *A. baumannii* warrants new approaches to understanding its virulence mechanisms and pathogenicity. As a first step in infection, *A. baumannii* can attach to host cells, providing a surface for the bacteria to grow and perhaps facilitating biofilm formation and subsequent tissue invasion. In this study, we evaluated antibiotic resistance and characterized biofilm formation, attachment, invasion, and surface protein RNA transcription profiles of *A. baumannii* clinical isolates. Some isolates were resistant to commonly prescribed antibiotics, with six of the seventeen showing MDR. We found that 16 of the 17 strains produce biofilms in varying amounts; all strains were able to adhere to and invade A549 pulmonary cells in high levels; and some of the strains exhibited genes associated with biofilm formation, attachment, and invasion. Levels of biofilm formation, attachment to A549 cells, and invasion of A549 were not associated with the presence or absence of target genes *ompA*, *abaI*, *pga* operon, *bap*, *csuE*, or $bla_{PER\text{-}1}$. Virulence of *A. baumannii* clinical isolates increases due to their ability to produce biofilms and to attach to and invade host cells. It is important to elucidate further mechanisms of virulence in order to better treat *A. baumannii* infections and to prevent transmission and future outbreaks. We aimed to elucidate new information on the pathogenicity of *A. baumannii* that could lead to new therapeutic treatments for these infections.

INDEX WORDS: *Acinetobacter baumannii*, biofilm formation, attachment, invasion, *ompA*, *abaI*, *pga* operon, *bap*, *csuE*, bla_{PER-1}

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"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales." -Marie S. Curie

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INTRODUCTION

Acinetobacter baumannii is a gram-negative bacterium commonly found on the skin, in the soil, and on abiotic surfaces such as plastics, counters, and floors (King et al., 2013). It can normally inhabit healthy people as a commensal. In individuals with compromised immune systems, however, *A. baumannii* can cause severe infections such as urinary tract infections, pneumonia, bacteremia, bronchitis, meningitis, and skin infections (Loehfelm et al., 2007). These infections can be life-threatening and difficult to treat, especially in the elderly population.

During the Iraq war, there was an increase in *A. baumannii* infections among wounded soldiers, gaining it the nickname "Iraqibacter" (Maragakis and Perl, 2008). In 2017, the World Health Organization named *A. baumannii* at the top of its clinical priority list for the development of novel treatments. The rise of multi-drug resistant (MDR) *A. baumannii* strains necessitates new treatments and preventative measures to combat outbreaks. There are approximately 45,000 *Acinetobacter* infections in the U.S. every year and more than 1 million cases globally (Spellberg and Rex, 2013). Mortality rates due to *A. baumannii* infections have risen in recent years; twenty-three percent of hospitalized patients and 43% of patients in intensive care units infected with *A. baumannii* die from those infections (Falagas et al., 2006).

Transmission

A. baumannii has the ability to persist on environmental surfaces for long periods of time (Maragakis and Perl, 2008; McDonald et. al, 1999). In some cases, it has been found viable on surfaces months or even years after an outbreak has occurred (Fournier and Richet, 2006; Houang et al., 1998). This poses a particular problem in hospital settings because of high patient turnover

and the need to disinfect surfaces between patients to prevent transmission. Its ability to persist on abiotic surfaces makes *A. baumannii* readily transmissible from patient to patient, as well as from healthcare worker to patient. This creates situations in which outbreaks are imminent and makes current outbreaks very difficult to contain.

A. baumannii is normally transmitted to patients by contaminated hands of healthcare workers and contaminated surfaces. In some cases, however, *A. baumannii* can be transmitted through the air around infected individuals. One study found that nearly 25% of infected patients' rooms contained air contaminated with carbapenem-resistant *A. baumannii* (CRAB). All patients in the study were infected with CRAB and no contamination was found in the airducts, suggesting it originated with the patients (Munoz-Price et al., 2013; Spellberg and Bonomo, 2013).

A. baumannii is also resistant to disinfection, and treatment with ethanol has been shown to result in increased tolerance of high salinity environments and increased virulence *in vivo* as shown in *Galleria* larvae*,* a type of moth used for bacterial animal infection models (Smith et al., 2004; Nwugo et al., 2012). Ethanol is commonly used for disinfecting surfaces and has previously been thought of as a very effective disinfectant. This study with *Galleria* shows that ethanol is not necessarily as effective as it was once regarded. In some cases, like this study showed, ethanol could aid in bacterial persistence by increasing its tolerance to a wider range of environments and make disinfecting patient's rooms ineffective.

RecA is an enzyme in *A. baumannii* that repairs damaged DNA, including that caused by desiccation, high heat, or oxidative burst following phagocytosis by macrophages, thereby playing a role in resistance to killing by macrophages and resistance to desiccation (Aranda et al., 2011). These characteristics together with the increase in MDR strains make *A. baumannii* infections

difficult to treat and even more difficult to contain when outbreaks occur, often within intensive care units (Maragakis and Perl, 2008).

Persistence, Pathogenesis, and Virulence Mechanisms

A. baumannii has the ability to readily absorb DNA from the environment and incorporate it into its genome, a bacterial phenomenon known as transformation. Bacteria with the ability to do this are termed as competent cells. *A. baumannii* also has the ability to acquire new resistance genes from nearby bacteria by transfer of plasmids via a sex pilus. This is known as conjugation (McQueary and Actis, 2011). R-plasmids are short, circular segments of DNA that can be transferred between bacterial species through conjugation. Antimicrobial resistance genes are commonly transferred through this mechanism, creating novel antibiotic resistant strains instantly. Furthermore, colonization of a host normally involves multiple genera and species of bacteria which may provide access to genes from other MDR organisms (Burton's Microbiology for the Health Sciences Tenth Edition, 2015). Patients who have undergone extensive antibiotic treatment are at increased risk for *A. baumannii* infections due to decreased competition from other commensal bacterial species and, because *A. baumannii* persists on surfaces for an extraordinary amount of time after an outbreak, MDR strains can readily spread from patient to patient (Aranda et al., 2011).

Biofilm formation is another important virulence mechanism in *A. baumannii*. Biofilms are composed of bacterial cells which adhere to a surface and produce an extracellular polysaccharide matrix that effectively protects the bacteria from antibiotic treatment and host immune responses (King et al., 2013). These biofilms also contribute to antibiotic resistance and immune evasion by shielding the bacteria from treatment (King et al., 2013; McQueary and Actis, 2011). Patients being treated within a hospital are at elevated risk of acquiring *A. baumannii* infections for many

reasons. Indwelling devices such as urinary catheters, ventilators, and intravenous devices provide a mode of entry into the body and a surface on which pathogenic bacteria may attach and produce biofilms (Costerton et al., 1999; Donlan and Costerton, 2002).

Many patients admitted to hospitals and ICUs also have compromised immune systems compounded with the problem of preexisting diseases. Furthermore, nosocomial strains— those found in a hospital setting— are more likely to be MDR and can make hospital-acquired infections extremely difficult to treat and to contain (Costerton et al., 1999; McQueary and Actis, 2011).

An observational study examining ICU patients who had been intubated with endotracheal tubes (ETTs) monitored and tested tubes for biofilm formation. Multi-species biofilms were found on the ETTs in 71 out of 75 patients. *A. baumannii* composed 32% of the isolated biofilms, and mature biofilms were found on ETTs as soon as 24 hours after intubation (Gil-Perotin et al., 2012). They also found that biofilm formation was not associated with antibiotic treatment, length of hospital stay, immunosuppression, or selective digestive tract decontamination as a preventative treatment. Additionally, *A. baumannii,* along with *Pseudomonas aeruginosa,* persisted in 78% of patients with ventilator-associated pneumonia following broad-spectrum antibiotic treatment.

Another study performed by Wand et al. found that differences in biofilm formation between *A. baumannii* strains did not correlate with virulence in *Galleria* moth larvae but that dispersed bacteria harvested from fully formed biofilms were more virulent than bacteria grown planktonically (Wand et al., 2012). This suggests that different virulence mechanisms are acquired or expressed within a biofilm than in planktonic form. Another study by Rumbo-Feal et al. studying gene expression found 1621 genes that were overexpressed when *A. baumannii* was grown in biofilms including 55 genes expressed exclusively in biofilm-associated bacteria (2013). Many proteins associated with virulence were found to be upregulated in biofilm formation

including Csu pili proteins, efflux pump proteins, and secretion proteins (Rumbo-Feal et al., 2013). Furthermore, these studies indicate that it is not the amount of biofilm produced that affects virulence, but merely the presence of biofilm that establishes higher virulence.

Host cell adherence and subsequent invasion are important first steps in infection (Beachey, 1981). Many clinical isolates have the ability to attach to host cells but only some have the ability to invade host cells (Choi et al., 2008). Bacterial invasion of host cells provides protection from host immune factors, allowing pathogens such as *A. baumannii* to persist in the host (Choi et al., 2008). It is currently unclear, however, whether host cell attachment and invasion correlates with virulence in *A. baumannii* (Wand et al., 2012; Zimbler et al., 2013; Giannouli et al., 2013).

Immune Evasion

The host innate immune system is imperative in clearing *A. baumannii* infections. Together, three innate immune mechanisms—neutrophil killing, macrophage clearance, and killing by complement—combat and clear the pathogen, preventing spread of the infection (Bruhn et al., 2015). In strains with specialized capsules, however, some MDR strains have become resistant to complement activity (King et al., 2013; Bruhn et al., 2015). Capsules are a polysaccharide-based structure that coats the outside of some bacterial cells and protects them from immune detection. There has also been a rise in strains resistant to macrophage and neutrophil clearance and it is hypothesized that the capsule plays a large role in evasion of neutrophil and macrophage activities (Bruhn et al., 2015).

Without intact innate immune defenses, infections with *A. baumannii* can progress to sepsis, characterized by a "cytokine storm" caused by lipopolysaccharide (LPS), otherwise known as endotoxin (Bruhn et al., 2015). In gram negative bacteria, LPS is embedded in the cell

membrane with a portion that extends into the extracellular matrix where the host immune system recognizes LPS as foreign and mounts an immune response against it. In cases of septic shock, gram negative organisms have replicated in the blood and LPS binds to toll-like receptors (TLRs) on dendritic cells and macrophages resulting in over-production of cytokines including interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α). Upregulation of these two cytokines causes massive, wide-spread inflammation that leads to hypotension, fever, diarrhea, sporadic blood clotting, and organ failure (Kuby Immunology Seventh Edition pg 135, 2018). If left untreated, septic shock can result in death (Bruhn et al., 2015; Wong et al., 2017). Recent mortality rates for patients suffering from septic shock have reached approximately 40% (Mayo Clinic, 2018).

Neutrophils are also crucial to an effective immune response. In one study, investigators found phenylalanine presence to be crucial to neutrophil recruitment to the site of *A. baumannii* infection in zebrafish. Strains that express the *paaA* gene have the ability to catabolize phenylalanine and, as phenylalanine is broken down rapidly, fewer neutrophils are recruited to the infection site. This could be an important virulence mechanism to evade host innate immune responses (Bhuiyan et al., 2016). Lastly, *A. baumannii* is particularly motile and can evade macrophage and neutrophil killing simply by "out-running" the immune cells (McConnell et al., 2013; Clemmer et al., 2011).

Genes and proteins involved in biofilm formation, adherence, and invasion

Many proteins have been implicated in biofilm formation in *Acinetobacter baumannii* including CsuA/BABCDE pilus usher-chaperone assembly system proteins, biofilm-associated protein (Bap), and outer membrane protein A (OmpA) (Pakharukova et al., 2018; Breij et al.,

2009). The Csu pilus consists of four protein subunits and has been shown to be involved in attachment to abiotic substrates (Pakharukova et al., 2018). The subunit forming the tip of pili, CsuE, creates three finger-like loops that attach to hydrophobic surfaces such as the plastics that constitute indwelling devices residing in hospitalized patients. This is important for *A. baumannii* attachment to and biofilm formation on other surfaces in hospitals as well such as plastic containers and gloves. It has been shown that deletion of CsuE led to significantly decreased biofilm formation on plastics as compared to the wildtype (Pakharukova et al., 2018). It has also been shown that the Csu pili is not involved in adherence to epithelial cells (Breji et al., 2009).

Another set of genes associated with virulence and biofilm formation is the *pgaABCD* operon which controls poly-β-1,6-Nacetylglucosamine (PNAG) synthesis. Under stressful environmental conditions, the synthesis of peptidoglycan is upregulated via increased transcription of the *pgaABCD* operon and results in a thicker cell wall for protection and desiccation resistance (Choi et al., 2009). It has also been shown to play a significant role in colony adherence to abiotic surfaces to allow for biofilm production (Bossé et al., 2010).

A homolog of Bap originally characterized in *Staphylococcus aureus* has been found in many clinical isolates of *A. baumannii* (Loehfelm et al., 2007). It is involved in intracellular cohesion within a biofilm and is imperative to forming mature biofilm. In a *bap*-deficient mutant, biofilm formation decreased and biofilms were unable to reach maturity. Loehfelm et al. screened 98 strains isolated from infected soldiers overseas and 43% of the strains were positive for *bap* (2007). Bap has also been shown to play a role in attachment to human cells. Brossard and Campagnari showed that a *bap*-deficient mutant had both decreased biofilm formation and decreased attachment to normal human bronchial epithelial and normal human neonatal

keratinocyte cells (2011). This indicates that Bap may play a role in binding to host cells and this adhesion may be related to higher biofilm production on host tissues as well as on abiotic surfaces.

Other proteins have been implicated in cell attachment and invasion as well. OmpA has been associated with attachment to and invasion of host cells (Choi et al., 2008; Choi et al., 2005). In a study performed by Gaddy et al., they found that OmpA plays a role in biofilm formation and is required for attachment to A549 cells (2009). Another protein, PER-1, has been linked to adhesion to bronchial cells and may aid in invasion of host cells (Lee et al., 2008). AbaI is an autoinducer synthase involved in quorum sensing (Niu et al., 2008). It is has also been shown to play a necessary role in biofilm formation and may play an important role in the late stages of biofilm maturation and attachment (Lui et al., 2016). Many studies have investigated adhesion and invasion of host cells using laboratory strains, but few studies have characterized clinical specimens with these genes.

Lui et al. performed a retroactive study in China in which 122 sputum samples were collected from patients with *A. baumannii* infections (2016). Of the 122 clinical isolates collected, *abaI* and *csuE* were detected in 59.8%, *ompA* was detected in 100%, and bla_{PER-1} was not detected in any isolates. This study compared four biofilm-related genes and antibiotic resistance. They did not characterize biofilm formation or investigate bacterial adhesion or invasion. There have been no such studies to date that have characterized biofilm formation, adhesion to host cells, invasion of host cells, and presence of genes related to these virulence mechanisms in a large cohort of clinical strains of *A. baumannii*.

The purpose of this study was to characterize biofilm formation, attachment to and invasion of human cells, and to investigate the presence of genes encoding surface proteins shown to be involved in these virulence mechanisms of 17 *A. baumannii* clinical isolates (Table 1). The surface

protein RNA transcription profiles included screening for presence of genes encoding CsuABABCDE, Bap, PER-1, AbaI, Pga loci, and OmpA. By assessing the presence and transcription of these genes associated with biofilm formation, adherence, and invasion, we can better understand the complex virulence seen in *A. baumannii*. Many virulence mechanisms of *A. baumannii* are still not well understood and elucidating these mechanisms could lead to discovery of new treatments for *A. baumannii* infections.

METHODS

Clinical Isolates. 17 clinical isolates of *A. baumannii* were obtained from St. Francis Hospital in Columbus, Georgia. Identification and antibiotic susceptibility testing were performed on each strain at St. Francis Hospital using a MicroScan WalkAway 96 plus System Instrument (Beckman Coulter). Each isolate was grown overnight in 10 ml of LB broth and aliquots were frozen in mid-log phase in 20% glycerol for later use. Each isolate was renamed in the order they were acquired: SF1-17. Six of the 17 isolates were resistant to three or more tested antibiotics and deemed MDR isolates (Table 1). The location that each strain was isolated from on patients and name of the clinical isolate are listed in Table 1.

Inoculum Preparation. A. baumannii was thawed from freezer stock and grown overnight on MacConkey agar at 37°C. Twenty-four hours following, 10 ml of LB broth was inoculated and incubated overnight for approximately 12-18 hours at 37°C. Absorbance measurements of inocula were read at an OD_{600} with a BioRad microplate spectrophotometer until mid-log phase was reached at an OD_{600} of 0.2-0.3. Aliquots of mid-log phase bacteria were frozen at -80°C, thawed,

and plated for CFU/ml enumeration. The quantified aliquots of *A. baumannii* isolates were used to standardize the number of bacteria used in adhesion and invasion assays. Each assay was performed in triplicate with five or three biological replicates.

Biofilm Crystal Violet Assay. Fifty µl of mid-log bacterial inoculum and 50 µl of sterile LB broth was added to the wells of a 96-well plate and incubated for 24 hours at 37°C. Following the incubation, wells were washed four times with distilled water, stained with 100 µl of 0.1% crystal violet, and incubated for 30 minutes at room temperature. Wells were again washed four times with distilled water and trapped crystal violet was eluted with 200 µl of 95% ethanol. 125µl of this eluent was transferred to a fresh plate, and the absorbance was read at 570 nm using a BioRad microplate spectrophotometer (King et al., 2013). Assays were done in triplicate with a total of five biological replicates. Absorbance measurements were analyzed using a one-way ANOVA and Tukey's test of multiple comparisons.

Tissue Culture Preparation. Cells of the human alveolar epithelial cell line A549 (ATCC CCL-185) were cultured in a T-75 culture flask at 37°C in Dulbecco's Modified Eagles Medium (DMEM) (Gibco BRL), supplemented with 2 mM L-glutamine, 1% PenStrep, and 10% fetal bovine serum (Gibco BRL), in a humidified atmosphere containing 5% CO_2. Cells were subcultured when reaching approximately 90% confluency. A549 cells used in assays were seeded at passage three and used for all assays.

Adhesion Assay. A549 cells in a T-75 flask were washed two times with Hank's Balanced Salt Solution (HBSS) (Colonetics) prewarmed to 37°C to remove drug media. Cells were then lifted

with 0.25% Trypsin-EDTA solution in HBSS (Sigma-Aldrich) and incubated at 37°C with 5% CO_2 for 20 minutes or until cells lifted. $1x10^5$ A549 cells were added to the wells of a 12-well tissue culture plate (Fisherbrand™) and allowed to adhere for 24 hours at 37°C with 5% CO_2. Cells were washed two times with HBSS and $1x10^7$ bacteria suspended in 1 ml of drug-free DMEM (dfDMEM) with 10% fetal calf serum (FCS) was added to each respective well and incubated for 30, 90, and 180 minutes at 37°C with 5% CO_2 (Brossard and Campagnari, 2011). Following incubation, cells were washed three times with HBSS and cells were lifted with 500 µl of 0.25% Trypsin-EDTA solution. 500 µl of dfDMEM was added to the 500 µl of trypsinized cells in the wells, serially diluted, and plated on MacConkey agar for colony-forming units per ml (CFU/ml) enumeration. Adhesion assays were performed in triplicate with three biological replicates. Values were analyzed using a One-way ANOVA and Tukey's test of multiple comparisons (SPSS).

Invasion Assay. $1x10^5$ A549 cells were added to the wells of a 12-well tissue culture plate (Fisherbrand™) and allowed to adhere for 24 hours at 37°C with 5% CO_2. A549 cells were washed with dfDMEM and inoculated with $1x10^7$ bacterial cells. Samples were incubated for 180 and 300 minutes at 37°C with 5% CO_2. Following incubation, the wells were treated with 300 µg/ml of Amikacin (SF1-SF14, SF16 and SF17) or 300 µg/ml of Ceftazidime (SF15) dissolved in dfDMEM and incubated for one hour to kill extracellular bacteria. Ceftazidime was used on SF15 due to resistance of the isolate to all other tested antibiotics. Wells were then washed two times with HBSS and A549 cells were lifted with 500 µl of 0.25% Trypsin-EDTA solution and incubated for 20 minutes or until cells lifted. Cells were lysed with 500 µl of 1.0% saponin solution, 500 µl of dfDMEM was added to the cells, and samples were serially diluted and plated on MacConkey agar

for CFU/ml enumeration (Brossard and Campagnari, 2011). Values were analyzed using a One-way ANOVA and Tukey's test of multiple comparisons (SPSS).

PCR Analysis. Target genes were detected using colony polymerase chain reaction (colony PCR) using Go Taq™ Master Mix (Promega™) following manufacturer protocols. In short, one colony of bacteria was added to a reaction with dNTPs, Taq DNA polymerase, forward and reverse primers, and reaction buffer. The reaction mixture was placed in a thermocycler and set with the following PCR cycling parameters. The various target gene primer sequences and product lengths used are shown in Table 2. Each amplification reaction included 2.5 ul of forward and reverse primers (10 nM concentration) and approximately one colony of the respective clinical isolate.
The PCR thermal cycling parameters for *abaI* and *csuE* were an initial denaturation at 95°C for 10 minutes; followed by 35 cycles of 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes (Lui et al., 2016). The PCR thermal cycling parameters for *ompA* and bla_{PER-1} were an initial denaturation at 95°C for 10 minutes; followed by 35 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes (Lui et al., 2016). The PCR thermal cycling parameters for *bap* were an initial denaturation at 95°C for 10 minutes; followed by 30 cycles of 95°C for 30 minute seconds, temperature gradient ranging from 50-60°C for 30 seconds per temperature, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes (Loehfelm et al., 2007). The PCR thermal cycling parameters for the *pga* locus were an initial denaturation at 95°C for 10 minutes; followed by 30 cycles of 95°C for 30 minute seconds, temperature gradient ranging from 55-65°C for 30 seconds per temperature each cycle, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes

(Choi et al., 2009). DNA bands were visualized using a Bio-Rad GelDoc Imaging System. By comparing the resulting bands and a DNA base pair marker following 1% agarose gel electrophoresis, products that showed the expected molecular weight were regarded as positive.

Reverse-transcription PCR. Primers listed in Table 2 were used for RT-PCR. Total RNA was isolated with PureLink™ RNA Mini Kit (Invitrogen™) following manufacturer protocols. RNA was treated with DNase and RT-PCR was performed on isolates positive for presence of the target genes using SuperScript™ IV One-Step RT-PCR System (Invitrogen™) following manufacturers protocol. The RT-PCR thermal cycling parameters were the same as above with an added reverse-transcription step at 55°C for 10 min before starting the initial denaturation step. cDNA of transcripts of target genes were analyzed on 1% agarose gel by gel electrophoresis. DNA bands were visualized using a Bio-Rad GelDoc Imaging System. Products that showed the expected molecular weight were regarded as positive for presence of the target gene transcript.

RESULTS

Clinical Isolates Produce Biofilm on Polystyrene

Most clinical isolates produced biofilms to some extent, with 13 strains producing low amounts of biofilm and 1 strain producing no biofilm (Figure 1). SF9 and SF17 produced significantly more biofilm than all other isolates (One-way ANOVA, $F_{16, 238}$= 58.02, P<0.0001; Figure 1). SF10 produced significantly more biofilm than SF2, SF4, SF5, SF6, SF11, SF12, SF14, SF15, and SF16 (One-way ANOVA, $F_{16, 238}$= 58.02, P<0.0001; Figure 1). All other strains showed no significant

difference in biofilm formation when compared to one another. SF16 produced such a small amount of biofilm that it was characterized as producing none.

Clinical Strains Adhere to A549 Cells

All clinical isolates were able to adhere to A549 cells in high levels (Figure 2). SF2 and SF13 adhered in significantly higher amounts than all other strains (One-way ANOVA, $F_{16, 136} = 21.34$, $P < 0.0001$; Figure 2). SF2 also exhibited higher adherence to pulmonary cells than most isolates with the exception of SF1, SF4, SF13, and SF15 (One-way ANOVA, $F_{16, 136} = 21.34$, $P < 0.0001$; Figure 2). SF1 and SF4 all exhibited higher adherence to A549 cells than 7 of the 17 isolates. SF3 exhibited significantly lower adherence than all isolates except SF5, SF6, SF7, SF11, and SF 12. SF 12 exhibited significantly lower adherence to pulmonary cells than all isolates except for SF3 and SF5 (One-way ANOVA, $F_{16, 136} = 21.34$, $P < 0.0001$; Figure 2). While all strains were able to adhere to pulmonary cells in high amounts, based on these results, we have designated SF1, SF2, SF4, SF13, and SF15 as more highly adherent than other strains and SF3 and SF12 as less adherent than other strains.

Clinical Strains Invade A549 Cells

All clinical strains were able to invade A549 pulmonary cells in high numbers (Figure 3). SF10 was significantly more invasive than 11 (SF1, SF2, SF3, SF4, SF7, SF8, SF9, SF11, SF12, SF14, SF16, SF17) of the 17 isolates (One-way ANOVA, $F_{16, 136} = 10.31$, $P < 0.0001$; Figure 3). SF3 was significantly less invasive than all other isolates except for SF1, SF12, and SF14. Based on the results, SF1, SF3, SF12, and SF14 exhibited low levels of invasion and SF5, SF6, SF10, SF13,

and SF15 exhibited high levels of invasion. All other isolates—SF2, SF4, SF7, SF8, SF9, SF11, and SF14— did not exhibit a significant pattern when compared to invasion of other isolates.

Some Clinical Isolates Encode Target Genes

Based on colony PCR and gel electrophoresis, SF1, SF2, SF13, SF15, and SF17 do not encode any of the target genes. *OmpA* was present in 7 of the 17 (41%) isolates (Figure 4 and Table 3). *AbaI* was present in 9 of the 17 (53%) isolates. *CsuE* was present in 3 (17.6%) of the isolates. *Bap* was present in 1 isolate. The *pga* locus was present in 7 (41%) of the isolates. No clinical strains exhibited presence of the $bla_{\text{PER-1}}$ gene.

Some Clinical Isolates Demonstrate Presence of Target Genes

RT-PCR was done on representative isolates to determine the presence of mRNA transcripts when grown planktonically All isolates exhibited cDNA products of target mRNA except for *ompA* in SF14 (Figure 5 and Table 4). *OmpA* was absent in SF14, which could indicate that the gene was not transcribed when grown in suspension.

DISCUSSION

Acinetobacter baumannii has risen in clinical relevance over the last two decades. *A. baumannii's* ability to resist killing by disinfectants, killing by antibiotic treatment, and killing by the immune system makes it a clinical priority in need of novel therapies and treatments. With the increase in *A. baumannii* cases in hospitals, it is imperative that we elucidate new possible virulence mechanisms that could lead to new treatments. Past research has investigated biofilm

production and attachment to pulmonary epithelial cells in lab strains of *A. baumannii*, but research has yet to be done on clinically relevant strains of *A. baumannii*. In this study, we aimed to characterize biofilm production, adhesion to human pulmonary cells, invasion of pulmonary cells, and to identify the presence of target genes associated with biofilm, attachment, and invasion in 17 clinical isolates of *A. baumannii*.

Many previous studies have demonstrated the ability of *A. baumannii* to produce biofilms on abiotic and biotic surfaces (King et al., 2013; King et al., 2014; McQueary and Actis, 2011; Costerton et al., 1999; Donlan and Costerton, 2002; Bossé et al., 2010; Loehfelm et al., 2007). Some of these studies have also suggested an association between the presence of genes like *ompA*, *bap*, bla_{PER-1}, *abaI*, *pga* operon, and *csuA/BABCDE* and increased biofilm formation. Results of our study confirm that 16 of the 17 clinical specimens obtained from infected patients were able to produce biofilms, with 3 (SF9, SF10, and SF17) of the isolates producing significantly more than the rest. Biofilms are an important virulence mechanism. They provide protection from the external environment, desiccation, antibiotics, and host defenses (McQueary and Actis, 2011). Biofilm production in 16 of our clinical isolates demonstrates the prevalence of biofilms in nosocomial strains of *A. baumannii*. The ability of these strains to produce biofilm makes subsequent infections caused by strains difficult to treat in hospitalized patients. The prevalence of plastics and metal surfaces is widespread in hospitals, providing surfaces for pathogenic strains of *A. baumannii* to attach to and produce biofilms.

As mentioned previously, the mere presence of biofilm is enough to increase virulence of clinical strains (Wand et al., 2012; Rumbo-Feal et al., 2013). In some cases, sensitivity of the strains to antibiotics is irrelevant, as the biofilm protects the bacteria from antibiotic penetration (Wand et al., 2012; Rumbo-Feal et al., 2013). This is especially relevant with our cohort of clinical

isolates since 11 of the 16 biofilm-producing strains were sensitive to most commonly prescribed antibiotics in planktonic form (Table 1). This suggests that these infections may be more difficult to treat when forming a biofilm and may be more readily transmissible from patient to patient, making outbreaks difficult to contain.

Our results further suggest the presence or absence of biofilm is not associated with any of the target genes, in contrast with previous studies (Pakharukova et al., 2018; Breij et al., 2009). Biofilms are complex and many proteins are involved in their formation. Because of the wide heterogeneity between strains of *A. baumannii*, it is a possibility that conclusions of previous studies were specific to the strains used in the studies. Furthermore, many studies testing gene-deficient mutants were performed using lab strains of *A. baumannii*. With rapid bacterial evolution and the high level of competence—gene transfer between bacterial strains and species—seen in *A. baumannii*, these lab strains may not be representative of the current population of *A. baumannii* strains seen in hospitals today.

Many lab strains of *A. baumannii* have been found to adhere to pulmonary cells, and it is widely acknowledged that attachment to host cells is the first step in infection (Beachey, 1981; Wand et al., 2012; Zimbler et al., 2013; Giannouli et al., 2013). This study demonstrates the ability of *A. baumannii* clinical strains to adhere to A549 alveolar cells *in vitro*. There does not seem to be an association, however, between the presence or absence of any of the target genes and adhesion. Isolates without any of the target genes were still able to adhere to pulmonary cells. One study found a positive correlation between biofilm production and attachment to host cells (Brossard et al., 2011) but we did not observe that. The difference in results between their study and this one could be attributed to a few key differences in their study design. Their study evaluated adhesion of one strain of *A. baumannii* (Ab307) to two different primary cell lines: normal human

bronchial epithelial cells and normal human neonatal keratinocytes. Eukaryotic cells express different receptors and surface markers depending on the cell type and bacterial strains express different proteins and adhesins that bind to cell surface markers on human cells (Kuby Immunology Seventh Edition, 2018). Our study evaluated adhesion of a larger cohort of clinical isolates to the A549 adenocarcinoma alveolar cell line. The inclusion of more clinical isolates, the genetic diversity of *A. baumannii* clinical isolates, and the use of an alternative cell line could all contribute to the divergence of our findings as compared to published findings. This study demonstrates that the bacterial proteins that bind to eukaryotic cells have yet to be fully elucidated. In the case of the Csu pili, it is specialized to bind to hydrophobic molecules like the ones found in polystyrene, a plastic. Many human cell receptors are hydrophilic so the Csu pili will not bind to them. In the same way, the target genes investigated in this study may not be the genes encoding adhesins that bind to human epithelial cells.

OmpA has been shown to be involved in invasion of host cells. All clinical strains used in this study were able to invade A549 pulmonary cells to high levels. This is a significant finding, as most other studies have shown strains that were only mildly invasive (Breji et al., 2009; Breji et al., 2010). Our study is the first to demonstrate a large cohort of clinical isolates of *A. baumannii* invading pulmonary cells to a great degree. Attachment to and invasion of host cells are often the first steps in infection. The ability to invade cells also increases the risk for systemic infection that could lead to septicemia (Bruhn et al., 2015; Wong et al., 2017). It is also worth noting that all isolates could invade host cells, regardless of the location at which the specimens were isolated. Further elucidation with other cell types is needed. The ability to invade different types of host epithelial cells without specificity to cell type could increase the virulence and transmissibility of these isolates. These strains may have the ability to translocate in an infected patient or could be

transmitted to another patient and cause a different infection (e.g. a strain causing ventilator-associated pneumonia and then causing catheter-associated urinary tract infection in a different individual).

While all strains were able to invade pulmonary cells at high levels, the level of invasion did not appear to have an association with biofilm formation, adhesion, or presence of *ompA* or other targets genes. It is worth noting that three of the 17 isolates exhibited both high levels of adhesion and high levels of invasion of host cells (SF10, SF13, SF15). Two of these strains (SF13 and SF15) were isolated from patients with catheter-associated urinary tract infections, and both of these isolates are MDR. Interestingly, two of the most adhesive and invasive isolates (SF13 and SF15) do not contain any of the target genes. This further suggests the need for the elucidation of more mechanisms of *A. baumannii* attachment to and invasion of host cells.

A. baumannii is an important pathogen in hospital settings, and novel therapeutic approaches are required to combat these infections. The increase in antibiotic resistance among bacterial species, especially in *A. baumannii*, has shifted the focus to the virulence of pathogens and the mechanisms used to evade antimicrobial killing and host immune responses. This study has shown the robustness of *A. baumannii* clinical isolates in their ability to produce different levels of biofilm, adhere to and invade pulmonary cells, and the differences in gene presence. In the future, a genome-wide assessment of gene targets could help elucidate the proteins involved in adhering to and invading host cells. It is likely that there are multiple genes involved in these processes and elucidating them could lead to the development of much-needed new treatments to combat this global epidemic.

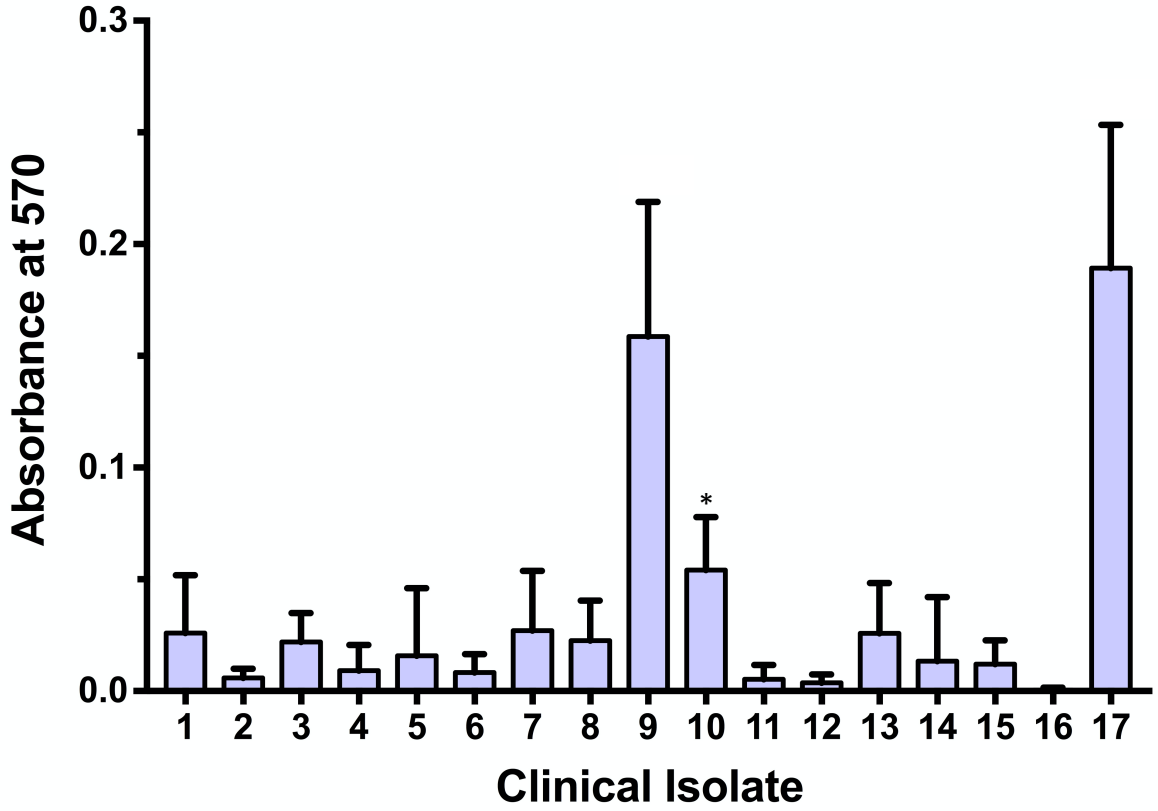

Figure 1. Average absorbance (+/- 1 SD) of crystal violet at 570nm representing biofilm formation of 17 St. Francis Clinical Strains. Isolates were grown to midlog phase, incubated in the wells of a 96-well plate overnight, stained with crystal violet and eluted with ethanol. Absorbances were read with a microtiter plate reader at a wavelength of 570nm. Absorbances were averaged and analyzed using a one-way ANOVA and Tukey's post-hoc test. Asterisks denote statistical significance. (One-way ANOVA, $F_{16, 238}$= 58.02, P<0.0001).

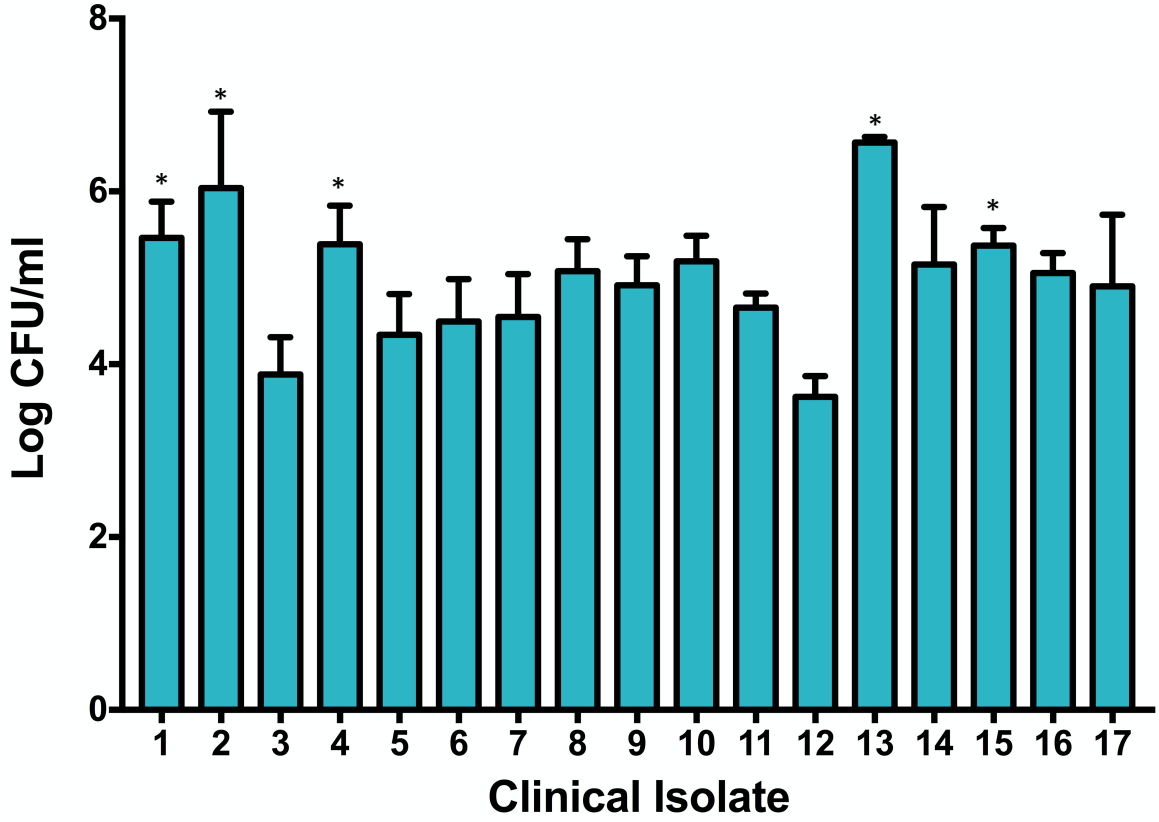

Figure 2. Average log CFU/ml (+/-1 SD) of cell-associated bacteria. A549 cells were inoculated with midlog bacteria at a MOI of 100:1 and incubated at 37°C for 30, 90, and 180 minutes. Average cell-associated log CFU/ml counts were averaged over the three time points and analyzed using a one-way ANOVA and Tukey's post-hoc test. Asterisks denote statistical significance. (One-way ANOVA, $F_{16, 136} = 21.34$, $P < 0.0001$).

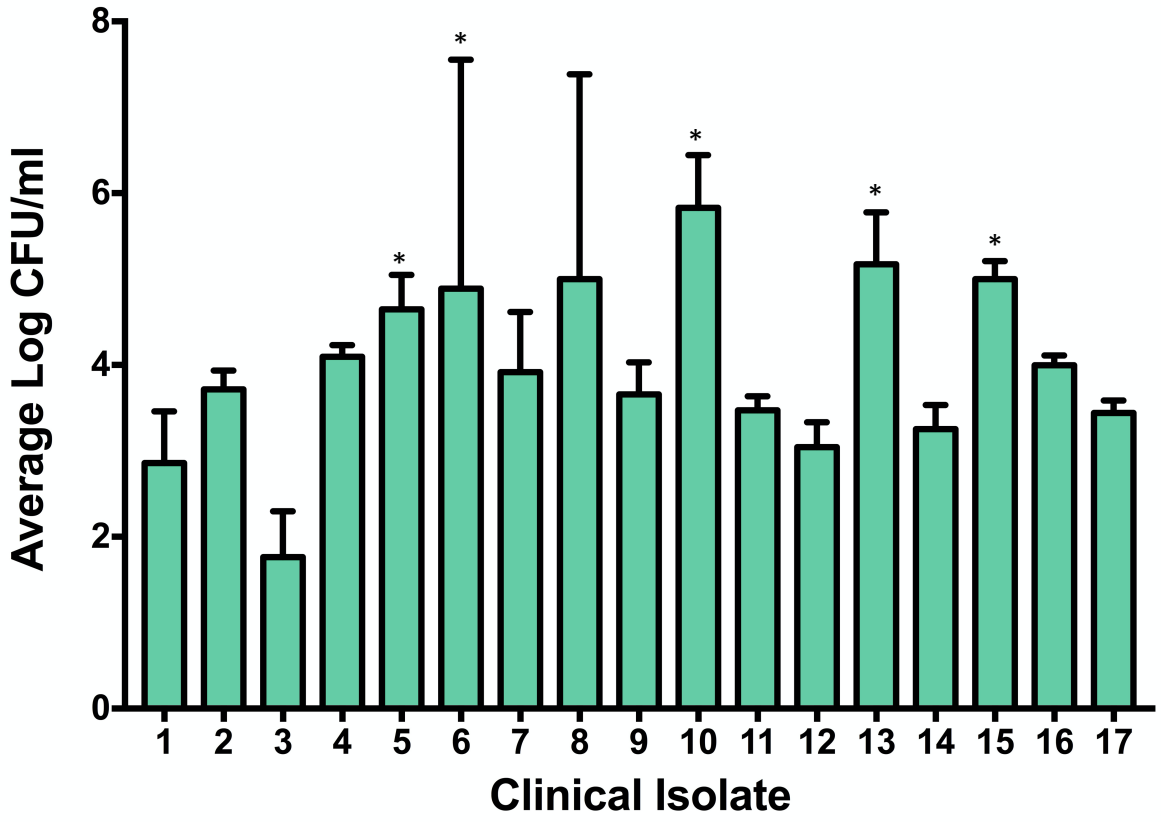

Figure 3. Average log CFU/ml (+/-1 SD) of invasive bacteria. A549 cells were inoculated with midlog bacteria at a MOI of 100:1 and incubated at 37°C for 180 and 300 minutes. Cells were treated with antibiotics and incubated for 1 hour to kill extracellular bacteria. Average intracellular log CFU/ml counts were averaged over the two time points and analyzed using a one-way ANOVA and Tukey's post-hoc test of multiple comparisons. Asterisks denote statistical significance. (One-way ANOVA, $F_{16, 136} = 10.31$, $P < 0.0001$).

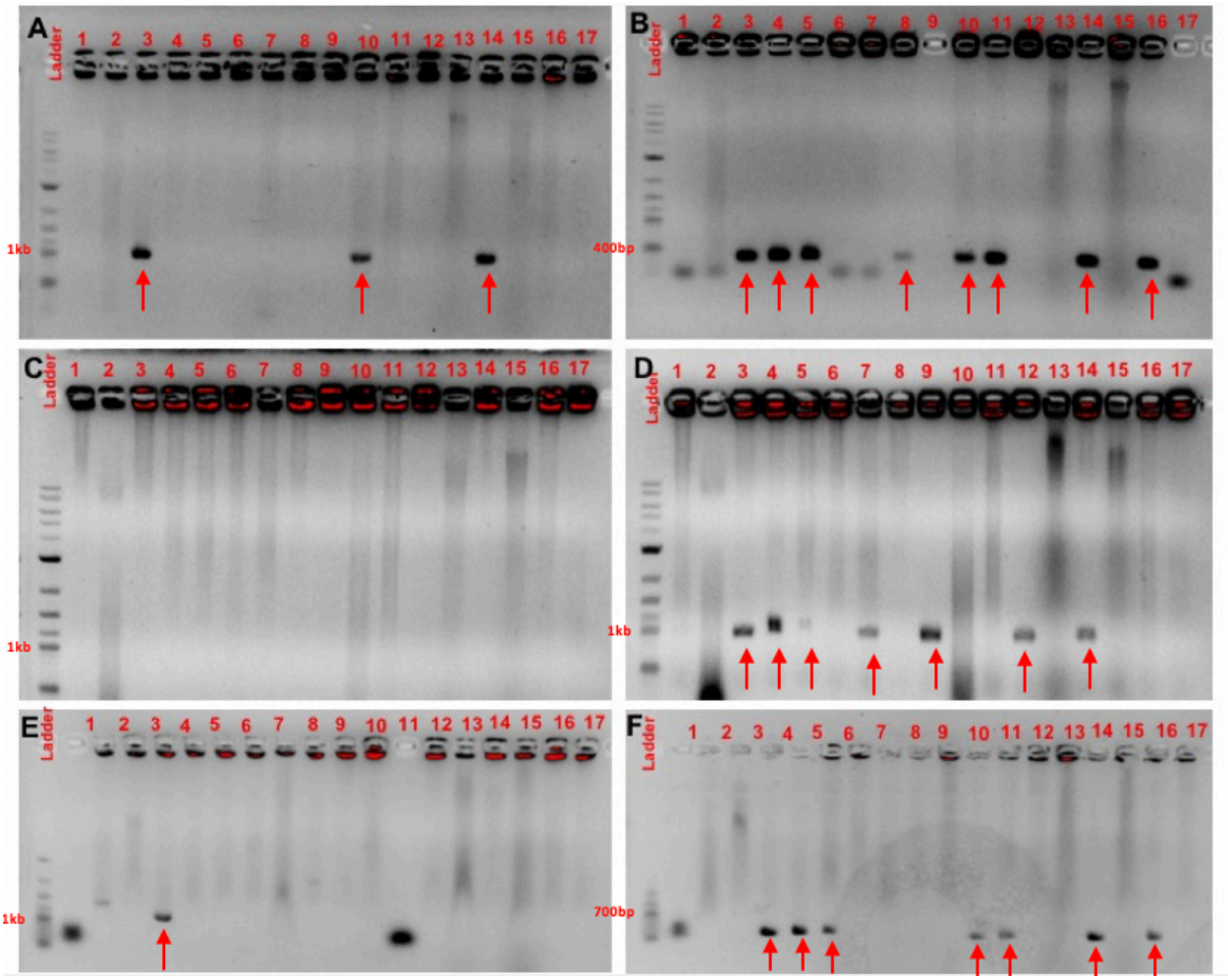

Figure 4. Colony PCR gel electrophoresis of target genes in 17 clinical isolates of *A. baumannii*: *csuE* (A), *abaI* (B), bla_{PER1} (C), *ompA* (D), *bap* (E), and *pga* operon (F). Presence of band at the correct molecular weight is considered positive.

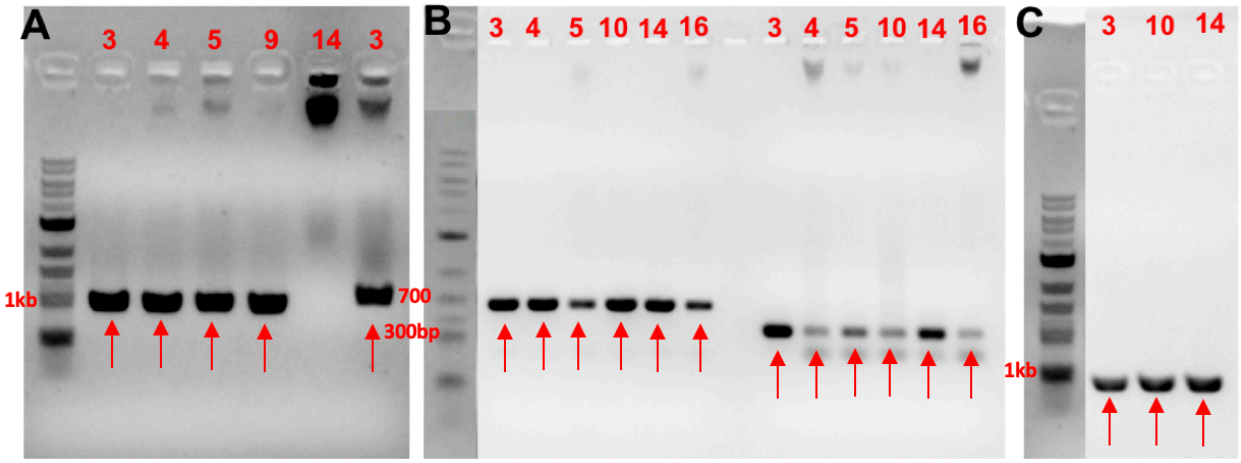

Figure 5. RT- PCR gel electrophoresis of target genes in 17 clinical strains of *A. baumannii*: *ompA* and *bap* respectively (A), *pga* operon and *abaI* respectively (B), *csuE* (C) Presence of a band at the correct molecular weight is considered positive for cDNA.

Table 1. List of clinical isolates and locations of infections collected from patients at St. Francis Hospital in Columbus, GA.

Isolate	Location of Infection
SF 1	Otitis media
SF 2	No data
SF 3	Midstream urine
SF 4*	Midstream urine
SF 5*	Bronchial wash
SF 6	Peritoneal fluid
SF 7	Blood
SF 8	Sputum
SF 9	Wound
SF 10	Wound/Ear
SF 11*	Bronchial wash
SF 12	Cath urine
SF 13*	Cath urine
SF 14	Wound
SF 15*	Cath urine
SF 16*	Wound
SF 17	Bronchial Wash

* Indicates MDR isolates.

Table 2. List of oligonucleotide primers used in PCR and RT-PCR.

Primer Name	Gene Product Target	Primer Squence	Product Length	Source
1124	5' region of *bap*	TCATACGTCTGAAAAATGGCGAG		Loehfelm et al., 2007
1125	5' region of *bap*	CATCAAGTGCTACTGTCGGCG	~1,019 bp	Loehfelm et al., 2007
csuE-1	*csuE*	ATGCATGTTCTCTGGACTGATGTTGAC		Lui et al., 2016
csuE-2	*csuE*	CGACTTGTACCGTGACCGTATCTTGATAAG	~976 bp	Lui et al., 2016
bla$_{PER-11}$*-1*	*bla-*$_{PER1}$	ATGAATGTCATTATAAAAGC		Lui et al., 2016
bla-$_{PER-1}$*-2*	*bla-*$_{PER1}$	AATTTGGGCTTAGGGCAGAA	~978 bp	Lui et al., 2016
ompA-1	*ompA*	CAATTGTTATCTCTGGAG		Lui et al., 2016
ompA-2	*ompA*	ACCTTGAGTAGACAAACGA	~966 bp	Lui et al., 2016
abaI-1	*abaI*	GTACAGTCGACGTATTTGTTGAATATTTGGG		Lui et al., 2016
abaI-2	*abaI*	CGTACGTCTAGAGTAATGAGTTGTTTTGCGCC	~382 bp	Lui et al., 2016
NdeI-F-pga	*pga* loci	GGGCCCCATATGTCGTATTTTACAAATGACA		Choi et al., 2009
BmtI-R-pga	*pga* loci	GGGCCCGCTAGCACTTGACATAATAAACAGATAATCAG	~641 bp	Choi et al., 2009

Table 3. Colony PCR-indicated presence of target genes.

Clinical Isolate	*abal*	*csue*	*ompa*	*bla*$_{PER-1}$	*bap*	*pga*
SF1						
SF2						
SF3	X	X	X		X	X
SF4	X		X			X
SF5	X		X			X
SF6	X					
SF7			X			
SF8	X					
SF9			X			
SF10	X	X				X
SF11	X					X
SF12			X			
SF13						
SF14	X	X	X			X
SF15						
SF16	X					X
SF17						

Table 4. RT-PCR-indicated presence of target genes.

Clinical Isolate	***abal***	***csue***	***ompa***	***bap***	***pga***
SF3	X	X	X	X	X
SF4	X		X		X
SF5	X		X		X
SF9			X		
SF10	X	X			X
SF12		X			
SF14	X	X			X
SF16	X				X

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