ESTABLISHING PCR FOR THE DETECTION OF PSEUDOMONAS AERUGINOSA FROM KERATITIS PATIENTS

by

Maria Elizabeth Hillenbrand

B.A. in General Biology, Washington and Jefferson College, 2008

Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Maria Elizabeth Hillenbrand

It was defended on

April 21, 2010

and approved by

Advisor and Committee Chair:
Regis P. Kowalski, MS (M)ASCP
Associate Professor
Department of Ophthalmology
School of Medicine
University of Pittsburgh

Graduate Thesis Advisor and Committee Co-Chair:
Velpandi Ayyavoo, PhD
Associate Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Committee Member:
Robert M.Q. Shanks, PhD
Associate Professor, Department of Ophthalmology
School of Medicine
University of Pittsburgh

Committee Member:
Jeremy J. Martinson, DPhil
Associate Professor, Departments of Infectious Diseases and Microbiology/Human Genetics
Graduate School of Public Health
University of Pittsburgh

Committee Member:
Robert M. Wadowsky, ScD
Director of Pediatric Molecular Microbiology
Children's Hospital of Pittsburgh
Professor, Departments of Pathology and Infectious Diseases and Microbiology
School of Medicine and Graduate School of Public Health
University of Pittsburgh

Regis P. Kowalski, M.S. (M)ASCP

ESTABLISHING PCR FOR THE DETECTION OF PSEUDOMONAS AERUGINOSA FROM KERATITIS PATIENTS

Maria Elizabeth Hillenbrand, M.S.

University of Pittsburgh, 2010

Introduction: *Pseudomonas aeruginosa* is a corneal pathogen and may cause corneal ulceration. The goal of this study was to determine the potential of PCR for detecting *P. aeruginosa* in corneal specimens from patients with keratitis.

Study Aims: 1) To establish a specific real-time PCR assay to detect *P. aeruginosa*. 2) To determine a secondary target for *P. aeruginosa* that may provide a universal target for other bacterial pathogens. 3) To validate both assays for diagnostic testing with true positive and true negative clinical samples.

Methods: 1) Analytical studies were conducted by testing *P. aeruginosa* and other bacteria isolated from patients with keratitis with a PCR assay designed to amplify the *ecfX* gene of *P. aeruginosa*. The outcome parameters were limit of detection, and amplification efficiency. 2) Similarly, *P. aeruginosa* isolates were tested for the 16S rRNA gene using the same parameters. 3) Validation of both assays was done by testing 20 cornea samples known to be positive for *P. aeruginosa* and 20 clinical samples known to be negative for *P. aeruginosa* DNA. Descriptive statistics were determined. PAGE analysis was performed to confirm the presence of amplified product.

Results: 1) Amplification efficiency of the *ecfX* assay was 96.6%, with a limit of detection of 33.6 copies of target DNA/µl. All 21 *P. aeruginosa* isolates were detected, with no detection of

the 35 non-*P. aeruginosa* isolates. 2) Amplification efficiency of the 16S rRNA assay was 103.4%, with a limit of detection of 8.12 copies /µl. All 21 *P. aeruginosa* isolates were detected. 3) The sensitivity, specificity, positive predictive value, negative predictive value, and efficiency for the *ecfX* and 16S rRNA assays were, [75%, 95%, 94%, 79%, and 85%], and [70%, 100%, 100%, 77%, and 85%], respectively. PAGE analysis supported specificity of the DNA amplified products.

Conclusions: Both real-time PCR assays used in this study detected *P. aeruginosa* DNA from keratitis patient samples. These results indicate that aside from culture, PCR may be a useful adjunct method in the diagnosis of keratitis patients.

Public H ealth R elevance: Real-time PCR can be used to detect *P. aeruginosa* from patients with keratitis to help preserve vision.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

Last, but not least, I want to thank my family for their constant support. To my mom and dad, thank you for having the confidence in me to succeed and always being there when I needed you. To my younger brothers, Brent and Clark, you two are truly my best friends. To my dogs, Sydney and Duncan, thank you for always being there for a hug after a tough semester. I love you all and feel very blessed to have you in my life.

1.0   INTRODUCTION

1.1   *PSEUDOMONAS AERUGINOSA* HISTORY

Since the mid-1800s, researchers and physicians have been observing and studying *P. aeruginosa*-related infections. *P. aeruginosa* was most likely first identified by Luke in 1862, when he found rod-shaped particles in pus-filled infections (25). Twelve years earlier, in 1850, Sédillot had made similar observations on surgical wound dressings (24). Gessard was the first person to actually isolate and culture this bacterium from infections, naming it *Bacillus pyocyaneus* in "On the Blue and Green Coloration of Bandages" (24, 25). In 1916, Freeman gave a more detailed description about the infection process of *P. aeruginosa* (24).

1.2   *PSEUDOMONAS AERUGINOSA* BIOLOGY

The bacterial species, *P. aeruginosa*, is a member of the Gamma Proteobacteria class of bacteria and falls under the bacterial family of Pseudomonadaceae (38). *P. aeruginosa* is a Gram-negative, oxidase-positive, non-fermenting, rod-shaped bacterium that lives freely in soil or water environments (38). *P. aeruginosa* is found in the form of a biofilm or as a single microorganism with a single flagellum in the environment. In culture, clinical samples normally have either a smooth or mucoid appearance growing on a blood agar plate. It grows optimally at

37°C, but it can grow at temperatures up to 42°C (38). This bacterium's ability to survive with a limited nutritional supply and ability to adapt to different physical conditions allow it to thrive in hospital and community environments (24). *P. aeruginosa* possesses two types of soluble pigments, which include a fluorescent pigment, pyoverdin and a blue pigment, pyocyanin (38). Pyoverdin gives this pathogen a fluorescent characteristic, allowing it to be identified under ultraviolet light, while pyocyanin is vital to iron metabolism (38).

Aside from being able to grow at these high temperatures, this pathogen is resistant to high concentrations of certain antibiotics, as well as salts/dyes and weak antiseptics. The high level of resistance to these antibiotics is due to the organism's structure, specifically the Gram-negative outer membrane. Some strains possess plasmids with antibiotic resistance genes that can be horizontally transferred to other bacteria, increasing the problem of multi-resistant *P. aeruginosa*. By nature, *P. aeruginosa* is an opportunistic pathogen, which means that it will infect an anatomical site only when the immune system has been weakened or the site has been compromised in some way or when it gains access to tissue or the bloodstream (38). For example, if the cornea becomes irritated from contact lens wear, this provides an opportunity for *P. aeruginosa* to infect that area.

1.3   *PSEUDOMONAS AERUGINOSA* PATHOGENESIS

The main infections that this pathogen causes include the following: urinary tract infections, bacteremia, bone and joint infections, gastrointestinal infections, and systemic infections. People at a high risk of infection are patients dealing with life-threatening burns, cancer, cystic fibrosis, or AIDS because their immune systems are not very strong at combating pathogens (38). In

addition to these types of cases, *P. aeruginosa* may cause keratitis, which can eventually result in corneal scarring and vision impairment (5). A break in the epithelial barrier provides *P. aeruginosa* with an opportunity to cause infection by way of its type 4 pili and flagellum (7). This pathogen possesses several virulence factors which play a role in the infection process. Exotoxin A blocks the protein biosynthesis and causes cell death by catalyzing ADP-ribosylation and inactivating elongation factor 2 (41). Exoenzyme S also catalyzes ADP-ribosylation like exotoxin A but instead, targets GTP-binding proteins (12). This action then leads to the breakdown of tissue in the lungs (29), which causes problems for patients suffering from cystic fibrosis. Two virulence factors that work together are phospholipase C and rhamnolipids. Rhamnolipids increase the solubility of the phospholipids of lung surfactant, which allows for phospholipase C to more effectively cause damage to the lungs (7). Alkaline protease has been studied and found to cause damage to the corneal surface of the eye, in addition to potentially acting as a colonization factor (11). In addition to these virulence factors, elastase B, protease IV, and *P. aeruginosa* small protease play an integral part to the infection process of *P. aeruginosa* (5). Figure 1 mentions several virulence factors involved in the infection process. Two different phenotypes of *P. aeruginosa* from corneal isolates exist—cytotoxic and invasive strains. The cytotoxic strains have been reported to cause corneal edema, while the invasive strains have led to corneal ulceration (22).

1.4   MICROBIAL KERATITIS

Infectious keratitis, a medical condition which can be caused by bacterial, fungal, amoeboid, or viral pathogens, refers to inflammation of the cornea, leading to damage of the epithelial tissue

of the cornea (15). If this condition is left untreated, patients can suffer long-term damage to their cornea or rarely lose their vision in only 24 hours (19); therefore, quick and effective treatment is crucial for preservation of a patient's eyesight. Symptoms associated with keratitis include redness in the eye, tenderness in the eye, altered vision, sensitivity to light, and tearing (28). Each year, it is estimated that about 500,000 people fall victim to ulcerative keratitis in the world, with about 30,000 of these cases occurring in the United States (42).

A leading risk factor of microbial keratitis involves people who wear contact lenses, with this group at a 10-fold higher risk than the rest of the population (28). Contact lens wear associated *P. aeruginosa* infections affects about 10 to 30 people per 100,000 people per year in the United States (19). Gram-negative isolates are related to 10-50% of bacterial keratitis cases in the United States (19). Patients suffering from Gram-negative infections will experience pus and mucus surrounding the eye, indicative of necrotic destruction of the corneal epithelium (10). *P. aeruginosa* is the leading Gram-negative isolate linked to bacterial keratitis cases (19).
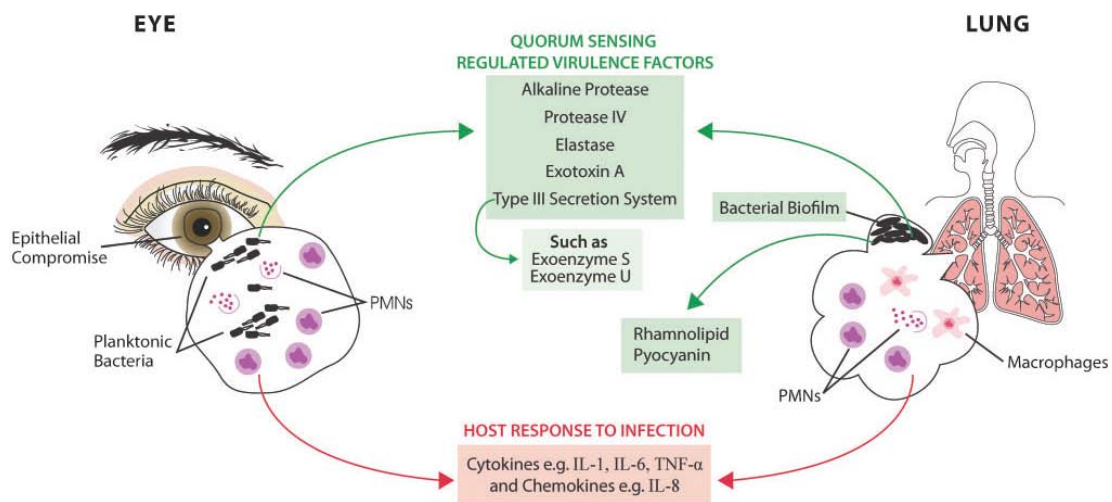
Figure 1. *P. aeruginosa* **virulence factors and corneal host response to keratitis infection (43).**

4

1.5   HOST RESPONSE TO KERATITIS

In a healthy individual, tears and blinking of the eyes protect people's eyes from bacterial infection, in addition to mucus on the tear layer (25). For those cases where opportunistic pathogens gain entry to the eye, the host reacts with an inflammatory response, which causes substantial damage while trying to combat the foreign pathogen (Figure 1). Toll-like receptors recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide which is found in Gram-negative bacteria, and inform the host to engage in phagocytosis or to activate pro-inflammatory pathways (1). Exoproducts released from host corneal cells and activated leukocytes cause the destruction of the corneal epithelial tissue (10). Polymorphonuclear leukocytes (PMN) have been studied extensively and have been shown to serve as a protective agent of the immune system in combating ocular infections. One study showed that when PMNs were selectively deleted from mice or when its expression was blocked and the mice were then exposed to *P. aeruginosa* in the eye, the mice died significantly quicker than mice with a normal functioning innate immune system (10). On the other hand, studies have shown that even though PMN helps in combating an ocular *P. aeruginosa* attack, they also cause damage to the ocular area through cytokine activity and their time of activation (10).

1.6   KERATITIS DIAGNOSIS AND TREATMENT

Accurate and quick diagnosis of keratitis is the first step to obtaining the proper treatment for this medical condition. Diagnostic tests targeting corneal pathogens are expected to have high sensitivity and specificity values, as well as simple sample collection methods, quick transport of

the specimen to a laboratory, and results in 24 to 72 hours (20). Physicians must make sure to obtain two corneal specimens from the infected patient's eye, in order for the clinical laboratories to examine one specimen for the presence of microorganisms and use the other specimen for staining, such as the Gram stain (20). In addition to antibiotic susceptibility testing, the laboratories will culture the isolated specimens and observe growth for at least a five day period (20). Clinical ophthalmic laboratories normally are able to positively identify a causative pathogen through culture in 40% to 60% of cases (17).

If physicians suspect keratitis, there are a few different tests they can perform to identify the causative agent after obtaining a past medical history from the patient. A typical eye exam can be performed on patients to test their vision, in addition to a visual acuity test to observe how well patients' eyes are capable at handling seeing from different distances (30). A slit-lamp test allows for the physician to observe the ocular surface, as well as the inner structures of the eye, with the use of a microscope (30). This type of test also utilizes fluorescein dye eyedrops, in order to more easily detect a defect on the corneal surface (40). The staining pattern of this dye on a patient's eye can help physicians determine the cause of the problem (28). Biopsies, and blood tests are two other types of tests that physicians can order to confirm a keratitis diagnosis (30). With the biopsy, a small piece of corneal tissue can be isolated from a patient with keratitis and then the cells can be viewed under a microscope, in order to identify any abnormalities. Blood tests can provide physicians with a better understanding of the immune system response to the corneal infection (30).

In terms of providing patients with proper treatment, antibiotic resistance has been an increasing problem for researchers and physicians over the past few decades. Bacteria, such as *P. aeruginosa*, have the ability to become resistant to antibiotics through the transfer of resistant

genes encoded in plasmids from one bacterium to another or through information encoded in chromosomes (24). Although antibiotic resistance is a growing concern, physicians usually treat patients who are suffering from a corneal infection with fluoroquinolones (5). Ciprofloxacin and ofloxacin, both second-generation fluoroquinolones, have been used since the 1990s to treat patients with bacterial keratitis (5).

1.7   PCR AS A DIAGNOSTIC TOOL

Culture is the gold standard when it comes to identifying the pathogen that has invaded a patient's cornea. In the past two decades, researchers have been utilizing PCR and determining different assays that may be able to produce equal or better sensitivity values than results from culture. Culture-based testing may take a few days to grow out properly on an agar plate for identification; therefore, PCR would be very helpful because results could be available in a few hours or at least in 18 to 24 hours (17). For example, a real-time PCR assay developed to identify bacterial DNA from biological fluid samples is capable of having a result in about four hours by utilizing melting-curve analysis to distinguish between different bacteria (16). Specific genes are targeted in these PCR assays, as well as different regions of the highly conserved universal 16S rRNA gene present in all bacterial species. By having species-specific genes as targets in a PCR assay, a patient sample could be screened for these genes, in order to identify if that particular pathogen is at the source of the infection. Also, universal primers would be able to determine if the infection is being caused by a bacterial source; moreover, if the PCR results are negative, then a bacterial pathogen could be ruled out and more tests could be run to determine the identity of the pathogen, whether it be viral or fungal, etc. Other advantages of

using PCR are that the materials necessary for these assays are relatively inexpensive and have a long shelf life (17). Also, since small amounts of reagents are used in each run and small amounts of sample are used, materials will last longer and samples can be saved for later testing.

Aside from hardware expense, risk of contamination is the main disadvantage of PCR. The following steps can be taken by researchers to try and prevent contamination: placing collection materials, microcentrifuge tubes, pipettes, pipette tips, reagents, and water under UV light; using a laminar-flow hood rather than a benchtop; and using separate rooms for different steps in the PCR process (17). Also, purchasing reagents and consumables that have been treated for nucleic acid contamination is another good laboratory practice to prevent contamination.

1.8   REAL-TIME PCR ASSAYS

Real-time PCR differs from conventional PCR by detecting the presence of amplified product in "real time" with the aid of a fluorescent molecule, as opposed to running amplified product on an agarose gel (4). In addition to sequence-specific primers, the TaqMan assay utilizes a specific probe, which possesses a fluorescent reporter at the 5' end and a quencher at the 3' end (Figure 2). The probe binds to the specific DNA sequence during the annealing PCR stage. Once the extension stage has been reached, the reporter is cleaved after a slight adjustment in the probe's position. Once cleaved, the reporter fluoresces; therefore, the amount of fluorescence is proportional to the amount of amplified DNA. This assay is highly specific, but a drawback is that the probe used in this assay is very expensive, making this assay more costly than other PCR assays (4).

During annealing, the TaqMan probe binds to the target sequence

Extension

During extension, the probe is partially displaced and the reporter is cleaved. The free reporter fluoresces

R   Reporter
Q   Quencher
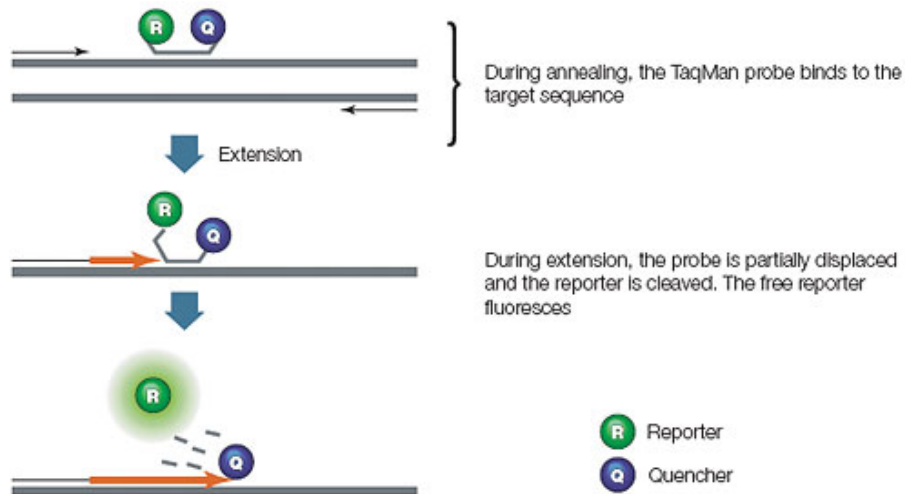
Figure 2. TaqMan assay (4).

An alternative to using a probe, the SYBR Green assay utilizes a DNA-binding dye, which binds nonspecifically to double-stranded DNA. When this dye is not bound to DNA, it does not fluoresce (Figure 3). Moreover, the fluorescence increases up to 1,000-fold once SYBR Green binds to double-stranded DNA (4). The fluorescence recorded by the real-time PCR unit is proportional to the amount of double-stranded DNA in the sample. This assay provides a melt-curve analysis option unlike the TaqMan assay, which helps to determine if nonspecific products were amplified. Unlike the TaqMan assay, SYBR Green is not as specific because the dye will bind to any double-stranded DNA present, while the probe in the TaqMan assay will only bind to a specific portion of DNA (4).
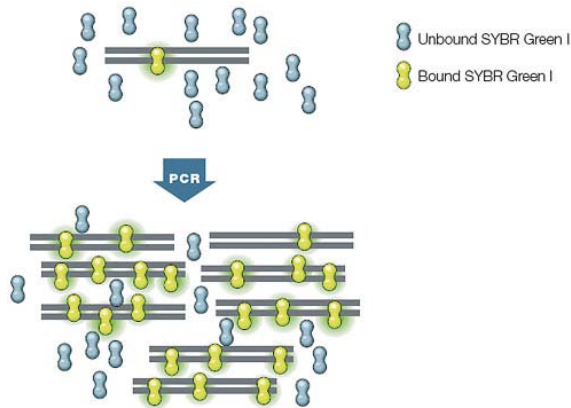
Figure 3. SYBR Green PCR assay (4).

1.9   EVALUATION OF PCR AS A DIAGNOSTIC TOOL

Five different descriptive statistics can be determined following a real-time PCR assay, which can be used for comparison against another diagnostic tool, such as culture. In this study, sensitivity refers to how well the PCR works at detecting samples that are true positive and how well the assay limits the occurrence of false negative results. Specificity values focus on the samples that should be true negatives in the assay and shows how well the PCR limits false positive results. Positive predictive value takes into account the occurrence of true positives and false positives, while negative predictive value focuses on true negative values and false negative values. The last calculation that can be used as comparison between PCR assays and diagnostic tools is efficiency, which takes into account true positives, true negatives, false positives, and false negatives, in order to determine how well the assay works overall (36).

The following shows how these calculations were mathematically determined:

Sensitivity = [True Positive / (True Positive + False Negative)] x 100%

Specificity = [True Negative / (True Negative + False Positive)] x 100%

Positive Predictive Value = [True Positive / (True Positive + False Positive)] x 100%

Negative Predictive Value = [True Negative / (True Negative + False Negative)] x 100%

Efficiency = [(True Positive + True Negative) / (True Positive + False Positive + True Negative + False Negative)]

*True Positive = of the "true positives", how many came up positive in PCR assay

*False Negative = of the "true positives", how many came up negative in PCR assay and supposed to be positive

*True Negative = of the "true negatives", how many came up negative in PCR assay

*False Positive = of the "true negatives", how many came up positive in PCR assay and supposed to be negative

2.0 GOALS AND SPECIFIC AIMS

The main goal of this project was to determine if PCR was capable of detecting *P. aeruginosa* from corneal specimens of bacterial keratitis patients. The Cepheid SmartCycler® II system (Sunnyvale, CA) was the real-time PCR system utilized in this project. Two PCR targets for two different real-time PCR assays were selected for detection of *P. aeruginosa*, one being the species-specific *ecfX* gene, and the other the bacterial 16S rRNA gene, which is highly conserved in prokaryotes. The *ecfX* gene is specific to *P. aeruginosa* and encodes an extracytoplasmic function sigma factor; moreover, this gene might act as a virulence factor or assist in haem-uptake (21). Specific primers and probe targeted at the *ecfX* gene, as well as universal primers and probe targeted at the 16S rRNA gene, were utilized in both PCR assays with the hope that they will detect *P. aeruginosa* from corneal specimens.

2.1 SPECIFIC AIM 1: TO ESTABLISH A SPECIFIC REAL-TIME PCR ASSAY TO DETECT *PSEUDOMONAS AERUGINOSA*

To establish this real-time PCR assay, the *ecfX* gene was cloned into a plasmid vector, followed by a plasmid dilution assay, in order to optimize the specific assay on the Cepheid SmartCycler® II real-time PCR system. The optimization of the assay allowed for the determination of

amplification efficiency and limit of detection. Once optimized, this real-time PCR assay was tested against *P. aeruginosa* isolates and non-*P. aeruginosa* isolates.

2.2 SPECIFIC AIM 2: TO DETERMINE A SECONDARY TARGET FOR *PSEUDOMONAS AERUGINOSA* THAT MAY PROVIDE A UNIVERSAL TARGET FOR OTHER BACTERIAL PATHOGENS

To establish this real-time PCR assay, the 16S rRNA gene was cloned into a plasmid vector, followed by a plasmid dilution assay, in order to optimize this assay on the Cepheid SmartCycler® II real-time PCR system. The optimization of the assay allowed for the determination of amplification efficiency and limit of detection. Once optimized, this real-time PCR assay was tested against *P. aeruginosa* isolates.

2.3 SPECIFIC AIM 3: TO VALIDATE BOTH ASSAYS FOR DIAGNOSTIC TESTING WITH TRUE POSITIVE AND TRUE NEGATIVE CLINICAL SAMPLES

Validation of both real-time PCR assays took place utilizing retrospective, excess de-identified ocular samples from patients with *P. aeruginosa* keratitis. Amplified PCR products were purified and subjected to restriction digestion, in order to confirm the presence of amplified product. The presence of amplified product was observed using PAGE analysis. Descriptive statistics were determined to assess these two real-time PCR assays.

3.0   MATERIALS AND METHODS

3.1   CONTAMINATION CONTROL

P. aeruginosa is a ubiquitous organism that has the ability to live in different types of environments, including the clinical laboratory; therefore, steps to prevent contamination were taken before experimentation. The laminar flow hood was sprayed down with DNA*Zap*™ (Ambion, Austin, TX) prior to handling the isolates or direct patient samples. DNA*Zap*™ consists of two different solutions, that when used together, have the ability to degrade contaminating DNA and RNA from surfaces (2). The barrels of the pipettors were also sprayed down with DNA*Zap*™ prior to handling. When not in use, the laminar flow hood and contents of the hood were exposed to UV light.

3.2   REAL-TIME PCR PRIMERS AND PROBE SETS

The primers and probe sets utilized in the real-time PCR assays are listed in Table 1. The set targeted for the 63 bp *ecfX* gene was found in the literature (3) and then ordered through Integrated DNA Technologies (IDT) (Coralville, IA). All forward and reverse primers were used at a concentration of 0.4 µM and the Taqman probe was used at a concentration of 0.2 µM.

The 16S rRNA 27F primer and probe set with a target size of 567 bp was used (32). MultAlin (6) software was used to design the forward primer F2, in order to produce a smaller target size of 144 bp and to have a forward primer that would work with the reverse primer and probe from the 27F set. The F2 forward primer was ordered from IDT. The BAK11W/BAK2 primer set was found in the literature (44) and then ordered through IDT, to provide another primer set that would amplify a 792 bp section of the 16S rRNA gene. All forward and reverse primers were used at a concentration of 0.4 µM and the Taqman probe was used at a concentration of 0.2 µM.

3.3   PLASMID DNA PREPARATION FOR OPTIMIZATION OF REAL-TIME PCR ASSAYS

In order to optimize the real-time PCR assays, a plasmid was constructed with the targeted genes of interest, the *ecfX* gene and the 16S rRNA gene. The plasmid containing the *ecfX* gene, pMQ236/PA1300, was constructed by Eric Kalivoda, from the lab of Robert Shanks, Ph.D. (34). The plasmid containing the 16S rRNA gene, pGEM®-T Easy/PA UNI, was constructed with the pGEM®-T Easy Vector System (Promega Corporation, Madison, WI), following the pGEM®-T Easy Vector System Protocol (Promega, Corporation, Madison, WI).

3.4 DETERMINATION OF AMPLIFICATION EFFICIENCY AND LIMIT OF DETECTION

Amplification efficiency refers to how well the real-time PCR unit is able to double one piece of DNA in each PCR cycle. Results from plasmid dilution assays allow for amplification efficiency of a real-time PCR assay to be calculated; therefore, the *ecfX* primer set and the F2 16S rRNA primer set from this study were used to detect the target genes in the constructed plasmids at varying dilution values. The *ecfX* specific primers and probe set directed at the *ecfX* gene were run against dilutions ranging from 10^{-3} to 10^{-9} of the pMQ236/PA1300 plasmid. The F2 primer set and probe targeted at the 16S rRNA gene were run against dilutions ranging from 10^{-3} to 10^{-9} of the pGEM®-T Easy/PA UNI plasmid. Regression analysis was performed using Minitab 10 statistical software (Minitab Inc., State College, PA) after recording the results from the plasmid dilution assays. Amplification efficiency was determined using the equation $E = 10^{(-1/slope)} - 1$, with the slope referring to the slope of the regression line, designated as the coefficient value next to 'X' in the $y = b + aX$ equation. Amplification efficiency values between 90% and 105% are considered to be in the acceptable range (4). Limit of detection values were calculated next using the following template:

Size of vector (bp)
+ Size of insert (bp)
  X (bp)

$1.0\ A_{260}$ units ds DNA $= 50\ \mu g/ml$

O.D. $= X_2$

X_2 x 50 x 80 $= X_3\ \mu g/ml$

$X_3\ \mu g/ml = Y\ ng/\mu l$

$1\ \mu g$ of 1000 bp is 9.1×10^{11} molecules

$1\ \mu g$ of X DNA is: $(9.1 \times 10^{11}) / (X^{-3}) = Z$ molecules (copy number per μg)

Per $1\ \mu l$ of DNA $= Z^{-3} \times Y\ ng/\mu l = A$ molecules

Dilution Factor: A x 1/ (lowest dilution amplified) $= B$ copies/μl

3.5 PREPARATION OF ISOLATES AND DIRECT SAMPLES PRIOR TO REAL-TIME PCR ASSAYS

3.5.1 *P. aeruginosa* Isolates and Positive Control Isolate

The specific real-time PCR assay for the *ecfX* gene and the real-time PCR assays for the 16S rRNA gene should be able to detect *P. aeruginosa* isolates. To test this we used DNA from retrospective, de-identified clinical isolates, which were part of a clinical bank. These isolates were used for validation and susceptibility monitoring, and were stored at -80°C. *P. aeruginosa* strain, ATCC 27853, was purchased from the American Type Tissue Collection and was used in both real-time PCR assays as a positive control. In addition to the ATCC control, 21 de-identified *P. aeruginosa* isolates were selected for the study.

3.5.2   Non-*P. aeruginosa* Isolates

The specific PCR assay for the *ecfX* gene found in *P. aeruginosa* should not be able to detect any non-*P. aeruginosa* isolates, since the *ecfX* gene is specific to *P. aeruginosa*. All retrospective, de-identified clinical isolates were part of a clinical bank, which is used for validation and susceptibility monitoring, and were stored at -80°C.

The following are the 35 de-identified (use for validation and susceptibility monitoring) non-*P. aeruginosa* isolates that were selected for the study: *Chlamydia trachomatis*, *Nocardia farcinica*, *Mycobacteria chelonae*, *Propionibacterium acnes*, *Bacillus* species, *Staphylococcus aureus*, *Streptococcus pneumoniae*, alpha haemolytic *streptococcus*, nutritionally variant *streptococcus*, *Enterococcus faecalis*, *Haemophilus influenzae*, *Moraxella lacunata*, *Escherichia coli*, *Serratia marcescens*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Acinetobacter baumanii*, *Stenotrophomonas maltophilia*, *Deltfia acidovorans*, *Achromobacter xylosoxidans*, *Chryseobacterium indologenes*, *Chryseobacterium meningosepticum*, *Ochrobacterium anthropi*, *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Burkholderia cepacia*. Fungal isolates, *Candida parapsilosis*, *Candida albicans*, *Aspegillus niger*, *Alternaria* species, and *Fusarium* species, were included as negative controls, in addition to free-living amoeba *Acanthamoeba* and *Hartmanella* species. Lastly, adenovirus (ADV) and herpes simplex virus (HSV) type 1 were utilized as negative controls, as well.

3.5.3   Direct Sample Collection

All retrospective, de-identified clinical samples were part of a clinical bank, which is used for validation and susceptibility monitoring, and collected as excess specimens. No additional

specimens were collected for this study. These samples, stored at -80°C, were collected for bacterial culture and/or viral testing. A sterile swab (Fisher Scientific, Houston, TX) or a kimura spatula were used to obtain corneal samples from patients, which were then added to 2.0 ml of Chlamydial Transport medium (Bartels, Bellevue, WA).

3.5.4   Preparation of De-identified Samples

A total of 40 de-identified patient samples were used in this study, following the establishment of both real-time PCR assays with *P. aeruginosa* and non-*P. aeruginosa* isolates. 20 true positives consisted of direct samples from the corneas of patients with keratitis that had *P. aeruginosa* isolated. 20 true negatives consisted of samples that did not have *P. aeruginosa* isolated; instead, these samples were spiked with these non-bacterial isolates: VZV, HSV, *Fusarium* species, *Candida albicans*, and *Aspergillus niger*. All of these samples were extracted using the EPICENTRE® extraction technique and were then run against both the specific *ecfX* primers/probe set and the F2 primers/probe set. Descriptive statistics were determined for both the *ecfX* gene PCR assay and the bacterial 16S rRNA gene PCR assay.

3.6   DNA EXTRACTION

The DNA from the *P. aeruginosa* and non-*P. aeruginosa* isolates, in addition to the de-identified, direct patient samples, had to be extracted prior to both real-time PCR assays, and different DNA extraction methods were utilized. The isolates were subjected to DNA extraction techniques in order to remove the DNA from the *P. aeruginosa* bacterial cells. Also, DNA

extraction removed fluorescein, a fluorescent dye present in the direct patient samples, which would have interfered with the fluorescent activity of the TaqMan probe in the real-time PCR assays.

3.6.1 Basic Heat Treatment Method

DNA from *P. aeruginosa* isolates was extracted using the DNA extraction protocol used in previous work (3). Bacterial colonies were grown out on blood agar plates and then a 1.0-McFarland suspension in sterile, nuclease-free water was carried out for each isolate. The suspensions were then placed in a heating block set at 100.0ºC for a 10 minute time period. Each suspension was then vortexed and centrifuged at 3000 rpm for 5 minutes. All of the suspensions were stored at -20ºC.

3.6.2 QIAamp DNA Mini Kit extraction

For the QIAamp DNA Mini Kit extraction protocol, *P. aeruginosa* colonies were selected from blood agar plates with an inoculation loop and then suspended in 180 µl of Buffer ATL. The loops were stirred several times in the buffer, in order to thoroughly suspend the bacteria colonies. 20 µl of Proteinase K was added to each suspension, followed by vortexing and incubation in a heating block at 56ºC for 3 hours. Following this lysis incubation period, the tubes were centrifuged quickly and then 200 µl of Buffer AL were added to each tube. All of the tubes were pulse-vortexed for 15 seconds and then incubated at 70ºC for 10 minutes. Following this second incubation, the tubes were centrifuged again and then 200 µl of 100% ethanol were added. All the tubes were pulse-vortexed for 15 seconds. The mixtures in the tubes were added

20

to individual QIAamp Spin Columns which each fit into a 2 ml collection tube. The columns were spun at 8000 rpm for 1 minute. The collection tubes were discarded, and the spin columns were placed into new collection tubes. Following this step, 500 µl of Buffer AW1 were added to each spin column, which was then centrifuged at 8000 rpm for 1 minute. The collection tubes were discarded, and the spin columns were placed into new collection tubes. Then 500 µl of Buffer AW2 were added to each spin column, which was then centrifuged at 13,000 rpm for 3 minutes. The collection tubes were discarded, and the spin columns were placed into new collection tubes. The tubes were then spun at 13,000 rpm for 1 minute. The collection tubes were thrown away, and the spin columns were then placed into clean 1.5 ml microcentrifuge tubes. Next, 100 µl of Buffer AE was added to each spin column twice, followed by a 5 minute incubation period at room temperature. Then the tubes were centrifuged at 8000 rpm for 1 minute. The final 200 µl extraction solutions were stored at -20ºC.

3.6.3   EPICENTRE® Extraction Technique

For the non-*P. aeruginosa* isolates, a 0.5-McFarland suspension of each isolate was carried out. Next, 300 µl were aliquoted, heated at 98ºC for 10 minutes, and put on ice for a few minutes, followed by the addition of 150 µl of MPC protein precipitation solution (EPICENTRE, Madison, WI). Each tube was vortexed for 10 seconds and then centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant from each tube was decanted into a clean tube that contained 500 µl of isopropanol (DNase, RNase, Protease free) (Acros Organics, Fisher Scientific, Pittsburgh, PA). Each new tube was inverted 30 to 40 times and then centrifuged at 10,000 rpm for 10 minutes at 4ºC. Following centrifugation, the supernatants in each tube were discarded, with 500 µl of 75% ethanol (Spectrum Chemical Mfg. Corp., Gardena, CA) being added to each

pellet. All tubes were spun and centrifuged at 10,000 rpm for 5 minutes at 4ºC. Then 500 µl of 75% ethanol was added to the pellets again and centrifuged at the same settings. The centrifugation step was followed by a 30 to 45 minute drying stage for the pellets using a vacuum system. After drying, the pellets were suspended in 35 µl of TE buffer (10mM Tris-HCL [pH 8.0], 1 mM EDTA, Epicentre, Madison, WI).

Direct patient samples were also extracted using the EPICENTRE® DNA extraction technique (Epicentre, Madison, WI) described above. The only change in the protocol that was implemented was that the final pellets were suspended in 45 µl of TE buffer rather than 35 µl, in order to utilize and store more extracted DNA from the samples.

3.7   SMARTCYCLER® II REACTION MIX

The Cepheid SmartCycler® II Real-Time PCR system (Sunnyvale, CA) was used for all real-time PCR runs, in addition to 25 µl SmartCycler® II tubes. The PCR reactions using the primers and probe targeted at the *ecfX* gene consisted of 18.1 µl of master mix and 6.9 µl of sample. The master mix used in this assay consisted of a forward primer, reverse primer, probe, and TaKaRa *Premix Ex Taq* (Otsu, Shiga, Japan). The concentration of the primers used in all of the different real-time PCR reactions was set at 0.4 µM, with probe concentration set at 0.2µM.

The PCR reactions for the 27F and F2 16S rRNA primers and probe sets consisted of 17.5 µl of master mix and 7.5 µl of sample. The master mix used in these assays consisted of a forward primer, reverse primer, probe, and TaKaRa *Premix Ex Taq*™ (Otsu, Shiga, Japan). The PCR reactions utilizing the BAK11W/BAK2 primer set consisted of 20.0 µl and 5.0 µl of sample. The master mix used in these reactions consisted of a forward primer, reverse primer,

DNAase-free water, and TaKaRa SYBR® *Premix Ex Taq*™ (Otsu, Shiga, Japan). The concentration of the primers used in all of the different real-time PCR reactions was set at 0.4 µM, with probe concentration set at 0.2µM.

3.8 SMARTCYCLER® II PCR THERMAL CYCLING

The PCR settings for the *ecfX* gene specific real-time PCR assay were set at the following parameters: Stage 1) 95.0°C for 900 seconds; Stage 2) 45 cycles at 95.0°C for 15 seconds and 60.0°C for 60 seconds.

The PCR settings for the 16S rRNA gene assays, involving the 27F and F2 primer sets, were optimized at the following parameters: Stage 1) 95.0°C for 900 seconds; Stage 2) 45 cycles at 95.0°C for 15 seconds and 60.0°C for 30 seconds. The PCR settings for the 16S rRNA gene assay involving the BAK11W/BAK2 primer set were optimized at the following parameters: Stage 1) 95.0ºC for 60 seconds; Stage 2) 40 cycles at 95.0ºC for 60 seconds, 51.0ºC for 60 seconds, and 72.0ºC for 35 seconds; and Stage 3) melt curve analysis.

3.9 AMPLIFIED PCR PRODUCT PURIFICATION

Before subjecting the amplified PCR products (from the de-identified patient samples) to restriction digestion and PAGE analysis, they first had to be purified to remove the primers and other impurities, in order to isolate the DNA. The QIAGEN QIAquick PCR Purification kit (Valencia, CA) was used to purify the amplified product from 15 true positive patient samples

from the *ecfX* gene assay and amplified product from the 14 true positive patient samples with the 16S rRNA gene assay. The protocol used can be found under the QIAquick PCR Purification Kit Protocol (using a microcentrifuge). Prior to using this kit, PCR products were transferred from the Smartcycler reaction tubes to a 1.5 ml Sarstedt tube (SARSTEDT Inc., Newton, NC). Quantities were measured for each patient sample. For each volume of PCR product isolated from the Smartcycler reaction tubes, five volumes of Buffer PB were added and mixed with each sample. The resulting solution was then transferred to a QIAquick spin column, which sat in a 2 ml collection tube. At room temperature, the spin columns were centrifuged at 13,000 rpm for one minute. The collection tubes were discarded and replaced with new 2 ml collection tubes. Then 750µl of Buffer PE were added to each column and centrifuged for one minute. The collection tubes were again discarded and replaced with new 2 ml collection tubes. The spin columns were centrifuged for another minute, in order to remove any residual ethanol. Spin columns were then removed from the collection tubes and placed into 1.5 ml microcentrifuge tubes. 30µl of DNAse free water were added to each sample and then were left in the hood for at least one minute, followed by centrifuging for one minute. The purified DNA samples were then stored at -20°C.

3.10   RESTRICTION DIGESTION OF AMPLIFIED PCR PRODUCTS

In order to confirm that the amplified PCR products (from the de-identified patient samples) in the real-time PCR assays were the expected amplified PCR products, restriction enzyme digestion was carried out. NEBcutter V2.0 (New England BioLabs, Inc. software) was used to determine the available restriction enzyme site options for the 63 bp *ecfX* gene target. The *ecfX*

gene target purified PCR products from the de-identified patient samples were cut with the restriction enzyme, *Nco*I (New England BioLabs, Inc. software), in order to obtain 44 bp and 19 bp fragments as predicted for the *ecfX* amplicon. The reaction consisted of the following reagents: 10 µl of DNA; 2 µl of Buffer 4; 7.5 µl of distilled water; and 0.5 µl of *Nco*I. Reactions were incubated overnight in a waterbath at 37°C to ensure full digestion.

NEBcutter V2.0 (New England BioLabs, Inc. software) was used to determine the available restriction enzyme site options for the 144 bp 16S rRNA gene target. The 16S rRNA gene target purified PCR products from the patient samples were cut with the restriction enzyme, *Sac*II (New England BioLabs, Inc. software), in order to obtain 97 and 47 bp fragments as predicted for the amplicon. The reaction consisted of the following reagents: 10 µl of DNA; 2 µl of Buffer 4; 7.5 µl of distilled water; and 0.5 µl of *Sac*II. Again, reactions were incubated overnight in a waterbath at 37°C to ensure full digestion.

3.11   POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

After the amplified PCR products had undergone restriction digestion overnight, they were subjected to PAGE analysis. 15% polyacrylamide gels were poured and positive control *P. aeruginosa* samples (ATCC 27853) were loaded to validate the presence of the 63 bp *ecfX* gene target, as well as the expected restriction digest patterns from the 15 positive patient samples.

A 15% and a 6% polyacrylamide gel were poured and positive control *P. aeruginosa* samples (ATCC 27853) were loaded to validate the presence of the 144 bp 16S rRNA gene target, as well as the expected restriction digest patterns. For these larger restriction digest fragments, a 6% gel was poured instead of a 15% for the de-identified patient sample amplified

PCR products, in order to allow for better visualization and separation on the gel; therefore, to analyze the 14 positive patient samples, 6% polyacrylamide gels were poured. All gels were run at 100V, ranging from 90 to 120 minutes in duration, and were stained with 10 µl of ethidium bromide prior to image collection using a Gel Logic Imaging System (Carestream Health Molecular Imaging, Woodbridge, CT).

4.0   RESULTS

4.1   AMPLIFICATION EFFICIENCY AND LIMIT OF DETECTION

In order to optimize the *ecfX* real-time PCR assay, the *ecfX* primers and probe were used against serial dilutions of the pMQ236/PA1300 plasmid construct. The lowest dilution value that was detected by the real-time PCR assay was used in the calculation of the amplification efficiency. The *ecfX* primer set detected the *ecfX* plasmid in a serial dilution ranging from 10^{-3} to 10^{-9}, during the specific *ecfX* gene PCR assay, which lasted for a 45 cycle duration (Figure 4). A sigmoidal, S-shaped curve appeared for each dilution value, which is a visual sign of an efficient real-time PCR reaction. The Ct values ranged from 18.51 to 38.67, with a cycle difference of about 3 between each measured dilution value (Table 2).

Figure 4. Plasmid dilution assay of real-time *P. aeruginosa ecfX* PCR Assay.
Optimization of the *ecfX* real-time PCR assay was determined using a plasmid dilution assay. Serial dilutions of the pMQ236/PA1300 plasmid were performed, ranging from 10^{-3} to 10^{-9}. Each dilution was performed in duplicate, in addition to the negative control (TE buffer), and were subjected to the *ecfX* PCR assay (45 cycles). The Ct values for the serial dilutions ranged from 18.51 to 38.67, with a cycle difference of about 3 between each measured dilution value. The negative control was not detected by real-time PCR.

The recorded Ct values at each dilution factor were plotted versus the set dilutions on a regression plot (Figure 5). The slope of this regression plot, a value of -3.40611, allowed for the calculation of an amplification efficiency of 0.966 or 96.6%. From this data, the limit of detection was calculated to be 33.6 copies of target DNA/µl.

Regression Plot

Y = 8.13243 - 3.40611X
R-Sq = 99.9 %

Figure 5. Real-time *P. aeruginosa ecfX* PCR Assay Regression Analysis.
Data obtained from the pMQ236/PA1300 plasmid dilution assay was used to produce a regression plot on Minitab 10 statistical software (Minitab Inc., State College, PA), with Ct values versus dilution values. The slope of the regression line was calculated to be -3.40611, with an R-square value of 99.9%. This slope value was used in the calculation of the amplification efficiency value, which was determined to be 96.6%.

In order to optimize the *ecfX* real-time PCR assay, the *ecfX* primers and probe were used against serial dilutions of the pGEM®-T Easy/PA UNI plasmid plasmid construct. The 16S rRNA primer set detected the 16S rRNA plasmid in a serial dilution ranging from 10^{-4} to 10^{-9} during the 16S rRNA gene PCR assay, which lasted for a 45 cycle duration (Figure 6). A sigmoidal, S-shaped type curve was present at each dilution value. The Ct values ranged from 18.89 to 35.69, with a cycle difference of about 3 between each measured dilution value (Table 2).
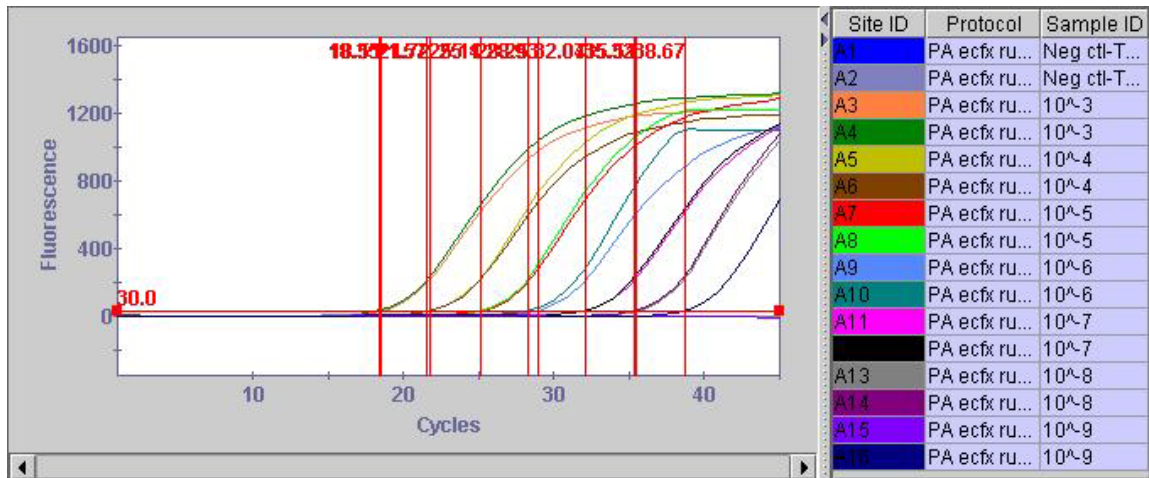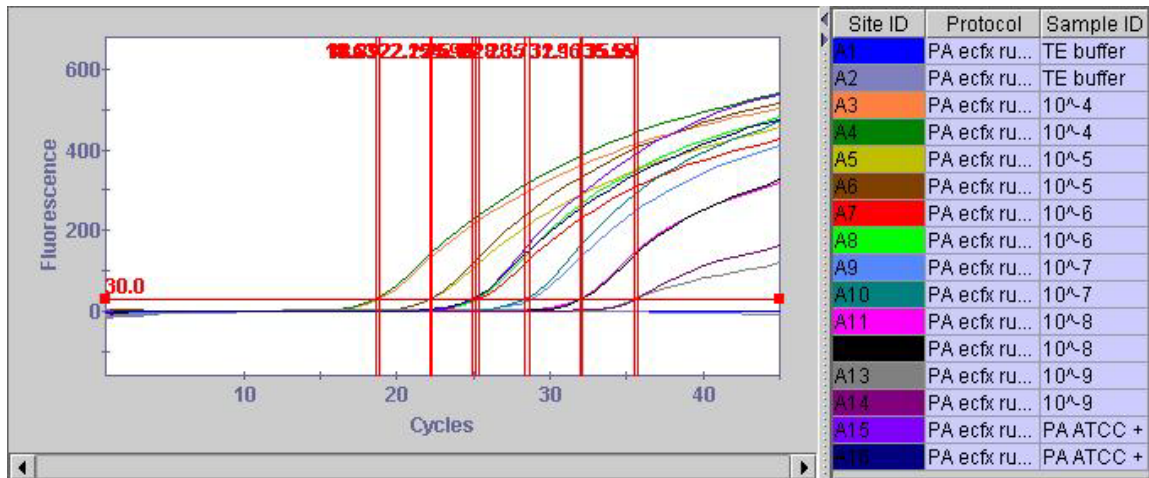
Figure 6. Plasmid dilution assay of real-time bacterial 16S rRNA PCR Assay.
Optimization of the F2 16S rRNA real-time PCR assay was determined using a plasmid dilution assay. Serial dilutions of the pGEM®-T Easy/PA UNI plasmid were performed, ranging from 10^{-4} to 10^{-9}. Each dilution was performed in duplicate, in addition to the negative control (TE buffer), and were subjected to the *ecfX* PCR assay (45 cycles). The Ct values for the serial dilutions ranged from 18.89 to 35.69, with a cycle difference of about 3 between each measured dilution value. The negative control was not detected by real-time PCR.

The recorded Ct values at each dilution factor were plotted versus the set dilutions on a regression plot (Figure 7). The slope of this regression plot, a value of -3.24314, allowed for the calculation of an amplification efficiency of 1.034, or 103.4%. From this data, the limit of detection was calculated to be 8.12 copies of target DNA/µl.
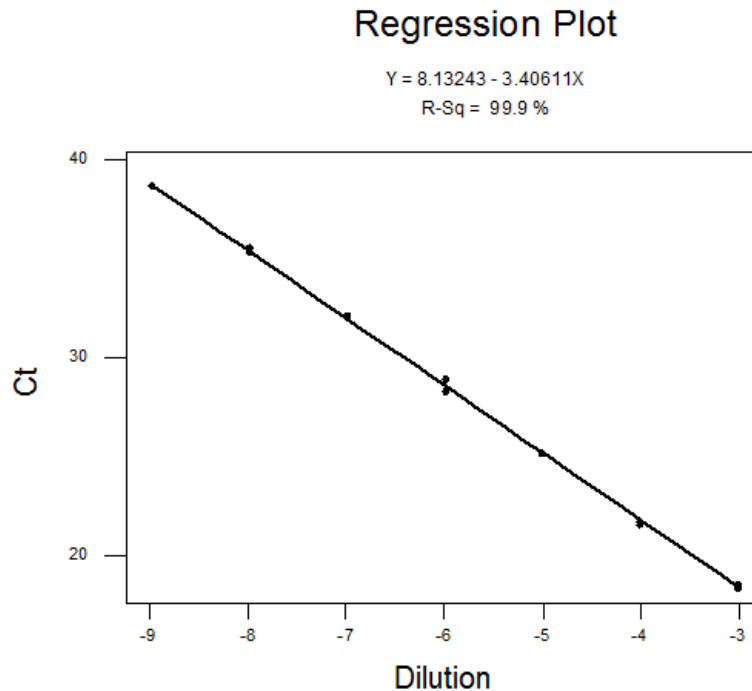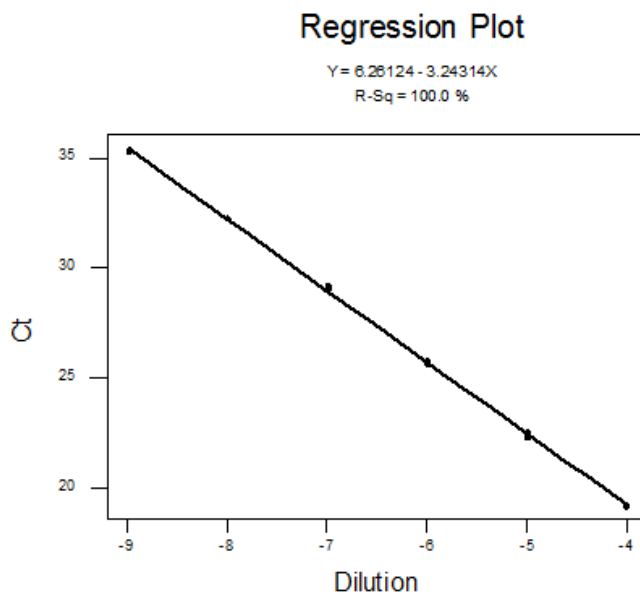
Figure 7. Real-time bacterial 16S rRNA PCR Assay Regression Analysis.
Data obtained from the pGEM®-T Easy/PA UNI plasmid dilution assay was used to produce a regression plot on Minitab 10 statistical software (Minitab Inc., State College, PA), with Ct values versus dilution values. The slope of the regression line was calculated to be -3.24314, with an R-square value of 100.0%. This slope value was used in the calculation of the amplification efficiency value, which was determined to be 103.4%.

4.2 REAL-TIME PCR ASSAYS WITH *P. AERUGINOSA* AND NON-*P. AERUGINOSA* ISOLATES

To test whether the *ecfX* primer set would detect *P. aeruginosa* and to test whether this primer set would not detect non-*P. aeruginosa* isolates, we performed the *ecfX* real-time PCR assay on the 21 *P. aeruginosa* isolates and 35 non-*P. aeruginosa* isolates. Following the DNA extraction protocols, it was found that 100% of the *P. aeruginosa* keratitis isolates (n=21) were detected with the *ecfX* real-time PCR assay, while none of the non-*P. aeruginosa* bacterial, fungal, amoeboid, and viral keratitis isolates (n=35) were detected (Figure 8).

31
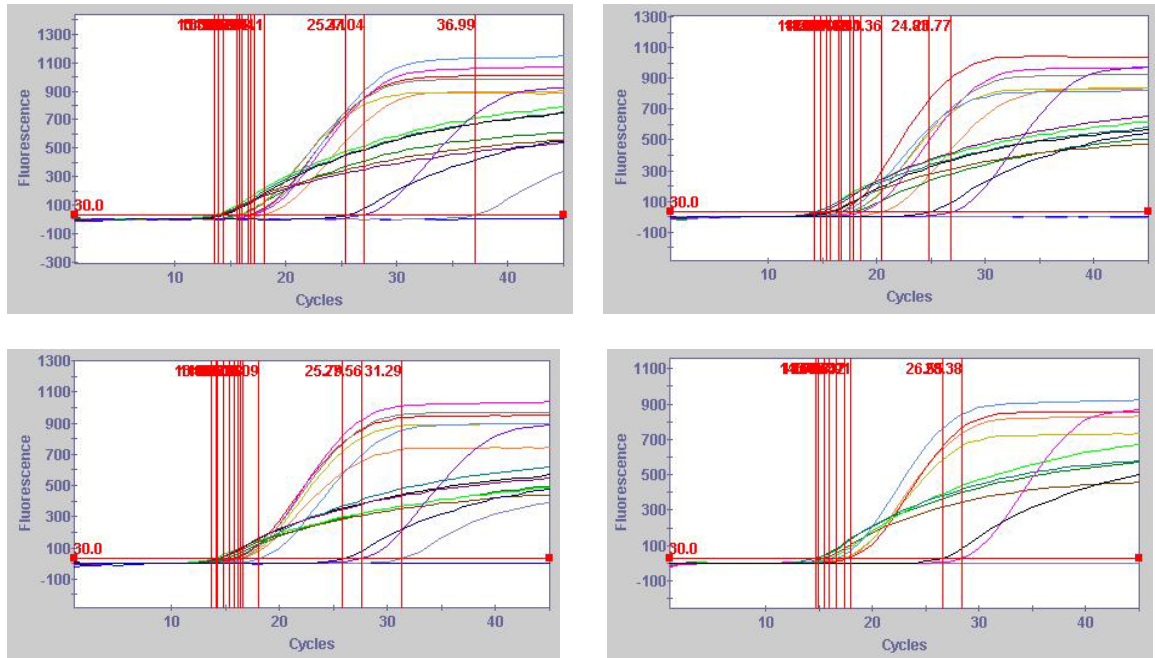
Figure 8. *P. aeruginosa* **positive isolates from** *ecfX* **and bacterial 16S rRNA real-time PCR assays.**
All 21 *P. aeruginosa* isolates were detected by both real-time PCR assays. The 35 non-*P. aeruginosa* isolates were not detected by the *ecfX* real-time PCR assay. The F2 primer set was used in the 16S rRNA real-time PCR assays. The negative controls (TE buffer) in each PCR run were not detected by the primer sets. The isolates that were detected by the assays produced similar Ct values to the positive control *P. aeruginosa* strain (ATCC 27853).

To test whether the 16S rRNA primer sets (Table 1) would detect *P. aeruginosa*, we performed the 16S rRNA real-time PCR assay on the 21 *P. aeruginosa* isolates. 100% of the *P. aeruginosa* keratitis isolates (n=21) were detected using the 27F primer set in this real-time PCR assay (32). After deriving the F2 forward primer from the MultAlin (6) software to be used with the 27F reverse primer and probe (32), 100% of the *P. aeruginosa* keratitis isolates (n=21) were detected with this real-time PCR assay (Figure 8). Twenty-one *P. aeruginosa* positive keratitis isolates were detected using the BAK11W/BAK2 primer set (44). In addition to these isolates, 22 non-*P. aeruginosa* positive keratitis isolates were detected using this assay.

4.3   REAL-TIME PCR ASSAYS OF DE-IDENTIFIED PATIENT SAMPLES

4.3.1   *P. aeruginosa ecfX* **gene PCR assay results**

To test whether the *ecfX* primer set would detect *P. aeruginosa* from direct patient samples, we performed this assay on twenty true positive and twenty true negative de-identified patient samples. Fifteen of the twenty true positive de-identified patient samples tested were detected as positive for *P. aeruginosa* in the *ecfX* specific real-time PCR assay. Each of the positive samples' real-time data showed a sigmoidal, S-shaped curve, which is an observational cue that the real-time PCR assay is working optimally (Figure 9). Ct values of these positive samples ranged from 24.89 cycles up to 44.86 cycles, out of a total of 45 cycles. This assay produced an efficiency value of 85%, sensitivity value of 75%, and specificity value of 95% (Table 4).
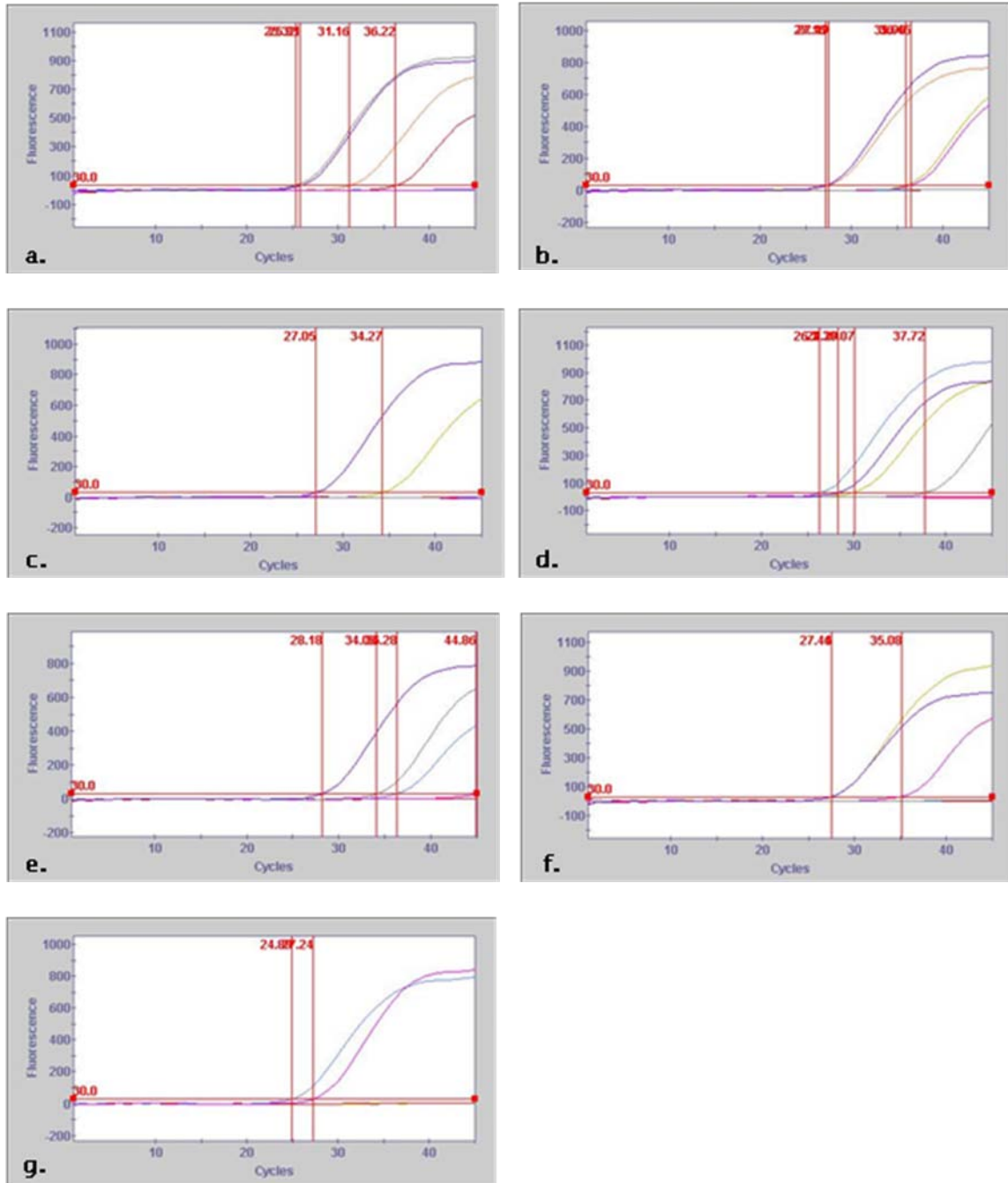
Figure 9. Keratitis de-identified patient samples *P. aeruginosa ecfX* PCR assay.
Fifteen of the twenty de-identified true positive patient samples were detected by the *ecfX* real-time PCR assay and include the following: 9893, 9516, 9742 (a.); T39, T44, T157 (b.); 9956 (c.); 8834, 9402, 9748 (d.); 9495, 9551 (e.); 9398, 9691 (f.); T273 (g.). The positive control *P. aeruginosa* strain (ATCC 27853) was also detected and depicted in each panel. The real-time data shows the sigmoidal, S-shaped curve, and Ct values for all samples ranged from 24.89 cycles up to 44.86 cycles, out of a total of 45 cycles.

4.3.2   Bacterial 16S rRNA gene PCR assay results

To test whether the F2 primer set would detect *P. aeruginosa* from direct patient samples, we performed this assay on twenty true positive and twenty true negative de-identified patient samples. Fourteen of the twenty true positive patient samples tested were detected as positive for *P. aeruginosa* in the 16S rRNA gene real-time PCR assay. Each of the positive samples' real-time data showed a sigmoidal, S-shaped curve or close resemblance to this type of curve (Figure 10). Ct values of these positive samples ranged from 25.04 cycles up to 39.41 cycles, out of a total of 45 cycles. This assay produced an efficiency value of 85%, sensitivity value of 70%, and specificity value of 100% (Table 4).
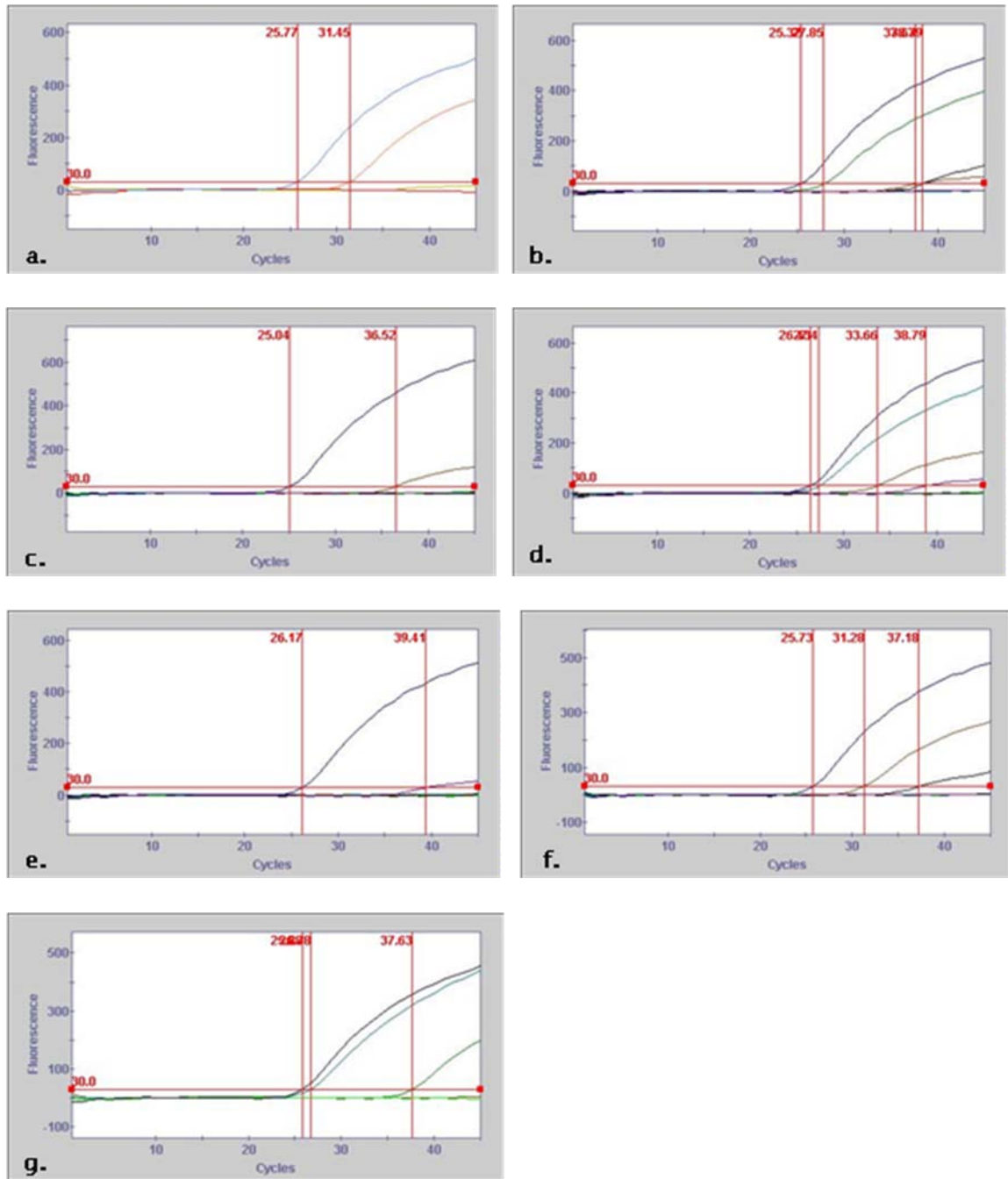
Figure 10. Keratitis de-identified patient samples 16S rRNA assay.
Fourteen of the twenty de-identified true positive patient samples were detected by the F2 16S rRNA real-time PCR assay (TaqMan) and include the following: 9893, 9742 (a.); T39, T44, T157 (b.); 9956 (c.); 8834, 9402, 9748 (d.); 9551 (e.); 9398, 9691 (f.); 9328, T273 (g.). The positive control *P. aeruginosa* strain (ATCC 27853) was also detected and depicted in each panel. The Ct values for all samples ranged from 25.04 cycles up to 39.41 cycles, out of a total of 45 cycles.

36

4.3.3 Validation of real-time PCR using PAGE analysis

To show that the amplified products from the de-identified patient samples in the *ecfX* real-time PCR assay were in fact the expected product sizes, we performed purification of these PCR products, followed by restriction digests with *Nco*I and PAGE analysis. In Figure 11, the positive control showed the expected 63 bp band on a 15% polyacrylamide gel in lanes 1, 3, and 6 for the *ecfX* real-time assay. Lanes 2, 4, and 7 showed the expected digest patterns with a 44 bp and a 19 bp band present in each lane (Figure 11). A 25 bp DNA ladder was loaded and run in lane 5 on the gel.
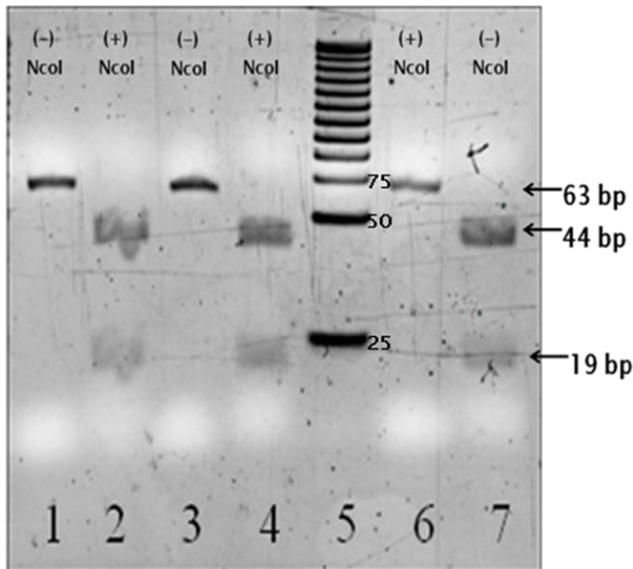
Figure 11. 15% polyacrylamide gel restriction digest of positive control *P. aeruginosa* (ATCC 27853) from *ecfX* assay.
The positive control strain of *P. aeruginosa* (ATCC 27853) was subjected to restriction digestion with the restriction enzyme *Nco*I in triplicate, after undergoing purification of the amplified product from the *ecfX* assay. The purified samples were run on a 15% polyacrylamide gel and restriction digest patterns were observed after exposure to ethidium bromide. Lanes 1, 3, and 6 showed the samples that were not cut with *Nco*I, while lanes 2, 4, and 7 showed the samples that were cut with *Nco*I. Lane 5 showed the 25 bp ladder used, with the 25, 50, and 75 bp bands marked. The uncut lanes showed the expected 63 bp fragment, while the cut lanes showed the expected 44 and 19 bp fragments.

37

In Figure 12, the fifteen *ecfX* purified PCR products from the patient samples that had come up as positive in the *ecfX* real-time PCR assay showed the presence of the expected 63 bp band, as seen in the positive control gel (Figure 11). After being cut with the *Nco*I restriction enzyme, fourteen of the fifteen samples showed the expected restriction digest patterns, with the sample in lane 7 (top gel pictured) showing no signs of the 44 bp and 19 bp fragments (Figure 12).
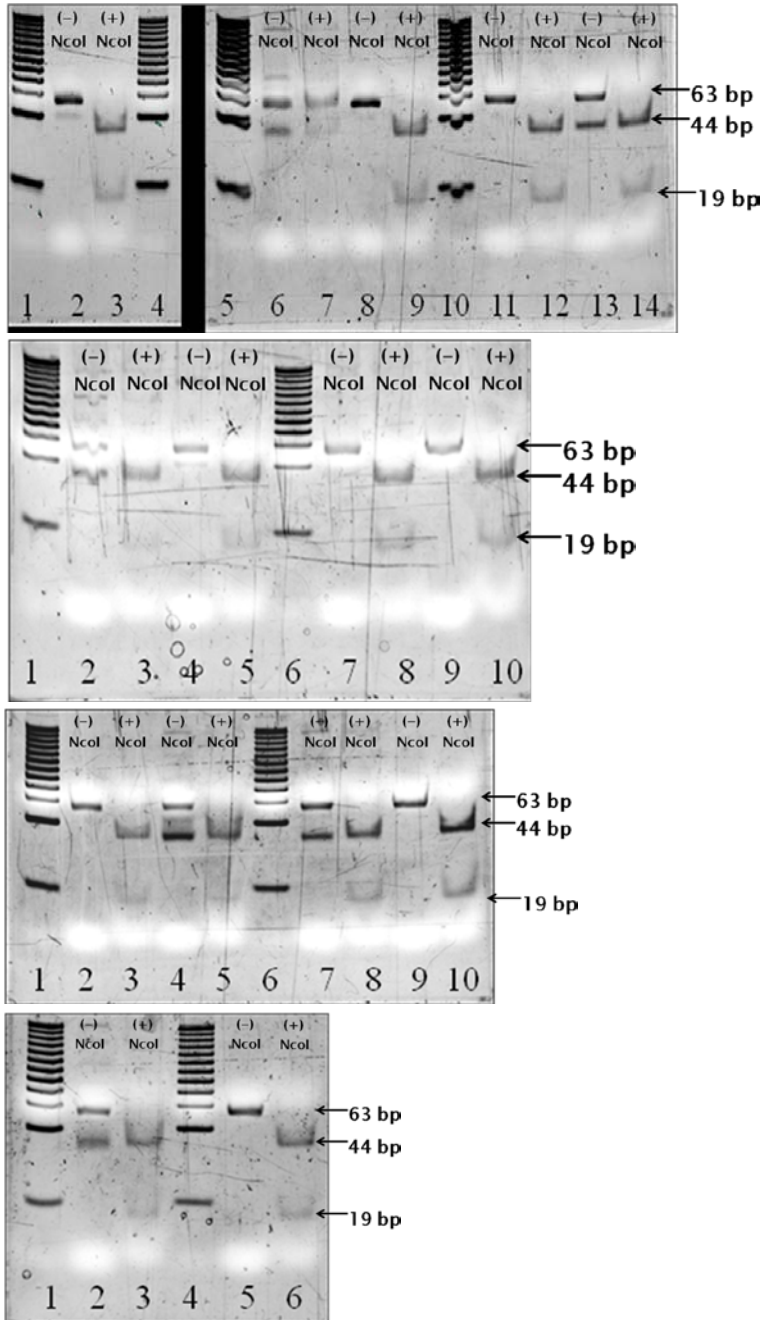
Figure 12. 15% polyacrylamide g el res triction d igest o f de-identified patient s amples f rom *P. aeruginosa ecfX* assay.

Of the 15 de-identified true positive patient samples that were detected by the *ecfX* real-time PCR assay, 15 showed the expected 63 bp band, while 14 showed the expected 44 and 19 bp bands after being subjected to restriction digestion with *Nco*I. 9893 (lanes 2 and 3), 9516 (lanes 6 and 7), 9742 (lanes 8 and 9), T39 (lanes 11 and 12), and T44 (lanes 13 and 14) in top gel. T157 (lanes 2 and 3), 9956 (lanes 4 and 5), 8834 (lanes 7 and 8), and 9402 (lanes 9 and 10) in second gel from top. 9748 (lanes 2 and 3), 9495 (lanes 4 and 5), 9551 (lanes 7 and 8), and 9398 (lanes 9 and 10) in third gel from top. 9691 (lanes 2 and 3) and T273 (lanes 5 and 6) in bottom gel. The de-identified sample 9516, in lane 7 (top gel), did not show the expected 44 and 19 bp bands.

39

To show that the amplified products from the de-identified patient samples in the 16S rRNA real-time PCR assay were in fact the expected product sizes, we performed purification of these PCR products, followed by restriction digests with *Sac*II and PAGE analysis. In Figure 13, the positive control showed the expected 144 bp band on a 15% polyacrylamide gel (lanes 2, 4, 7, and 9 from the top gel pictured) and 6% polyacrylamide gel (lanes 2, 4, 7, and 9 from the bottom gel pictured).   In addition to these observations, the expected digest patterns with 97 bp and 47 bp bands were visible on the 15% gel (lanes 1, 3, 6, and 8) and 6% gel (lanes 3, 5, 8, and 10). A 25 bp DNA ladder was loaded and run in lanes 5 and 10 on the 15% gel (top gel) and in lanes 1 and 6 on the 6% gel (bottom gel).
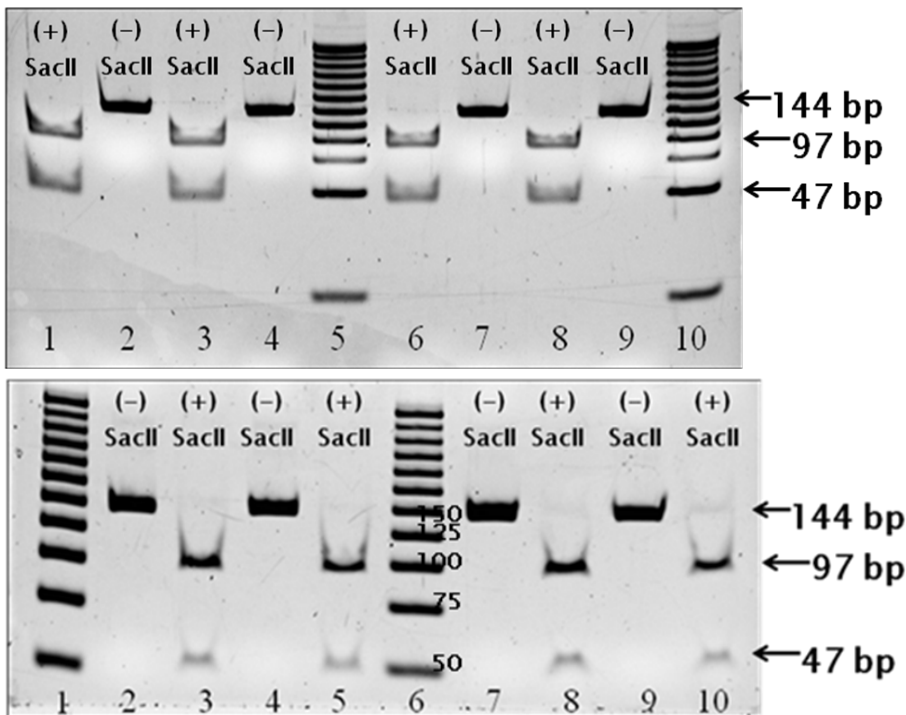
Figure 13. 15% and 6% polyacrylamide ge ls r estriction d igest of p ositive c ontrol *P. aeruginosa* (ATCC 27853) from bacterial F2 16S rRNA assay.
The positive control strain of *P. aeruginosa* (ATCC 27853) was subjected to restriction digestion with the restriction enzyme *Sac*II (repeated eight times), after undergoing purification of the amplified product from the F2 assay. The purified samples were run on a 15% polyacrylamide gel (top gel) and a 6% polyacrylamide gel (bottom gel) and restriction digest patterns were observed after exposure to ethidium bromide. Lanes 5 and 10 (top gel) and lanes 1 and 6 (bottom gel) showed the 25 bp ladder used, with the 25, 50, 75, 100, 125, and 150 bp bands marked in lane 6 (bottom gel). The uncut lanes, designated as (-) *Sac*II, showed the expected 144 bp fragment, while the cut lanes, designated as (+) *Sac*II, showed the expected 97 and 47 bp fragments.

40

In Figure 14, the fourteen 16S rRNA purified PCR products from the patient samples that had come up as positive in the 16S rRNA real-time PCR assay showed the presence of the 144 bp band, as seen in the positive control gel (Figure 13). All fourteen samples had the expected digest patterns, after being cut with the *Sac*II restriction enzyme, showing the presence of a 97 and a 47 bp fragment.
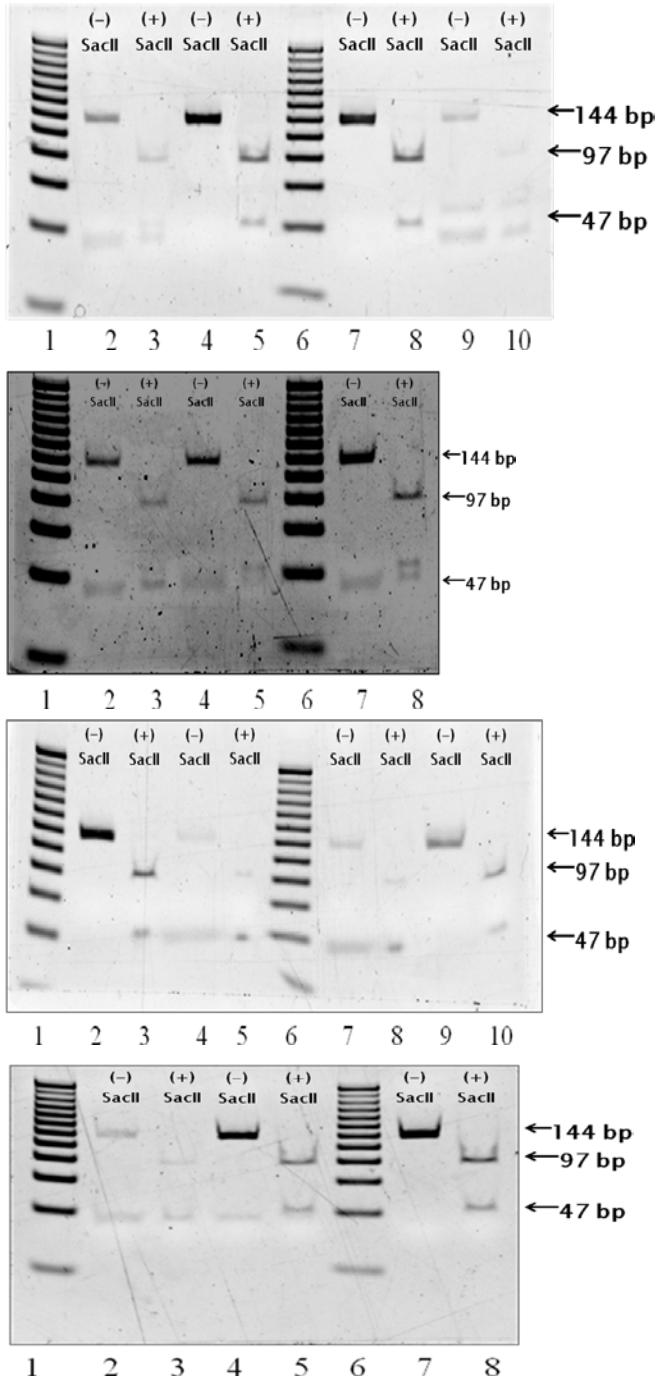
Figure 14. 6% polyacrylamide gel restriction digest of de-identified patient samples from bacterial 16S rRNA assay.

Of the 14 de-identified true positive patient samples that were detected by the F2 16S rRNA real-time PCR assay, all 14 showed the expected 144 bp band, and the expected 44 and 19 bp bands after being subjected to restriction digestion with *Sac*II. 9893 (lanes 2 and 3), 9742 (lanes 4 and 5), T39 (lanes 7 and 8), and T44 (lanes 9 and 10) in top gel. T157 (lanes 2 and 3), 9956 (lanes 4 and 5), and 8834 (lanes 7 and 8) in second gel from top. 9402 (lanes 2 and 3), 9748 (lanes 4 and 5), 9551 (lanes 7 and 8), and 9398 (lanes 9 and 10) in third gel from top. 9691 (lanes 2 and 3), 9328 (lanes 4 and 5), and T273 (lanes 7 and 8) in bottom gel.

42

5.0   DISCUSSION

Culture is the primary diagnostic tool in determining the identity of the infectious pathogen linked to individual keratitis cases in patients, but PCR has provided laboratories with an additional way to confirm the etiology of a keratitis infection. For example, in one study, isolates of *P. aeruginosa* were taken from patients with keratitis and subjected to PCR testing (35). This PCR assay focused on amplifying the 367 bp exotoxin-A gene from *P. aeruginosa* isolates with specific primers (35). It was concluded that PCR was a good complement to culture and biochemical tests in identification of pathogens (35).   Real-time PCR has become useful in detecting other corneal pathogens, such as *Acanthamoeba* (37). Thompson et al. compared two different real-time PCR assays, Qvarnstrom and Rivière, and found that the Qvarnstrom assay was able to detect more *Acanthamoeba* genotypes than the Rivière assay (37). This group concluded that PCR testing should be used alongside cultures and smears, in order to identify corneal pathogens (37).

In addition to common ocular pathogens, such as *P. aeruginosa* and *Acanthamoeba*, PCR has been used to identify more rare forms of these ocular pathogens involved in keratitis. Human cytomegalovirus is a difficult virus to study because since it is a species specific pathogen animal models are not ideal, although murine models have been used (14). This virus causes ocular damage in infected patients and has been linked to keratitis; therefore, a study was performed utilizing real-time PCR as a tool to diagnosis CMV in keratitis patients (14). Similar

43

to the *ecfX* PCR assay used in this study, this PCR assay had a small target size of 61bp and a TaqMan probe was utilized (14). The real-time PCR results were able to help calculate copy number values, which decreased over time as patients continued receiving treatment for their infection (14). Aside from CMV, PCR has also been used to detect microsporidial infections in keratitis patients (13).

The *ecfX* gene has been shown to be a target for identification of *P. aeruginosa* (3, 21), but to our knowledge, not yet been tested against keratitis isolates and direct patient samples from the cornea. In this study, 100% of the *P. aeruginosa* positive keratitis isolates tested were detected by the real-time *ecfX* PCR assay. This species-specific gene based PCR assay did not detect any non-*P. aeruginosa* isolates. In terms of testing direct de-identified patient samples, the *ecfX* primer set and specific probe produced a sensitivity of 75% and an efficiency of 85% (Table 4). For a clinical diagnostic test, the desired values for sensitivity and efficiency are 100%; therefore, future studies should further analyze the false negative samples. It can be concluded that the 63 bp target amplified by the *ecfX* primer set with the specific probe was shown to be a potential target for detection of *Pseudomonas aeruginosa* DNA in keratitis patients, but further studies are needed to produce a more sensitive real-time *ecfX* PCR assay.

Different studies have identified primer sets that have been considered universal, meaning that they have the ability to amplify a portion of the highly conserved 16S rRNA gene found in all bacterial species (26). These types of PCR assays have been used in the studies of the following infections: bacteraemia (23); bacterial contamination of platelets (8, 27); central vascular catheters (39); bacterial meningitis (18, 33); and bacterial endocarditis (9). More specifically, groups have started using these types of primer sets in keratitis research (17, 31). The 27F primer set was tested and was able to detect *P. aeruginosa* isolates through the

amplification of a 563 bp target; however, it was not effective at amplifying *P. aeruginosa* DNA extracted directly from the de-identified patient samples. The F2 forward primer was then designed to work with the reverse primer and probe from the 27F primer set and to amplify a smaller portion of the 16S rRNA gene, since the recommended target size for optimal real-time PCR results is between 75 and 200 bp (4). The 27F primer set was discontinued in the de-identified patient study and was replaced by this F2 primer set, which amplified a 144 bp segment of the 16S rRNA gene.

The F2 primer set had a sensitivity of 70% and efficiency value of 85% (Table 4). For a clinical diagnostic test, the desired values for sensitivity and efficiency are 100%; therefore, future studies should further analyze the false negative samples. Based on results from a SYBR Green real-time PCR assay, the F2 primer set was able to detect DNA from different bacterial species known to be common ocular pathogens (Table 3). This primer set used with SYBR Green, rather than with a specific probe, should be further studied because this assay can detect non-specific amplified products in the reaction tubes through melt curve analysis. Future testing of this primer set with bacterial species, aside from *P. aeruginosa*, will have to be performed. It can be concluded that the 144 bp target amplified by the F2 primer set with the specific probe was shown to be a potential secondary target for detection of *P. aeruginosa* DNA in keratitis patients, but further testing must be carried out to produce a more sensitive real-time PCR assay.

Following the de-identified patient sample study, the BAK11W/BAK2 primer set was tested because although the F2 primer set was able to detect *P. aeruginosa* from direct de-identified patient samples, it was found in further testing that this set was not effective at detecting some DNA from other types of bacterial isolates (data not shown). The real-time PCR assay results showed that the BAK11W/BAK2 primer set was able to detect 21 *P. aeruginosa*

isolates and 22 non-*P. aeruginosa* bacterial isolates. Melt-curve analysis showed that the amplified products were pure, with no nonspecific products being amplified. Even though the BAK11W/BAK2 set was able to detect *P. aeruginosa* and non-*P. aeruginosa* isolates, further testing of this primer set needs to be performed to evaluate how well this primer set will be able to amplify bacterial DNA from keratitis patient samples.

Table 5 shows the expected and actual results from the *ecfX* real-time PCR assay and the 16S rRNA PCR assay utilized in the de-identified patient study. Actual results that differ from the expected results are italicized. Seven different de-identified patient samples (9516; T192; T238; 9471; 9495; 9821; 9856; 9328) produced results that differed from the expected. The only de-identified patient sample that was not detected by the *ecfX* assay was 9328. The only de-identified patient samples that were not detected by the 16S rRNA assay included 9516 and 9495. Patient samples T192, T238, 9471, and 9856 were not detected by either of the two real-time PCR assays. Since all four of these samples were not detected by either assay, the possible ineffective collection of the samples from patient corneas must be taken into consideration. In addition to sampling error, the storage of the amplified PCR products and freeze-thaw issues could have affected the real-time PCR results. Also, DNA could have been lost during the DNA extraction process, leading to a concentration lower than that of the calculated limit of detection.

Aside from the issue of sample collection, another factor that was considered was PCR inhibition. In order to eliminate this factor as a possible reason for false negative results, the five false negatives from the de-identified patient sample *ecfX* assay were tested. Each of the five samples were spiked with a 10^{-4} dilution of the constructed *ecfX* plasmid and run against the *ecfX* real-time PCR assay. All five samples were detected by the assay and positive results were

recorded. This test showed that the PCR unit was working properly and that there were no inhibitors present during the de-identified patient study PCR assays.

Concentration levels of bacterial DNA and human DNA had to be considered, as well, with the de-identified patient sample study. The NanoDrop 2000 was utilized in measuring DNA concentrations of samples, but unfortunately, this machine did not have the ability to distinguish between bacterial and human DNA (Table 6). The samples that were not detected by the PCR assays may not have had an adequate amount of bacterial DNA present, but may have had human DNA present, which would not have been detected by the primer sets.

Validation of the amplified PCR products was important to ascertain, in order to confirm that the real-time assays had amplified the expected PCR products. All of the de-identified patient samples that had produced positive real-time PCR results in both assays underwent restriction digestion to confirm the positive result. NEBCutter V2.0 (New England BioLabs, Inc.) mapped out the possible restriction enzyme sites for each gene target. NcoI and SacII were selected based on the size of the fragments that would result following the restriction digests and the availability in the laboratory. With such small amplified products sizes, PAGE analysis was used rather than going through the process of DNA sequencing; moreover, the amplified PCR products, due to their small size, would have to be cloned into a plasmid vector in order to produce DNA sequencing results. All of the amplified PCR products tested from the de-identified patient samples, except for one, showed the expected digest patterns, as compared to the positive controls. An improperly setup restriction digest solution could have contributed to this one sample not cutting. Also, with this sample, a possible mutation could have been introduced during the PCR amplification process or this particular strain of *P. aeruginosa* could have possessed a mutation, leading to a negative PCR result. The presence of nonspecific

47

bands on the gels was observed with some of the patient samples, which could have been due to primer dimers.

6.0   CONCLUSIONS AND PUBLIC HEALTH RELEVANCE

In conclusion, real-time PCR provides clinical laboratories with a supplementary diagnostic tool to culture in the diagnosis of *P. aeruginosa* keratitis. Species-specific primers and primers targeted at the 16S rRNA gene have the ability to detect bacterial DNA extracted from isolates and direct patient samples. The *ecfX* real-time PCR assay (21) was adapted to the Cepheid SmartCycler® II real-time PCR system and was shown to detect *P. aeruginosa* isolates and *P. aeruginosa* from direct de-identified patient samples. Universal primer real-time PCR assays targeting the highly conserved bacterial 16S rRNA gene, once validated, will be able to detect bacteria from the cornea that do not grow out in culture. Although both assays detected different genes of *P. aeruginosa*, real-time PCR testing needs to produce higher sensitivity values for clinical diagnostic purposes. Restriction digests and PAGE analysis are tools that can be used to validate results from real-time PCR assays, as used in this study.

In terms of public health relevance, this study was important because real-time PCR was shown to serve as a supplemental diagnostic tool to culture in *P. aeruginosa* related keratitis. Patient corneas that are pretreated with antibiotics but still show no signs of improvement could be candidates for a broad-range PCR assay to identify if the corneal pathogen is of bacterial origin. By working to improve the sensitivity of PCR testing, clinical laboratories will have another diagnostic tool to use in diagnosing patients with keratitis.

APPENDIX: TABLES

Table 1. PCR Primer and Probe Sequences.

Pathogen	Target	Size (bp)	Primers and Probe
Pseudomonas aeruginosa	*ecfX* gene	63	Forward Primer ecfX-F: 5'-CCC ATG CCT ATC ACC CCT T-3' Reverse Primer ecfX-R: 5'-CAA CTG CCC AGG TGC TTG C-3' ecfX TM: 5'- /5G FAM/ATG CGG AGT TGC TGC GCT TCC T/3BHQ-1/-3'
Pseudomonas aeruginosa	16S rRNA	567	Forward Primer 27F: 5'-AGA GTT TGA TCM TGG CTC AG-3' Reverse 16S1RR-B: 5'-CTT TAC GCC CAR TRA WTC CG-3' 514-S: 5'- /56-FAM/TNT TAC CGC GGC TGC TGG CAC G/36-TAMSP/-3'
Pseudomonas aeruginosa	16S rRNA	144	Forward Primer F2: 5'-GCA CTT TAA GTT GGG AGG AA-3' Reverse Primer 16S1RR-B: 5'-CTT TAC GCC CAR TRA WTC CG-3' 514-S: 5'- /56-FAM/TNT TAC CGC GGC TGC TGG CAC G/36-TAMSP/-3
Pseudomonas aeruginosa	16S rRNA	144	Forward Primer F2: 5'-GCA CTT TAA GTT GGG AGG AA-3' Reverse Primer 16S1RR-B: 5'-CTT TAC GCC CAR TRA WTC CG-3' TaKaRa SYBR® *Premix Ex Taq*™
Pseudomonas aeruginosa	16S rRNA	792	Forward Primer BAK11W: 5'-AGTTTGATC(A/C)TGGCTCAG-3' Reverse Primer BAK2: 5'-GGACTAC(C/T/A)AGGGTATCTAAT-3'

Table 2. *P. aeruginosa ecfX* and bacterial 16S rRNA Plasmid Dilution Assay Ct values.

ecfX Dilution Value	Ct Value	16S r RNA Dilution Value	Ct Value
10^{-3}	18.51	10^{-4}	18.89
10^{-3}	18.35	10^{-4}	18.63
10^{-4}	21.57	10^{-5}	22.25
10^{-4}	21.72	10^{-5}	22.15
10^{-5}	25.14	10^{-6}	25.32
10^{-5}	25.10	10^{-6}	25.15
10^{-6}	28.93	10^{-7}	28.70
10^{-6}	28.25	10^{-7}	28.35
10^{-7}	32.04	10^{-8}	31.96
10^{-7}	32.07	10^{-8}	32.13
10^{-8}	35.51	10^{-9}	35.55
10^{-8}	35.32	10^{-9}	35.69
10^{-9}	0		
10^{-9}	38.67		

Table 3. SYBR Green Melting Temperatures of Selected Bacterial Specimens from using the F2 primer set.

Sample	Melting Temperature (°C)
P. aeruginosa ATCC 27853	84.91
P. aeruginosa K1582	84.66
P. aeruginosa K1582	84.81
P. aeruginosa K1581	84.61
P. aeruginosa K1581	84.77
P. aeruginosa K1668	84.72
P. aeruginosa K1668	84.64
Escherichia coli	84.79
Escherichia coli	84.97
Serratia marcescens	85.18
Serratia marcescens	84.74
Moraxella lacunata	85.39
Moraxella lacunata	85.45
Staphylococcus aureus	85.01
Staphylococcus aureus	84.75

Table 4. Descriptive statistics from de-identified patient sample study.

Statistic	Direct *P. aeruginosa* PCR assay	16S rRNA gene F2 PCR assay
True-positives	20	20
True-negatives	20	20
Sensitivity	75% (15/20)	70% (14/20)
Specificity	95% (19/20)	100% (20/20)
Positive Predictive Value	94% (15/16)	100% (14/14)
Negative Predictive Value	79% (19/24)	77% (20/26)
Efficiency	85% (34/40)	85% (34/40)

Table 5. De-identified patient study sample IDs with real-time PCR results from both assays.

De-identified Patient Sample ID#	Expected results: True positive (P) or true negative (N)	Actual results: *ecfX* PCR assay	Actual results: F2 16S rRNA PCR assay
9893	**P**	P	P
1410	**N**	N	N
9516	**P**	P	*N*
8753	**N**	N	N
1829	**N**	N	N
9742	**P**	P	P
T39	**P**	P	P
T44	**P**	P	P
T501	**N**	N	N
586	**N**	N	N
T157	**P**	P	P
1229	**N**	N	N
T192	**P**	*N*	*N*
9956	**P**	P	P
T454	**N**	N	N
T238	**P**	*N*	*N*
T545	**N**	N	N
611	**N**	N	N
T675	**N**	N	N
8834	**P**	P	P
T525	**N**	N	N
9402	**P**	P	P
1118	**N**	N	N
9748	**P**	P	P
9471	**P**	*N*	*N*
1333	**N**	N	N
T902	**N**	N	N
9495	**P**	P	*N*
9821	**N**	*P*	N
9551	**P**	P	P
T625	**N**	N	N
9398	**P**	P	P
9856	**P**	*N*	*N*
575	**N**	N	N
9691	**P**	P	P
321	**N**	N	N
9328	**P**	*N*	P
T607	**N**	N	N
602	**N**	N	N
T273	**P**	P	P

Table 6. Total DNA concentration values (calculated using the NanoDrop 2000) of purified de-identified patient samples, which were detected by the *ecfX* and F2 16S rRNA real-time PCR assays.

De-identified Patient Sample ID#	DNA Concentration (ng/µl) *ecfX* PCR purified product	DNA Concentration (ng/µl) F2 16S rRNA purified product
9893	15.5	6.5
9516	13.2	-
9742	11.2	11.5
T39	11.7	9.2
T44	11.3	5.6
T157	14.5	9.1
9956	14.7	11.3
8834	16.0	7.9
9402	14.7	9.9
9748	8.7	5.9
9495	10.7	-
9551	12.1	5.1
9398	14.6	6.4
9691	10.2	6.9
9328	-	7.0
T273	10.9	10.5

BIBLIOGRAPHY

1. **Aderem, A., and R. J. Ulevitch.** 2000. Toll-like receptors in the induction of the innate immune response. Nature **406:**782-7.
2. **Ambion, I.** 2010. DNAZap.
3. **Anuj, S. N., D. M. Whiley, T. J. Kidd, S. C. Bell, C. E. Wainwright, M. D. Nissen, and T. P. Sloots.** 2009. Identification of Pseudomonas aeruginosa by a duplex real-time polymerase chain reaction assay targeting the ecfX and the gyrB genes. Diagn Microbiol Infect Dis **63:**127-31.
4. **Bio-Rad Laboratories, I.** 2006. Real-Time PCR Applications Guide.
5. **Choy, M. H., F. Stapleton, M. D. Willcox, and H. Zhu.** 2008. Comparison of virulence factors in Pseudomonas aeruginosa strains isolated from contact lens- and non-contact lens-related keratitis. J Med Microbiol **57:**1539-46.
6. **Corpet, F.** 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res **16:**10881-90.
7. **Delden, C. V., and B. H. Iglewski** 1998, posting date. Cell-to-Cell Signaling and Pseudomonas aeruginosa Infections. Volume 4 Number 4. [Online.]
8. **Dreier, J., M. Stormer, and K. Kleesiek.** 2004. Two novel real-time reverse transcriptase PCR assays for rapid detection of bacterial contamination in platelet concentrates. J Clin Microbiol **42:**4759-64.
9. **Goldenberger, D., A. Kunzli, P. Vogt, R. Zbinden, and M. Altwegg.** 1997. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. J Clin Microbiol **35:**2733-9.
10. **Hazlett, L. D.** 2004. Corneal response to Pseudomonas aeruginosa infection. Prog Retin Eye Res **23:**1-30.
11. **Howe, T. R., and B. H. Iglewski.** 1984. Isolation and characterization of alkaline protease-deficient mutants of Pseudomonas aeruginosa in vitro and in a mouse eye model. Infect Immun **43:**1058-63.
12. **Iglewski, B. H., J. Sadoff, M. J. Bjorn, and E. S. Maxwell.** 1978. Pseudomonas aeruginosa exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc Natl Acad Sci U S A **75:**3211-5.
13. **Joseph, J., S. Sharma, S. I. Murthy, P. V. Krishna, P. Garg, R. Nutheti, J. Kenneth, and D. Balasubramanian.** 2006. Microsporidial keratitis in India: 16S rRNA gene-based PCR assay for diagnosis and species identification of microsporidia in clinical samples. Invest Ophthalmol Vis Sci **47:**4468-73.
14. **Kandori, M., T. Inoue, F. Takamatsu, Y. Kojima, Y. Hori, N. Maeda, and Y. Tano.** 2010. Prevalence and features of keratitis with quantitative polymerase chain reaction positive for cytomegalovirus. Ophthalmology **117:**216-22.

15. **Keay, L., K. Edwards, T. Naduvilath, H. R. Taylor, G. R. Snibson, K. Forde, and F. Stapleton.** 2006. Microbial keratitis predisposing factors and morbidity. Ophthalmology **113:**109-16.

16. **Klaschik, S., L. E. Lehmann, A. Raadts, M. Book, J. Gebel, A. Hoeft, and F. Stuber.** 2004. Detection and differentiation of in vitro-spiked bacteria by real-time PCR and melting-curve analysis. J Clin Microbiol **42:**512-7.

17. **Knox, C. M., V. C evellos, a nd D. Dean.** 1998. 16S ribosomal DNA typing for identification of pathogens in patients with bacterial keratitis. J Clin Microbiol **36:**3492-6.

18. **Kotilainen, P., J. Jalava, O. Meurman, O. P. Lehtonen, E. Rintala, O. P. Seppala, E. Eerola, an d S. N ikkari.** 1998. Diagnosis of meningococcal meningitis by broad-range bacterial PCR with cerebrospinal fluid. J Clin Microbiol **36:**2205-9.

19. **Krachmer, J. H., M. J. Mannis, and E. J. H olland.** 2004. CORNEA, Second Edition ed, vol. Volume One. Elsevier Mosby.

20. **Krachmer, J. H ., M . J. M annis, an d E . J. Holland.** 2010. Practical Ophthalmic Microbiology for the Detection of Corneal Pathogens, vol. 1. Elsevier.

21. **Lavenir, R., D. Jocktane, F. Laurent, S. Nazaret, and B. Cournoyer.** 2007. Improved reliability of Pseudomonas aeruginosa PCR detection by the use of the species-specific ecfX gene target. J Microbiol Methods **70:**20-9.

22. **Ledbetter, E. C ., J. J . M un, D . K owbel, a nd S . M . F leiszig.** 2009. Pathogenic phenotype and genotype of Pseudomonas aeruginosa isolates from spontaneous canine ocular infections. Invest Ophthalmol Vis Sci **50:**729-36.

23. **Ley, B. E., C. J. Linton, D. M. Bennett, H. Jalal, A. B. Foot, and M. R. Millar.** 1998. Detection of bacteraemia in patients with fever and neutropenia using 16S rRNA gene amplification by polymerase chain reaction. Eur J Clin Microbiol Infect Dis **17:**247-53.

24. **Lister, P. D ., D . J. Wolter, an d N . D . Hanson.** 2009. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev **22:**582-610.

25. **Lyczak, J. B ., C . L . C annon, an d G . B . Pier.** 2000. Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist. Microbes Infect **2:**1051-60.

26. **Millar, B. C., J. Xu, and J. E. Moore.** 2002. Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. J Clin Microbiol **40:**1575-80.

27. **Mohammadi, T ., H . W. R eesink, C . M. Vandenbroucke-Grauls, an d P . H . Savelkoul.** 2003. Optimization of real-time PCR assay for rapid and sensitive detection of eubacterial 16S ribosomal DNA in platelet concentrates. J Clin Microbiol **41:**4796-8.

28. **Mueller, J. B ., an d C . M. M cStay.** 2008. Ocular infection and inflammation. Emerg Med Clin North Am **26:**57-72, vi.

29. **Nicas, T . I., D . W. Frank, P . S tenzel, J. D . L ile, an d B . H . I glewski.** 1985. Role of exoenzyme S in chronic Pseudomonas aeruginosa lung infections. Eur J Clin Microbiol **4:**175-9.

30. **PDRhealth** 2009, posting date. Keratitis. [Online.]

31. **Rudolph, T ., C . We linder-Olsson, L . L ind-Brandberg, and U . Stenevi.** 2004. 16S rDNA PCR analysis of infectious keratitis: a case series. Acta Ophthalmol Scand **82:**463-7.

32. **Schabereiter-Gurtner, C., M. Nehr, P. Apfalter, A. Makristathis, M. L. Rotter, and A. M. Hirschl.** 2008. Evaluation of a protocol for molecular broad-range diagnosis of culture-negative bacterial infections in clinical routine diagnosis. J Appl Microbiol **104:**1228-37.

33. **Schuurman, T., R. F. de Boer, A. M. Kooistra-Smid, and A. A. van Zwet.** 2004. Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for diagnosis of bacterial meningitis in a clinical setting. J Clin Microbiol **42:**734-40.

34. **Shanks, R. M., D. E. Kadouri, D. P. MacEachran, and G. A. O'Toole.** 2009. New yeast recombineering tools for bacteria. Plasmid **62:**88-97.

35. **Song, K. P., T. K. Chan, Z. L. Ji, and S. W. Wong.** 2000. Rapid identification of Pseudomonas aeruginosa from ocular isolates by PCR using exotoxin A-specific primers. Mol Cell Probes **14:**199-204.

36. **Taylor, R. N., A. Y. Huong, K. M. Fulford, V. A. Przybyszewski, and T. L. Hearn.** 1979. Quality Control for Immunologic Tests, vol. U.S. Department of Health and Human Services.

37. **Thompson, P. P., R. P. Kowalski, R. M. Shanks, and Y. J. Gordon.** 2008. Validation of real-time PCR for laboratory diagnosis of Acanthamoeba keratitis. J Clin Microbiol **46:**3232-6.

38. **Todar, K.** 2008, posting date. Pseudomonas aeruginosa. [Online.]

39. **Warwick, S., M. Wilks, E. Hennessy, J. Powell-Tuck, M. Small, J. Sharp, and M. R. Millar.** 2004. Use of quantitative 16S ribosomal DNA detection for diagnosis of central vascular catheter-associated bacterial infection. J Clin Microbiol **42:**1402-8.

40. **WebMD** 2009, posting date. Slit Lamp Examination. [Online.]

41. **Wick, M. J., A. N. Hamood, and B. H. Iglewski.** 1990. Analysis of the structure-function relationship of Pseudomonas aeruginosa exotoxin A. Mol Microbiol **4:**527-35.

42. **Wilhelmus, K. R.** 2002. Indecision about corticosteroids for bacterial keratitis: an evidence-based update. Ophthalmology **109:**835-42; quiz 843.

43. **Willcox, M. D., H. Zhu, T. C. Conibear, E. B. Hume, M. Givskov, S. Kjelleberg, and S. A. Rice.** 2008. Role of quorum sensing by Pseudomonas aeruginosa in microbial keratitis and cystic fibrosis. Microbiology **154:**2184-94.

44. **Zucol, F., R. A. Ammann, C. Berger, C. Aebi, M. Altwegg, F. K. Niggli, and D. Nadal.** 2006. Real-time quantitative broad-range PCR assay for detection of the 16S rRNA gene followed by sequencing for species identification. J Clin Microbiol **44:**2750-9.