LABORATORY DIAGNOSIS OF *ACANTHAMOEBA* KERATITIS USING THE CEPHEID SMARTCYCLER® II AND THE EFFECTS OF TOPICAL OPHTHALMIC DRUGS ON REAL-TIME PCR

by

Paul Thompson

BMedSc, Charles Sturt University, Australia, 2000

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

2007

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Paul Thompson

It was defended on

August 23rd, 2007

and approved by

Thesis Advisor:
Regis P. Kowalski, MS, [M] ASCP
Associate Professor
Department Ophthalmology
School of Medicine
University of Pittsburgh

Thesis Co-advisor:
Velpandi Ayyavoo, Ph. D
Assistant Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

Committee Member:
Paul R. Kinchington, Ph. D
Associate Professor
Departments of Ophthalmology and Molecular Genetic and Biochemistry
School of Medicine
University of Pittsburgh

Committee Member:
Jeremy J. Martinson, Ph. D
Assistant Professor
Departments of Infectious Disease and Microbiology and Human Genetics
Graduate School of Public Health
University of Pittsburgh

Committee Member:
Robert M. Wadowsky, Sc. D,
Professor
Departments of Pathology and Infectious Disease and Microbiology
School of Medicine and Graduate School of Public Health
University of Pittsburgh

Regis P Kowalski, MS, [M] ASCP

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Paul Thompson, MS

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Introduction: *Acanthamoeba* keratitis (AK) infection needs to be diagnosed definitively to optimize therapy in order to avoid possible visual impairment.

Aims: 1) To optimize two noted Real-time PCR (RT-PCR) TaqMan methods (Rivière and Qvarnstrom) using the Cepheid SmartCycler® II system. 2) To identify potential inhibitory effects from topical drugs on RT-PCR. 3) To validate and compare the two assays using ocular clinical samples.

Methods: 1) Primers and probes were optimized for both assays to detect genus-specific *Acanthamoeba* 18S rDNA. 2) Thirteen topical ophthalmic drugs were diluted to determine the level of inhibitory effect present. The lowest non-inhibitory concentrations were then used to determine RT-PCR amplification efficiency. 3) Excess clinical samples (139) were processed for culture and assayed by both assays on the SmartCycler® II and the results were compared.

Results: 1) The Rivière RT-PCR plasmid DNA, cyst and trophozoite limits of detection and amplification efficiency were 10.13 copies/10μl, 0.7/300μl, 2.3/300μl, 94% respectively. The Qvarnstrom RT-PCR plasmid DNA, cyst and trophozoite limits of detection and amplification efficiency were 43.8 copies/10μl, 0.7/300μl, 2.3/300μl, 92% respectively. 2) Out of the thirteen topical drugs, the most noteworthy result was that of Polyhexamethylene biguanide (PHMB).

The non-inhibitory dilution and RT-PCR efficiency were 1/2560 and 72.7%. 3) The results of the clinical validation indicated that 134/139 (96.4%) results correlated between the two assays of which 4/134 samples were culture negative but RT-PCR positive.

Conclusions: The two RT-PCR assays were optimized successfully on the SmartCycler® II system with comparable results in detecting genus - specific *Acanthamoeba* DNA. In examining the effects of thirteen topical drugs on RT-PCR, PHMB was demonstrated to both inhibit the reaction at a high dilution and reduce amplification efficiency substantially. Ocular samples (139) were tested using both assays and results thus far indicate that both could be used to diagnose AK in the laboratory.

Public health relevance: RT-PCR can be used to rapidly diagnose AK. Commencement of AK specific therapy earlier will substantially reduce the patients the pain and suffering. Also by examining the effects of topical ophthalmic drugs on RT-PCR, the potential for false negative results and result delays could be minimized.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

'Two roads diverged in a wood, and I-

I took the one less traveled by,

And that has made all the difference.'

Robert Frost - A Road Not Taken

I definitely feel like I have traveled the road not taken in completing my Master's degree and in a lot of ways these words of Robert Frost, are in effect, how I have lived my life. But like most things in life it's never easy doing anything on your own so I'd like to take this opportunity to thank and acknowledge the people who have helped me survive the past four years.

Firstly I need to thank the Charles T. Campbell Foundation who provided financial support for the SmartCycler® II project. Without this funding this project would not have been possible.

I'd like to thank the University of Pittsburgh Medical Center who gave me the opportunity to work in the United Sates by sponsoring my visa, and for assisting with tuition throughout my Masters program.

To the UPMC Department of Ophthalmology and the University of Pittsburgh Ophthalmology and Visual Sciences Research Center. It is wonderful being part of such a dynamic team, dedicated to the common goals of trying to prevent and cure blindness. In

particular thanks must go to Dr Paul Kinchington who provided me with many resources including access to the Molecular Core Grant for Vision Research. I must also thank JP Vergnes and Dr Robert Shanks who assisted me with the preparation of the VZV and *Acanthamoeba* plasmid DNA. My thesis would have taken a lot longer without there help and advice.

To the staff and management of the Charles T. Campbell Microbiology Laboratory. I will be forever grateful for the opportunity to work in this 'small but mighty' laboratory. I'd like to thank Dr Jerold Gordon MD for the opportunities he has given me over the last three years and for his inspirational words of wisdom. I'd like to also thank Regis P Kowalski for his mentorship and friendship. Without his guidance and council things would have been much harder.

To my lecturers and fellow students at the Graduate School of Public Health and in particular the Department of Infectious Disease and Microbiology. Thank you for enhancing my knowledge and passion for IDM. It has been a fantastic experience and one that I can build on in the future.

To my Masters Committee members: Regis P. Kowalski, Dr Velpandi Ayyavoo, Dr Paul Kinchington, Dr Jeremy Martinson and Dr Robert Wadowsky. Thank you for having enough confidence in my abilities to acquiesce in helping with my thesis. Your support and input has been priceless.

There are a few individuals I should also like to thank. Dr Charles Leiter from Leiter's Pharmacy, San Jose, Ca. Thank you for supplying the off label formulations we needed to study PCR inhibition. I would not have been able to do this without your assistance.

To Dr Frederick Schuster of the California Department of Health Services. Unwittingly you sparked my interest in *Acanthamoeba.* Thank you for your time over the past few years. The

xiii

1.0   INTRODUCTION

1.1   *ACANTHAMOEBA* BIOLOGY

Acanthamoeba is classified as a free-living amoeba (does not need a host for replication) belonging to the kingdom Protista and its name is derived from the Greek prefix *acanth* meaning spikes added to the suffix amoeba (meaning to change). *Acanthamoeba* was first reported in 1930 by Castellani as a contaminant in a culture of *Cryptococcus pararoseus* (13) but was not classified until 1931 by Volkonsky (54) when it was placed in the genus *Hartmannella* (the genus at that point was split into three groups based on cyst characteristics: *Hartmannella*, *Glaeseria* and *Acanthamoeba*). After 40 years of conjecture and debate as to the designation of this genus, in 1975 Sawyer and Griffin (44) established the family Acanthamoebidae (Figure 1). It should be noted that the International Society of Protozoologists are updating the traditional hierarchical system from 'kingdom', 'phylum', 'class', 'subclass', 'superorder', 'order' to a new schema for Eukaryotes called 'Super Groups' (3). These include Amoebozoa, Opisthokonta, Rhizaria, Archaeplastida, Chromalveolta and Excavata. *Acanthamoeba* will belong to the Amoebozoa super group.

Acanthamoeba were originally characterized into three groups based on cyst morphology (38): Group I were designated on the basis of having a large cyst in comparison to that of cysts in

1
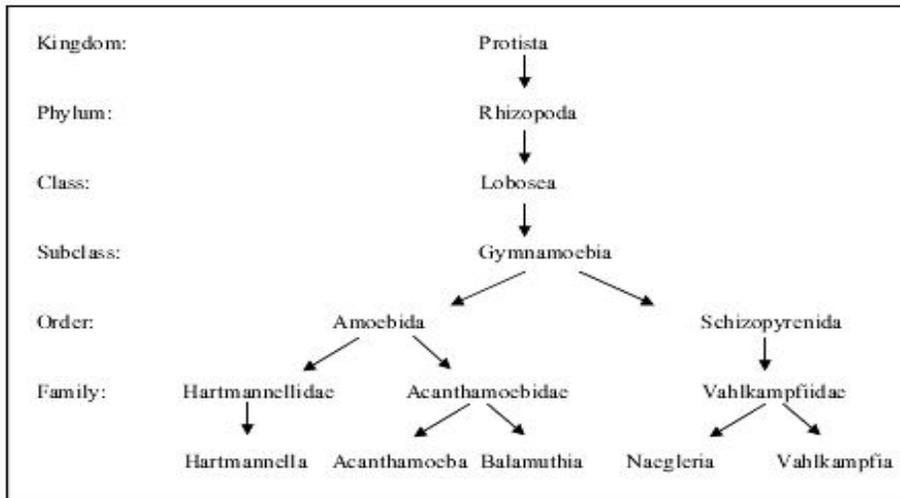
Figure 1 Kingdom Protista: Free-living Amoeba Associated with Disease
(Adapted from Khan, 2006)

the other groups. Group II is characterized as having a wrinkled ectocyst and an endocyst which could be stellate, polygonal, triangular, or oval. Group III typically have a thin, smooth ectocyst and a round endocyst (Figure 2). Unfortunately, this method of classification whilst useful was not foolproof so in an era of molecular based technologies, Gast et al (20) amongst others developed a classification scheme based on nuclear rDNA gene sequences (the 2300bp 18S rDNA). The rDNA gene is a popular target because it is part of the ribosomal gene repeat unit of which there are approximately 600 copies in *Acanthamoeba* (12). Stothard et al (50) then went on to use this technique to classify 53 *Acanthamoeba* isolates based on 12 rDNA sequence types (Rns genotypes) which at the time were designated into types T1 to T12. This has since been expanded to include T13-T15 (46). Mitochondrial DNA has also been used to type *Acanthamoeba* successfully (29) and in one study the authors felt that 'mitochondrial riboprinting may have an advantage over nuclear 18S rDNA sequencing because the mitochondrial small subunit rDNAs do not appear to have introns that are found in the 18S genes of *Acanthamoeba* that distort phylogenetic analyses'. (60)

2
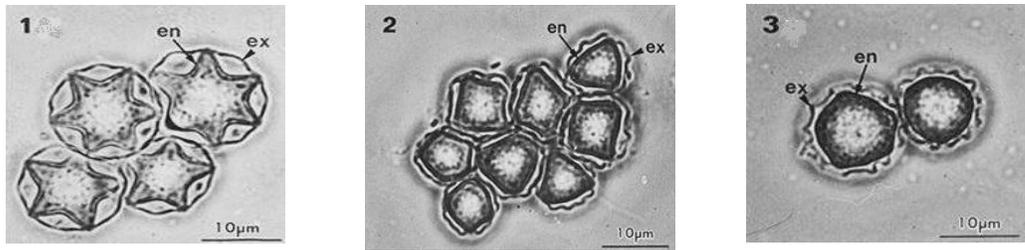
Figure 2 *Acanthamoeba* **Cyst Morphology (35)**

The life cycle of *Acanthamoeba* includes two stages: a dormant cyst stage and a motile trophozoite stage (Figures 3 and 4). The trophozoites range in size from 12-45 μm in diameter but size varies substantially between genotypes (16). They are characterized by spine-like structures on their surface called acanthopodia which function in adhesion to surfaces, in cellular movement or capturing prey or other food sources (phagocytosis and pinocystosis). The trophozoites contain a single, centralized nucleus about one-sixth of the size of the cell which contains a large dense nucleolus. *Acanthamoeba* during this phase ingest many food sources including bacteria, algae, yeast or other organic products which maybe seen in contractile
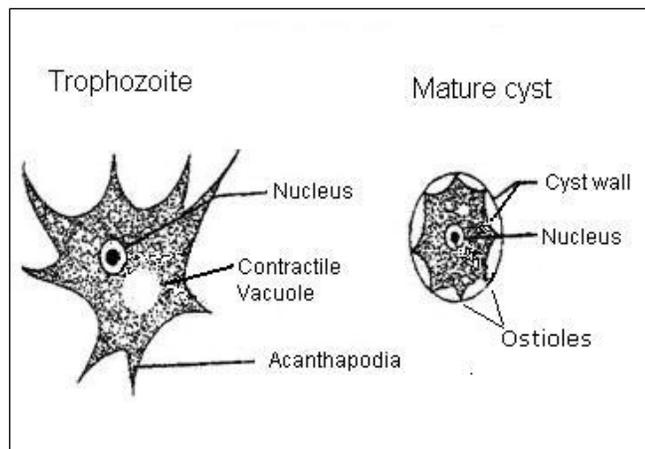
Figure 3 *Acanthamoeba* **Trophozoite and Cyst (23)**

vacuoles in the cytoplasm. The *Acanthamoeba* trophozoites divide asexually by binary fission in which the nuclear membrane and nucleolus disappear during cell division. The trophozoite state can be maintained provided that the environmental conditions are suitable. These include a steady food supply, optimal temperature, pH and osmolarity.
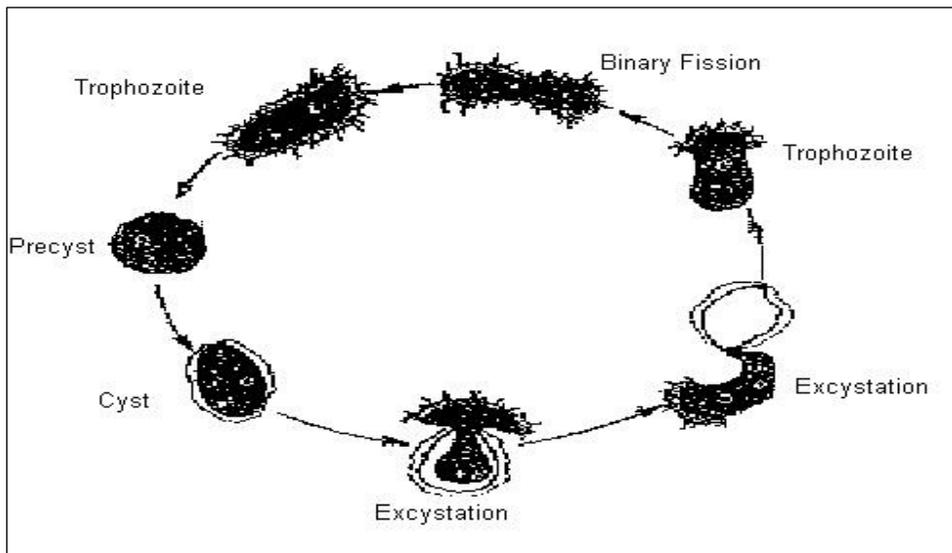
Figure 4 *Acanthamoeba* **Lifecycle (52)**

Once these conditions become adverse, the trophozoite firstly condenses into a single walled rounded state called the precyst, followed by the double-walled state. The wrinkled and proteinaceous outer wall is known as the ectocyst and the cellulose containing inner wall is known as the endocyst. The double walls provide the cyst with a defense to resist the extreme changes it may experience in its microscopic ecosystem. Cellular levels of RNA, proteins, triacylglycerides and glycogen have been shown to be reduced during encystment so cell volume and dry weight is decreased compared to the trophozoites (56). The cyst is slightly smaller than

4

the trophozoite at 5-20 µm in diameter but like the trophozoites the size can vary according to genotype (16). They also possess pores or ostioles which are formed at the points where the ectocyst and endocyst localize. Ostioles have the function of monitoring the environment outside the cyst so if the environmental conditions become favorable, excystation occurs and a trophozoite is formed leaving the outer wall behind.

Acanthamoeba are ubiquitous in the environment and have been isolated from numerous sites including swimming pools, garden soil, freshwater ponds, well water, hospital tap water, bottled water, seawater, beaches, air-conditioning units, air, sewage, compost, vegetables, surgical equipment, contact lenses and cases (26). It is not surprising with the prevalence of *Acanthamoeba* in the environment that eventually clinical disease would occur. However, the first reported human case of *Acanthamoeba* granulomatous encephalitis (AGE) was not reported until 1972 (24) and *Acanthamoeba* keratitis (AK) not until 1974 (34) but the potential for more disease is apparent with studies showing that 50-100% of normal individuals have antibodies to *Acanthamoeba*-specific antigens (15). Other etiology in *Acanthamoeba* disease have been reported in the literature including cases of cutaneous infection (19) and sinusitis (9).

1.2 *ACANTHAMOEBA* KERATITIS

AK is an extremely painful condition that if not treated effectively could potentially result in the patient requiring a corneal transplantation or worsen to the point where the patient's sight is threatened. Symptoms can take days or weeks to develop depending on the amount of *Acanthamoeba* present initially at the site as well as the degree of corneal injury. Early in the infection the symptoms will include eyelid ptosis, conjunctival hyperemia, blurred vision, pain,

tearing and photophobia and signs of epithelial irregularities, epithelial opacities, epithelial micro erosions, microcystic edema and patchy anterior stromal infiltrates (6). As the disease progresses, dendriform epitheliopathy can occur which is one of the reasons why AK is occasionally misdiagnosed as Herpes simplex virus (HSV) keratitis. Also the classic ring infiltrate (Figure 5) maybe seen in up to 83% patients by the second month (40). Stromal opacities are seen, there is a decreased corneal sensation and radial keratoneuritis may occur. In late stages of the disease corneal melting and perforation can occur. Complications of AK could be dacryoadenitis, sclerokeratitis, hypopyon, cataract, secondary glaucoma and reactive ischemic retinitis. AK usually occurs in one eye but can occur bilaterally.
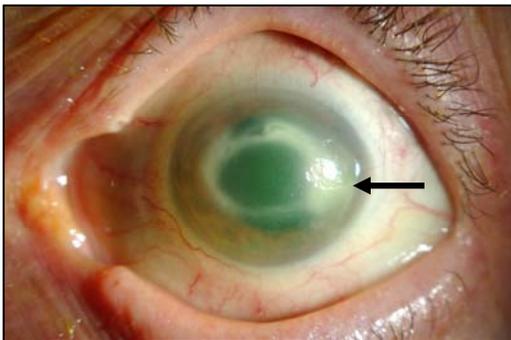
Figure 5 Classic Ring Infiltrate *(Photo Courtesy of UPMC Eye Center)*

Thus far several species of *Acanthamoeba* have been associated with keratitis. These include: *A. polyphaga, A. castellanii, A. hatchetti, A. culbertsoni* (Diamond), *A. rhysodes, A. griffini, A. quina and A. lugdenensis* (33). It should be noted that two different strains of *A. culbertsoni* been described in the literature. *A. culbertsoni* 'Diamond' strain belongs to the T4 genotype and has been reported to cause keratitis and *A. culbertsoni* A-1 'Lily' strain which actually belongs to the T10 genotype and has not been associated with corneal disease.

1.3   *ACANTHAMEOBA* MECHANISMS OF PATHOPHYSIOLOGY

Throughout the literature multiple authors refer to the benefits of a healthy intact cornea as being the best defense against *Acanthamoeba* infection (6, 36). The innate immune system also plays an important role in controlling the infection. Innate immune cells such macrophages and neutrophils confront any *Acanthamoeba* trophozoites or cysts that come into contact with the external ocular surface. Also, mucosal secretions such as mucus and tears, contain antimicrobial proteins, including, lysozyme, lactoferrin and defensins which may protect against *Acanthamoeba (15)*. As part of the adaptive immune system, *Acanthamoeba*- specific IgA also found in tears has been shown to block the binding of *Acanthamoeba* to corneal epithelial cells (32). Unfortunately some types of *Acanthamoeba* have been able to overcome this by producing specific proteases that will degrade the host's IgA (31). Also a group of individuals with *Acanthamoeba* infection have been shown to have lower than normal levels of IgA in their tears indicating the importance of antibody to block the organism (5).

The cornea tissue is a rigid barrier to the external environment. However if its integrity is compromised either by trauma or corneal abrasion, the corneal epithelium will respond by upregulating mannosylated glycoproteins as part of its injury response. *Acanthamoeba* trophozoites are able to bind to the mannosylated glycoproteins via a 136 kDa mannose-binding receptor which in turn activates the *Acanthamoeba* to produce a 133 kDa protease called mannose-induced protein (MIP) 133 (16). MIP 133 leads to cytolysis of corneal epithelium, and the eventual breachment of the Bowman's membrane. Trophozoites continue to produce MIP 133, a 65 kDa cysteine protease, an elastase, and a matrix metalloproteinase. The combination of these enzymes allow for the continued degradation of the corneal stromal layer. Trophozoites have been found to sequester around corneal nerves, producing radial keratoneuritis and extreme

pain. For unknown reasons *Acanthamoeba* trophozoites rarely breach the corneal endothelium to produce intraocular infections.

1.4 *ACANTHAMOEBA* KERATITIS INCIDENCE

AK can occur in healthy and immunocompromised hosts but in western countries the majority of cases occur in immunocompetent patients that wear contact lenses. In the United States, 85% (49) of AK were in patients who use contact lenses with an incidence rate of 1-2 cases per million contact lens wearers(45). In the United Kingdom the rate is much higher, at 17-21 cases per million and this has been attributed to the storage of potable water in rooftop tanks (27). In most cases, the patient has admitted to having exposure to contaminated water such as lakes, swimming pools, hot tubes or they may have cleaned or stored their contact lenses with tap water or non-disinfecting solutions. In some cases, even when the contact lens user is compliant, infection can still occur. In recent times a manufacturer of contact lens cleaning solution was being investigated by the FDA and CDC for possible *Acanthamoeba* contamination in their product AMO Complete Moisture Plus (1). AK in patients who are not contact lens wearers typically have a history of trauma to their eye either through surgery or accident (7). In contrast to western countries, in places such as India 40% of AK cases are caused by trauma to the eye (48).

1.5   *ACANTHAMOEBA* TREATMENT

Treatment of AK relies on antiamoebic agents that are cell and tissue toxic disinfectants and are not licensed to be used as eye medications in the United States. Based on anecdotal evidence patients who are diagnosed early in infection have a better recovery than those patients who have tolerated the symptoms of AK without treatment for a longer period of time (22). The reason for this is that the *Acanthamoeba* trophozoites have not had sufficient opportunity to embed deeper into the corneal stroma to form cysts and a more chronic infection. Cysts are more resistant to antiamoebic agents than trophozoites but the treatment efficacy may also be affected due to the drug not penetrating deep enough into the cornea to exert high enough cysticidal concentrations (6).

There are a number of antiamoebic formulations that are available for use topically. The more popular formulations are polyhexamethylene biguanide (PHMB), 0.02%, chlorhexidine. digluconate, 0.04%-0.06% and propamidine isethionate, 0.1% (not approved in the United States). They have been used in various combinations and have shown some success in with antifungals such as topical clotrimazole, 1%-2%, and miconazole 0.1% (18). As a last resort corneal transplant may need to be used to save the eye from the damage and secondary effects of the infection process. Recurrence of infection can occur as cysts may have survived in residual cornea or sclera.

1.6 *ACANTHAMOEBA* DIAGNOSIS

Diagnosis of AK in the laboratory has traditionally been through the use of different stains (Giemsa, Calcofluor white and Gram) to visualize the cysts and trophozoites, and planting of the patient sample on non-nutrient agar with a fresh overlay of either *E. coli* or *E. aerogenes* to cultivate the *Acanthamoeba* over several days. Both methods in their own right can be insensitive and in the case of culture potentially slow (up to 10 days for a negative culture result). Since the early 1990s, *Acanthamoeba* DNA detection by polymerase chain reaction (PCR) has been utilized by some select laboratories and found to be more sensitive then culture (53). However because of the infrequency of cases it is not cost effective for many facilities to add it to their molecular diagnostics armamentarium.

PCR has proven itself to be an invaluable tool in clinical diagnostics. It is ideal for detecting organisms that are unculturable such as *Tropheryma whipplei* (41); organisms that are of public health importance but are slow to grow such as *Mycobacterium tuberculosis* (2); and organisms that can cause rapid, life-threatening disease and require quick identification to institute treatment, such as HSV encephalitis (37). *Acanthamoeba* in its own right is an ideal candidate for routine PCR testing because it can be a slow growing organism or not grow at all on the non-nutrient agar. AK causes a painful, sight threatening disease that requires correct diagnosis and specific therapy. The wrong diagnosis and incorrect therapy could cost the health system a substantial amount through lengthy hospital stays and costly procedures. The cost of running a PCR is far less.

PCR technology has become a much broader field since its discovery by Dr Kary Mullis in 1986. One area that has evolved is that of Real-time PCR (RT-PCR). Instead of the amplification and detection being two separate processes, RT-PCR incorporates them into one. It

does this either by utilizing an intercalating dye such as SYBR green to bind the amplified double stranded DNA or by utilizing a complementary fluorescent probe that binds downstream of either the forward or reverse primer. As Taq polymerase adds dNTPs to the 3' end of either primer it will eventually reach the probe and cleave the fluorophore into solution. The Real-time system is designed to measure the amount of fluorescence produced after each cycle. The amount of amplified DNA increases exponentially as more bound probe is liberated into solution. The RT-PCR system is also designed to heat and cool the reactions more efficiently so a result can be achieved in 1-2 hours with the results of each amplification cycle shown in 'Real-time' on the computer display.

RT-PCR has now been applied to detecting genus-specific *Acanthamoeba* DNA. Rivière and colleagues published the first Real-time PCR utilizing TaqMan technology in January 2006 using the ABI 7700 system (42). The assay targeted a portion of the 18S rDNA and was shown to have good amplification efficiency against extracted trophozoites and cysts, showed no non-specific PCR amplification against other free-living amoebae and has sensitivity that could detect as low as 10 cysts or trophozoites per reaction. However a possible short fall of this paper was that they designed their primers and probe against six characterized strains of *Acanthamoeba* that belonged to the T4 genus (common to many keratitis isolates) and only performed their experiments on one of those strains (*A. castellanii* ATCC 30243).

Later in 2006, Qvarnstrom and colleagues (39) published the second Real-time PCR paper to detect *Acanthamoeba* DNA utilizing TaqMan technology (using Stratagene Mx3000P system). This paper was somewhat different because they were able to design a triplex RT-PCR assay for *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri*. They found the amplification efficiency to be appropriate, there was no cross-reactivity between the other free-

living amoebae and the assay was sensitive, having a limit of detection of less than one trophozoite per total amount of sample processed. This paper however designed its primers and probe against 40 different 18S rRNA sequences included seven cultured genotypes from T1, T4, T7 and T10. Qvarnstrom ran the seven isolates with the Rivière TaqMan assay and found that 1/1 T1 isolate was PCR positive, 3/4 T4 isolates were positive (the one negative was an environmental strain), 0/1 T7 isolate was negative and 0/2 T10 isolates were also negative. Figures 6 and 7 show multi-alignments (Multalin Software, INRA, France) of nine GenBank sequences of Acanthamoeba 18S rDNA regions showing the conserved regions for the Qvarnstrom RT-PCR but the unconserved region toward Rivière's reverse primer which will affect detection of some strains. One of the conclusions that Qvarnstrom made from this was that Rivière's assay '*may still be useful for detecting keratitis strains*'. A possible limitation of the Qvarnstrom RT-PCR, was their clinical evaluation. Only tested 9 clinical samples of cerebrospinal fluid and brain (no cornea samples) were tested. None of the samples were positive for *Acanthamoeba*. In this thesis a more thorough clinical evaluation will be conducted looking strictly at ocular clinical samples for *Acanthamoeba* DNA using both the Rivière and Qvarnstrom RT-PCR assays.

Figure 6 Qvarnstrom 18S rDNA Target

The Qvarnstrom target area is very conserved and shows perfect homology across a number of different *Acanthamoeba* genotypes.

Figure 7 Rivière 18S rDNA Target

The Rivière target is not quite as conserved as the Qvarnstrom target especially in the region of the reverse primer.

1.7 CEPHEID SMARTCYCLER® II SYSTEM

There are many RT-PCR systems currently on the market all having different features to suit different laboratory environments and workloads. The Cepheid SmartCycler® II instrument (Cepheid, Sunnyvale, CA) is a small, closed PCR system that can be used for conventional PCR and RT-PCR assays to detect DNA or RNA. It utilizes its microprocessor-controlled I-CORE® (<u>I</u>ntelligent <u>C</u>ooling/Heating <u>O</u>ptical <u>Re</u>action) module to amplify and detect target DNA or RNA. Each processing block contains sixteen I-CORE modules so for a small laboratory either research or clinical, it requires little space and is cost effective. A total of six processing blocks can be daisy chained together if required (14).

  The SmartCycler® II utilizes a unique reaction tube, designed to be very thin so as to allow for rapid rates of heating and cooling of the reaction mixture. This allows for rapid amplification. The tube also has two optical windows at 90° to each other along the bottom edges. These windows interface with the I-CORE optical blocks to allow fluorescence excitation and emission detection to occur.

1.8 LOCKED NUCLEIC ACID PROBE

Another aspect of PCR that is being revolutionized is research into primers and probes that bind to DNA or RNA with greater affinity. One such area is the use of Locked Nucleic Acids (LNAs) instead of regular dinucleotides (dNTPs) (17).
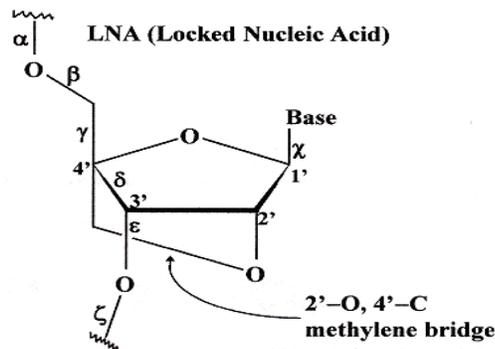
Figure 8 LNA structure

LNAs are different to regular nucleic acids because they contain nucleosides that are characterized by the presence of a methylene bridge between the 2'-O and the 4' C atoms of the ribose ring (Figure 8). The methylene bridge reduces conformational possibilities, and therefore increases the stability between probe and target nucleic acid. The advantage of this for RT-PCR probes is two-fold. Firstly, a probe can be designed more easily to have a higher melt temperature than the primers (an important criteria in RT-PCR assay design) and secondly, the probe will bind with greater affinity to the target.

1.9   PCR INHIBITION

As the demand for PCR testing increases there is more pressure on the clinical laboratory to test samples from many different anatomical sites. PCR technology is advancing quickly and more sensitive methods maybe available but the sensitivity is only as good as the quality of the sample. It has been established that certain exogenous and endogenous factors known as

inhibitors can interfere with the PCR process and result in false negative reactions. They can do this by: interfering with the cell lysis necessary for extraction of DNA, interfering by capture or degradation of nucleic acid, or inhibiting polymerase activity for amplification of target DNA (58). Reported endogenous inhibitors are hemoglobin, lactoferrin and immunoglobulin (Ig) G (4) and a well known exogenous inhibitor is heparin (43).

In Ophthalmology, the push to develop and utilize PCR to diagnose microbial infection over conventional methods is forthcoming (30). However unlike other anatomic sites, the exposed surfaces of the eye in patients, who have ocular infection or are being examined for ocular disease, are continuously flooded with topical medications and dyes to assist in treatment or diagnosis. Dyes such as lissamine green, rose bengal (47) and flourescein which are used to stain epithelial defects (21) have been proven to cause inhibition. Flourescein can also interfere with the detection of the amplified product in RT-PCR TaqMan assays as this technology utilizes fluorescent probes. Topical drugs are also a potential problem. Oxybuprocain, a topical anesthetic, has been recently shown in inhibit PCR (21). Exogenous inhibitors have also been found in ocular samples. Weidbrauk and colleagues reported the inhibitory affects of vitreous samples on PCR (57) however at that time no specific factor/s were identified. Further studies need to be conducted to examine the effects of other topical drugs such as antibiotics and steroids and their effects on PCR.

2.0   GOALS AND SPECIFIC AIMS

The goals of this project were to utilize two published RT-PCR TaqMan methods for the detection of genus – specific *Acanthamoeba* 18S rDNA and adapt them for use on the Cepheid SmartCycler® II system. The SmartCycler® II is a compact, closed system for RT-PCR that is ideal for use in smaller clinical and research laboratories. In adapting the assays to the SmartCycler® II, the two assays would need to be optimized and their limit of detection determined. Additionally the effects of different topical drugs on RT-PCR based detection will also be investigated.

If the assays were shown to display appropriate amplification efficacy then clinical samples will be used to validate the assays for clinical use. A comparison will be made to determine if one assay is more appropriate for use to detect *Acanthamoeba* 18S rDNA from ocular clinical samples than the other.

2.1   SPECIFIC AIM 1: TO OPTIMIZE TWO REAL-TIME PCR ASSAYS TO DETECT *ACANTHAMOEBA* DNA USING THE CEPHEID SMARTCYCLER® II SYSTEM

In order to optimize the two RT-PCR assays, the target DNA for both assays will be cloned using a commercial plasmid vector kit. The vector will be inserted into competent cells and the

competent cells cultured. The target plasmid DNA is isolated from the cells and quantitated. The target plasmid DNA can now be used to optimize the assays for use on the Cepheid SmartCycler® II RT-PCR system and the limit of detection determined. The limit of detection for the *Acanthamoeba* trophozoites and cysts will also be determined.

2.2 SPECIFIC AIM 2: TO EVALUATE THE EFFECTS OF COMMONLY USED TOPICAL OPHTHALMIC DRUGS ON REAL-TIME PCR PERFORMANCE

Many different topical drugs are used in ophthalmology to treat infectious and non-infectious keratitis. Thirteen commonly used drugs were selected. The Rivière RT-PCR assay will be used to determine the possible effects of topical ophthalmic drugs on RT-PCR performance

The first step will be to determine the non-inhibitory drug titer using the Rivière target plasmid DNA. Following this, the second step will be to use the determined non-inhibitory drug titer to serially dilute the target DNA and calculate whether RT-PCR amplification efficiency is being compromised.

2.3 SPECIFIC AIM 3: TO VALIDATE AND COMPARE THE TWO REAL-TIME PCR ASSAYS BY TESTING A NUMBER OF OCULAR CLINICAL SAMPLES

To validate the two RT-PCR assays, retrospective and prospective excess ocular clinical samples from patients with a differential of keratitis will be analyzed. No additional samples were required. DNA extraction will be performed on two aliquots from an individual sample. One

aliquot will be used as *Acanthamoeba* test sample and the second aliquot will act as an internal control. Results for both assays will be tabulated and compared to find out if one assay is performing better than the other.

3.0 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

RT-PCR was performed on excess samples of clinical ophthalmic relevance that were submitted to the laboratory in conjunction with an *Acanthamoeba* culture. No additional samples were collected for the RT-PCR. Routine cornea, conjunctiva and eyelid samples were collected with a sterile cotton tipped swab (Fisher Scientific, Houston, TX) or by scraping the area of infection with a kimura spatula and placed in 2.0ml of Chlamydial Transport medium (Bartels, Bellevue, WA). Tissue or contact lens paraphernalia for *Acanthamoeba* culture was planted directly onto non-nutrient agar and RT-PCR was performed from aliquots taken from the transport medium where viral culture was requested. Contact lens/cases for *Acanthamoeba* RT-PCR were prepared by saturating a sterile cotton tipped swab with the case solution and placing it into 2.0ml of Chlamydial Transport medium. Contact lens solution bottles were prepared by centrifuging at 3000 rpm for 5 minutes, up to 15.0ml of solution, decanting the supernatant and using a sterile cotton tipped swab to absorb any residual liquid precipitate. The swab was then placed into 2.0ml of Chlamydial Transport medium. Samples were stored at -80°C until DNA extraction.

3.2   DNA EXTRACTION

Samples for RT-PCR were thawed and two aliquots from each sample were prepared (300µl for the test sample and 100µl for an internal control). 1µl of Varicella zoster virus (VZV) plasmid DNA containing approximately $3.81x10^5$ copies/µl (prepared in a previous RT-PCR study (30)) was used to act as the internal control target. Aliquots were heated for 10 minutes at 98° C and then placed on ice. The protein was extracted from boiled samples with 150µl MPC protein precipitation solution (Epicentre, Madison, WI) (28). The mixture was vortexed for 10 seconds and centrifuged at 10,000 rpm for 10 minutes at 4° C. The supernatant was transferred to a clean tube. The DNA was extracted from the supernatant by adding 500µl of isopropanol (DNAse, RNAse, Protease free) (Acros Organics, Fisher Scientific, Pittsburgh, PA), inverting the tube 30-40 times, and centrifuging at 10,000 rpm for 10 minutes at 4° C. The supernatant was discarded and the pellet containing the DNA was washed twice with 75% ethanol (500µl) using centrifugation at 10,000 rpm for 5 minutes at 4° C. The remaining pellets were dried under vacuum to eliminate any residual ethanol. The final DNA pellet was resuspended in 45µl of TE buffer (10 mM Tris-HCL [pH 8.0], 1 mM EDTA, Epicentre®, Madison, WI).

3.3   PREPARATION OF CONTROLS

3.3.1   Negative Controls

The non-*Acanthamoebic* negative control isolates utilized, were known to cause ocular infection. They were obtained from the Charles T Campbell Ophthalmic Microbiology Clinical Tissue

Bank. The bacterial and fungal isolates (*Staphylococcus aureus* (4), *Pseudomonas aeroginosa* (4), *Haemophilus influenzae* (3), *Streptococcus pneumoniae* (3), *Nutrionally variant streptococcus* (2), *Streptococcus viridans* (2), *Moraxella species* (1), *Enterococcus faecalis* (1), *Mycobacteria chelonae* (1), *Serratia marcescens* (1), *Escherichia coli* (1), *Bacillus species* (1), *Nocardia farcinica* (1), *Fusarium species* (1), *Alternaria* (1), *Penicillium species* (1), *Aspergillus niger* (1)) used as the non-acanthamoebic negative controls were prepared by suspending overnight growth from solid agar medium (5% sheep's blood or chocolate agar) (BD BBL™, Sparks, MD) to a 0.5 McFarland standard in trypticase soy broth (BD BBL™, Sparks, MD), and pipetting 50µl of the suspension in 500µl of Chlamydia transport medium. 300µl of the transport media was then removed for DNA extraction

Viral isolates *Adenovirus* (ADV) (3), *Herpes simplex virus* (HSV) type 1 (4), VZV (1) were grown in A549 cells (Viromed Laboratories, Minnetonka, MN) at 37° C until cytopathic effect was evident. 300µl of the culture media was then removed for DNA extraction.

A single isolate of *Hartmannella species* was grown axenically in $25cm^2$ tissue culture flask (TPP, Switzerland) containing YPD broth (Teknova Inc, Hollister, CA). It was cultivated at 30° C until 40-50% confluency was present, then 300µl of the YPD broth was then removed for DNA extraction.

3.3.2   Positive Controls

The positive *Acanthamoeba* controls associated with keratitis were obtained from the Charles T Campbell Ophthalmic Microbiology Clinical Tissue Bank (7 strains). In addition, another 7 strains were purchased through the University of Pittsburgh purchasing department from the American Type Tissue Collection (ATCC): *A. lugdenensis* ATCC 24050 (T4), *A. polyphaga*

ATCC 30461 (T4), *A. hatchetti* ATCC PRA-113 (T11), *A. castellanii* ATCC 30010 (T4), *A. rhysodes* ATCC 50368 (T4), *A. culbertsoni* A-1 ATCC 30171 (T10), *A. griffini* TIO:H37 ATCC 50702 (T3). Strains were grown axenically in 25cm^2 tissue culture flask (TPP, Switzerland) containing YPD broth (Teknova Inc, Hollister, CA). They were cultivated at 30° C until 40-50% confluency was present, then 300μl of the YPD broth was then removed for DNA extraction.

To determine the ability of the assays to work in the midst of other DNA, a number of the negative controls were spiked with a low concentration of *Acanthamoeba* plasmid DNA. One isolate each of *Staphylococcus aureus, Pseudomonas aeroginosa, Adenovirus, Herpes simplex virus, Haemophilus influenzae, Streptococcus pneumoniae, Nutritionally variant streptococcus, Moraxella species, Alpha haemolytic streptococcus, Enterococcus faecalis, Mycobacteria chelonae, Serratia marcescens, Hartmannella species, Bacillus species* were used. 20μl of extracted isolate DNA was mixed with 1μl of $1x10^{-6}$ dilution of *Acanthamoeba* plasmid DNA (Rivière $1.13x10^5$ copies/reaction, Qvarnstrom $4.38x10^3$ copies/reaction).

3.4   PRIMERS AND PROBES

Table 1 describes the primers and probes for detecting *Acanthamoeba spp* using two different RT-PCR assays and VZV RT-PCR (internal control), *Acanthamoeba spp* for conventional PCR and the *Acanthamoeba* internal sequencing primers. All primer and probe sequences were verified theoretically through the GenBank database prior to being synthesized by Integrated DNA Technologies (Coralville, IA). The TaqMan probes for the two *Acanthamoeba spp* RT-PCR assays were constructed using locked nucleic acids (LNA).

3.5   SMARTCYCLER® II REACTION MIX

All PCR reactions were carried out on the Cepheid SmartCycler® II RT-PCR system (Cepheid, Sunnyvale, CA). Each PCR reaction contained 15μl of master mix and 10μl of patient or control sample placed in 25μl SmartCycler® II tubes. The master mix for two reactions comprised of a forward primer, reverse primer, probe, DNAase-free water, and an OmniMix® HS bead (3 units TaKaRa Hot Start Taq™ polymerase, 200μM dNTP, and 4mM $MgCl_2$ in 25mM HEPES buffer, pH 8.0±0.1). The concentrations of forward primer, reverse primer, and probe in the final reaction tube for the two *Acanthamoeba* spp RT-PCR assays were determined to be the same at 0.4μM, 0.4μM, and 0.3μM, respectively; VZV were 0.3μM, 0.3μM, and 0.2μM, respectively (after testing a number of concentration combinations at the specific melt temperatures); *Acanthamoeba spp* conventional PCR forward and reverse primers were 0.3μM, 0.3μM, respectively; and *Acanthamoeba* internal sequencing primers 3.4pmole.

3.6   SMARTCYCLER® II PCR THERMAL CYCLING

The Qvarnstrom RT-PCR settings were set for *Acanthamoeba spp* in two stages: Stage 1) 95°C for 2 minutes to activate the "Hot Start" Taq polymerase, and Stage 2) 45 cycles of 95°C for 15 seconds (denaturing); 55°C for 30 seconds (annealing); and, 72°C for 30 seconds (extension) with a temperature increase of 0.5°C per second.

The Rivière RT-PCR for *Acanthamoeba spp* and VZV RT-PCR settings were set in two stages: Stage 1) 95°C for 2 minutes to activate the "Hot Start" Taq polymerase, and Stage 2) 45

cycles of 95°C for 15 seconds (denaturing); 60°C for 30 seconds (annealing); and, 72°C for 30 seconds (extension).

The conventional PCR settings for the Booton PCR (larger PCR product for *Acanthamoeba* sequencing): Stage 1) 95°C for 2 minutes to activate the hot-start Taq-polymerase, and Stage 2) 45 cycles of 95°C for 60 seconds (denaturing); 60°C for 60 seconds (annealing); and, 72°C for 120 seconds (extension).

3.7 *ACANTHAMOEBA* SEQUENCING

Samples that were RT-PCR positive by both methods but were culture negative were PCR assayed again using conventional PCR to amplify a larger target region on the 18S rDNA (10). The amplified product was confirmed by 6% Polyacrylamide Gel Electrophoresis (PAGE) and visualized by 0.5 μg/ml ethidium bromide. The remaining amplified product was purified using the QIAquick® PCR cleanup kit (Qiagen Sciences, Valencia, CA) and prepared for sequencing at the University of Pittsburgh Core DNA Sequencing facility maintained by the School of Medicine's Biomedical Research Support Facility. The facility uses the ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) for this purpose. Preparation of each reaction include the addition of the sequencing primer 3.4pmol (Table 1), Nuclease-free, PCR quality water, and the amplified DNA to a total reaction volume of 13μl. Chromatogram results were emailed back from sequencing facility and examined using Sequencher™ software (Gene Codes Corporation, Ann Arbor MI) and results were then compared to catalogued *Acanthamoeba* sequences in GenBank (NCBI, Bethesda, MD).

25

3.8 SMARTCYCLER® II RESULT INTERPRETATION

The Cycle Threshold (Ct) is the cycle that denotes a significant amplification of target DNA over the background threshold and is deemed positive. Results that do not cross the threshold are deemed negative. These results are shown in 'Real-time' on the instruments display.

In this study, each sample was initially tested individually, with a test sample (Rivière and Qvarnstrom RT-PCR assays) and an internal control (VZV RT-PCR assay). If the test sample was negative for *Acanthamoeba* DNA and the internal control was positive then the sample was considered 'Negative'. If the test sample was positive for *Acanthamoeba* DNA by one or both RT-PCR assays, it was repeated in duplicate. If the result was still positive then the sample was considered to be 'Positive'. However if the result could not be reproduced then the sample was considered to be 'Equivocal'. If the test was negative for *Acanthamoeba* DNA and the internal control was also negative, the sample was re-extracted and tested again. If the internal control was still negative, the sample was considered to be 'Inhibitory'.

3.9 PLASMID DNA PREPARATION

All plasmid DNA (Rivière *Acanthamoeba* target DNA, Qvarnstrom *Acanthamoeba* target DNA, Watzinger VZV target DNA) was prepared using the pGEM®-T Easy Vector System (Promega Corporation, Madison, WI). The target DNA was amplified using the Smartcycler® II and the PCR product ligated into the pGEM®-T Easy plasmid (14°C overnight). DH10B™ competent cells (Invitrogen, Carlsbad, CA) were transformed by electroporating (*E.coli* Pulser, Bio-Rad, Hercules, CA) them with the ligated plasmid (Incubated for 45 minutes at 37°C in shaking

incubator). The DH10B™ cells were then plated onto LB / Ampicillin agar plates (prepared in house with LB agar, Ampicillin (200 µg/ml), 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) (x1000), and 3% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) (x375)) which were incubated aerobically at 35^oC overnight. If target DNA had been inserted correctly into the plasmid and the plasmid electroporated into the DH10B™ cells then white colonies would be present. Blue colonies indicate no insertion. Next day, white colonies were inoculated into LB/Ampicillin liquid media and cultured overnight in the shaking incubator at 37^oC. The plasmid DNA was extracted using the Wizard Miniprep kit (Promega Corporation, Madison, WI). The DNA was linearized from the plasmid using the restriction enzyme EcoR1 (New England Biolabs, MA) by incubating for 2 hours in a 37^oC water bath. Electrophoresis (Bio-Rad, Hercules, CA) using 6% PAGE run at 100v for about 45 minutes and stained with 0.5 µg/ml Ethidium bromide (Sigma-Aldrich, St. Louis, MO) and viewed using a UV trans-illuminator (Bio-Rad, Hercules, CA) to show the presence of correct weight plasmid DNA. More of the plasmid was purified using the Wizard plus Miniprep kit the next day. Concentration of the DNA was measured using a Beckman spectrophotometer (Beckman Coulter Inc, Fullerton, CA) and calculated using the constant 1.0 A_{260} unit ds DNA = 50 µg/ml. PCR optimization, amplification efficiency and the limit of detection of the primers and probes for the two *Acanthamoeba* assays were validated using serial dilutions of the respective cloned PCR product. Amplification efficiencies (E) for the two *Acanthamoeba* assays were calculated using the equation: $E = 10^{(-1/slope)} -1$. The slopes were determined from regression plots (Ct versus dilution of Target DNA) using the known amounts of target plasmid DNA. Regression plots and slope were calculated using Minitab 10 statistical software (Minitab Inc., State College, PA). From the line equation, Y

= b + aX, "a" is the slope of the regression line. Acceptable amplification efficiency is between 90%-105%. The VZV plasmid used had been validated in a previous study (30).

3.10 TROPHOZOITE DILUTION

To determine the detection limit of the two RT-PCR *Acanthamoeba* assays using *A. polyphaga* (ATCC 30461) trophozoites, the trophozoites were grown in YPD broth until flask was confluent (24–48 hours). YPD broth was removed and trophozoite monolayer was washed twice with fresh YPD to remove any cysts. Flask was placed in ice for about 30 minutes to 1 hour to detach the trophozoites (modified method from Qvarnstrom et al, 2006). Contents of the flask were transferred to a chilled 15ml Falcon tube and centrifuged for 5 minutes at 2500 rpm at 4°C. Supernatant was removed and trophozoites were washed in 2ml 0.85% saline and centrifuged for 5 minutes at 2500 rpm at 4°C. Step was repeated. Trophozoites were counted using a Hemocytometer (Bright-Line, Buffalo, NY). Concentration was adjusted to 1x10^{4} trophozoites/ml. Trophozoites were serially diluted twofold down to less than one *Acanthamoebic* trophozoite per 300μl sample (300μl chosen because it was the starting extraction volume) in Bartels Chlamydia transport media.

3.11 CYST DILUTION

To determine the detection limit of the two RT-PCR *Acanthamoeba* assays using *A. polyphaga* (ATCC 30461) cysts, firstly the trophozoites were grown in YPD broth until flask was confluent

(24–48 hours). YPD was decanted and 10ml of 8% Glucose in RPMI 1640 (Invitrogen Corp., NY) is added (to induce encystment). Flask was incubated at $30^{\circ}C$ for up to 48 hours. To ensure that only cysts are present, sodium dodecyl sulfate (SDS, 0.5% final concentration) was added (SDS will lyse trophozoites) (26). Contents of the flask was transferred to a 15ml falcon tube and centrifuged at 2500 rpm for 5 minutes. Media was decanted and cysts washed in 2ml of 0.85% saline, followed by centrifugation at 2500 rpm for 5 minutes. Saline was decanted and cysts washed again in 2ml of 0.85% saline followed by another spin at 2500 rpm for 5 minutes. The saline was decanted and 2ml of Bartels Chlamydia transport media was added. Cysts were counted using a Hemocytometer. Volume was adjusted to 1×10^4 cysts/ml. The cysts were then serially diluted twofold down to less than one *Acanthamoebic* cyst per 300µl sample (300µl chosen because it was the starting extraction volume) in Bartels Chlamydia transport media.

3.12   EFFECTS ON RT-PCR EFFICIENCY BY NON-INHIBITORY DRUG TITER

3.12.1   Determination of Non-Inhibitory Drug Concentration using RT-PCR

Each of the ophthalmic drugs were serially diluted with TE buffer to include dilution factors of 1, 10, 20, 40, 80, 160, 320 (640, 1280, 2560, 5120, 10240, 20480, 40960 as needed). To each drug 1µl of 1×10^{-6} dilution of Rivière *Acanthamoeba* plasmid DNA (1.13×10^5 copies/µl) was added. The drugs used were Proparacaine[®], 0.5% (Falcon Pharmaceuticals, Fort Worth, TX), Zymar[®], 0.3% (Allergan, Irvine, CA), Vigamox[®], 0.5% (Alcon Laboratories, Fort Worth, TX), Econopred Plus[®], 1.0% (Alcon Laboratories, Fort Worth, TX), Xibrom[™], 0.09% (ISTA Pharmaceuticals, Irvine, CA), Nevanac[™], 0.1% ((Alcon Laboratories, Fort Worth, TX),

Tobramycin, 1.4% (MP Biomedicals, Solon, OH), Cefazolin, 5.0% (MP Biomedicals, Solon, OH), Amphotericin B, 0.15% (MP Biomedicals, Solon, OH), Trifluridine®, 1.0% (Falcon Pharmaceuticals, Fort Worth, TX), Brolene®, 0.1% (Aventis Pharma, Auckland, New Zealand), Polyhexamethylene biguanide (PHMB), 0.02% (Leiter's Pharmacy, San Jose, CA), and Chlorhexidine, 0.02% (Leiter's Pharmacy, San Jose, CA). Corneal tissue was also used also to see if it contained any endogenous inhibitors. The corneal tissue was obtained from donor cornea rims. It was weighed (0.172 g/ml), then prepared by homogenizing with the Pro 200 tissue homogenizer (PRO Scientific Inc, Monroe, CT). When an even suspension was evident, dilutions were made using TE buffer.

Samples that had non-inhibitory dilutions of ≥1/20 were deemed to be noteworthy and would require a repeat non-inhibitory dilution with pre and post DNA extraction determination. ≥1/20 was deemed to be important because it is felt that in vivo, a lower drug dilution would be diluted out during the specimen collection process or removed during the DNA extraction process. Also the concentration of drug penetrating the cornea tissue would be much less then the concentrations of drug found in vitro.

3.12.2 Effect of Non-Inhibitory Drug Concentration on RT-PCR Amplification Efficiency

Once the non-inhibitory drug dilution was determined, the concentrated Rivière *Acanthamoeba* plasmid DNA (381.2 μg/ml) was diluted using the lowest uninhibited drug titer. The amplification efficiency was then determined. The plasmid DNA dilutions were: $1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$, $1x10^{-6}$, $1x10^{-7}$, $1x10^{-8}$, and $1x10^{-9}$. 10μl was used in the Rivière RT-PCR assay and the

RT-PCR was run in duplicate. The regression plot and amplification efficiency were determined as previously described in 3.7.

4.0   RESULTS

4.1   THE OPTIMIZATION OF TWO REAL-TIME PCR ASSAYS TO DETECT *ACANTHAMOEBA* DNA USING THE CEPHEID SMARTCYCLER® II SYSTEM

4.1.1   Rivière RT-PCR Assay: Optimization, Limit of Detection and Amplification Efficiency

Stock primers and probe were reconstituted when received from IDT. A number of different primer/probe concentrations were assayed on the Smartcycler® II and the final combination selected was a working primer concentration of 0.4μM and working probe concentration of 0.3μM (Figure 9). Working concentrations were based on the criteria of an early Ct value and the appearance of a sigmoidal (S) shape curve (indicative of an efficient RT-PCR reaction).

Figure 9 Rivière RT-PCR Optimization

32

The plasmid DNA was serially diluted to determine the limit of detection. 1×10^{-11} was the lowest positive dilution of *Acanthamoeba* plasmid DNA detected. The starting concentration of plasmid DNA was determined to be 381.2 µg/ml based on the initial O.D value from the spectrophotometer. Taking into account the size of the pGEM®-T vector and target DNA, the starting concentration and the lowest dilution amplified, the copy number and the limit of detection for the *Acanthamoeba* target were calculated to be 11.3 copies/10µl and 38.12 attograms DNA.

The RT-PCR efficiency was determined using the serially diluted plasmid DNA (Figure 10). The determined Ct values and dilution factor were used to calculate the linear regression in the Minitab statistical software (Figure 11). The correlation coefficient was calculated by Minitab to be 99.8% indicating good correlation between the points and the slope (-3.47196) was used to calculate the amplification efficiency of 94%.

Figure 10 Rivière RT-PCR Amplification Efficiency

Figure 11 Rivière RT-PCR Regression Analysis

4.1.2   Qvarnstrom RT-PCR Assay: Optimization, Limit of Detection and Amplification Efficiency

Stock primers and probe were reconstituted when received from IDT. A number of different primer/probe concentrations were assayed on the SmartCycler® II and the final combination selected was a working primer concentration of 0.4μM and working probe concentration of 0.3μM (Figure 12). Working concentrations were based on the criteria of an early Ct value and the appearance of a sigmoidal (S) shape curve (indicative of an efficient RT-PCR reaction).
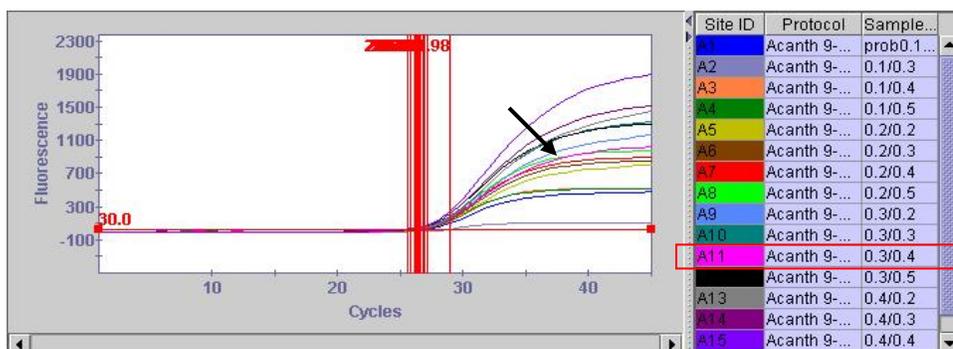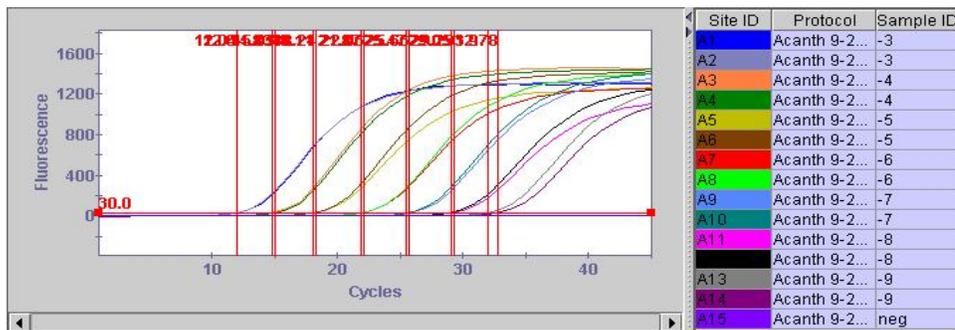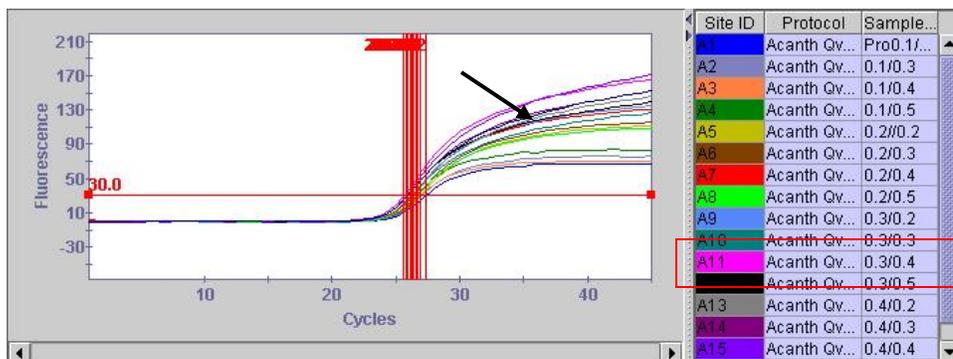

Figure 12 Qvarnstrom RT-PCR Optimization

The plasmid DNA was serially diluted to determine the limit of detection. 1×10^{-9} was the lowest positive dilution of *Acanthamoeba* plasmid DNA detected. The starting concentration of plasmid DNA was determined to be 15.375 μg/ml based on the initial O.D reading from the spectrophotometer. Taking into account the size of the pGEM®-T vector and target DNA, the starting concentration and the lowest dilution amplified, the copy number and the limit of detection for the *Acanthamoeba* target were calculated to be 43.8 copies/10μl and 153.75 attograms DNA.

The RT-PCR efficiency was determined using the serially diluted plasmid DNA (Figure 13). The determined Ct values and dilution factor were used to calculate the linear regression in the Minitab statistical software (Figure 14). The correlation was calculated to be 99.2% indicating good correlation between the points and the slope (-3.53123) was used to calculate the amplification efficiency of 92%.

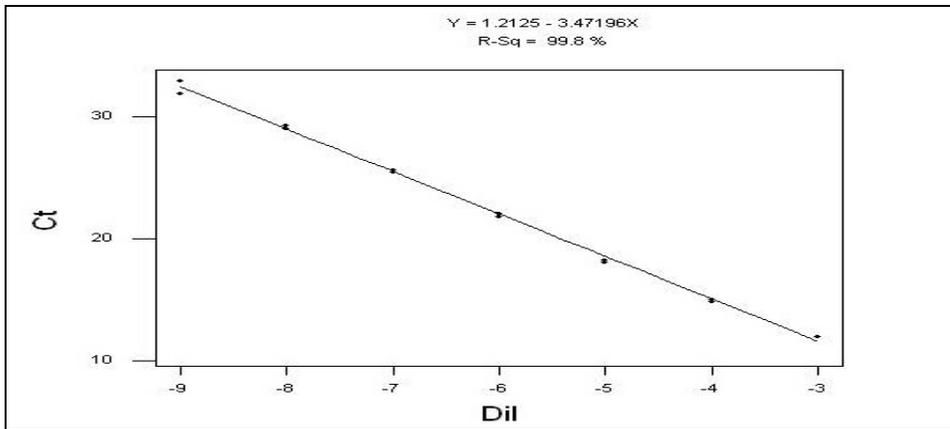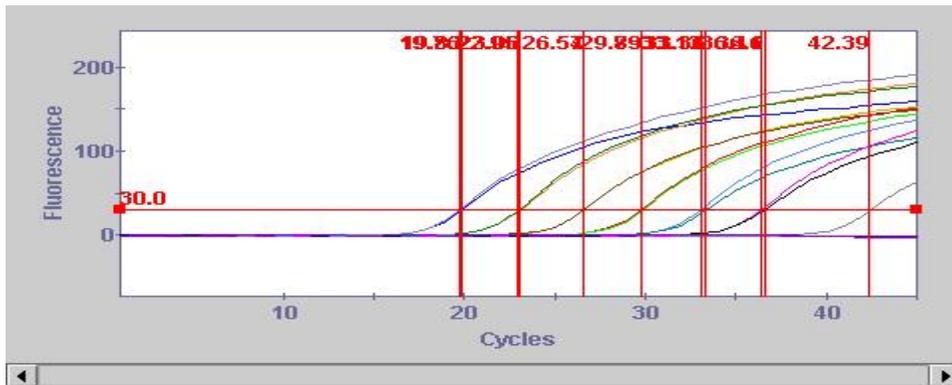**Figure 13 Qvarnstrom RT-PCR Amplification Efficiency**

Y = 8.87522 - 3.53123X
R-Sq = 99.2 %
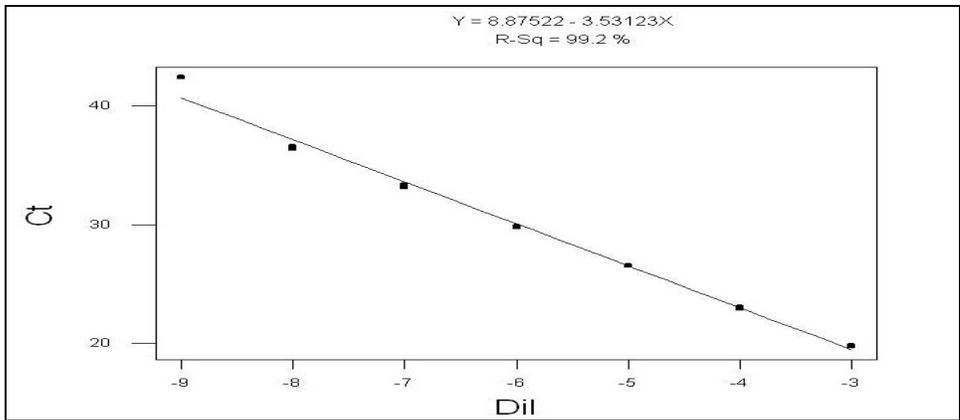
Figure 14 Qvarnstrom RT-PCR Regression Analysis

4.1.3   Rivière and Qvarnstrom RT-PCR: Trophozoite Limit of Detection

A. polyphaga ATCC 30461 trophozoites were prepared as per the materials and methods including growth in axenic culture, trophozoite serial dilution and DNA extraction of 300µl. RT-PCR was then run for both assays. The limit of detection for both assays was determined to be the same at 2.3 ± 1.7 trophozoites per 300µl (Figure 15 and 16).
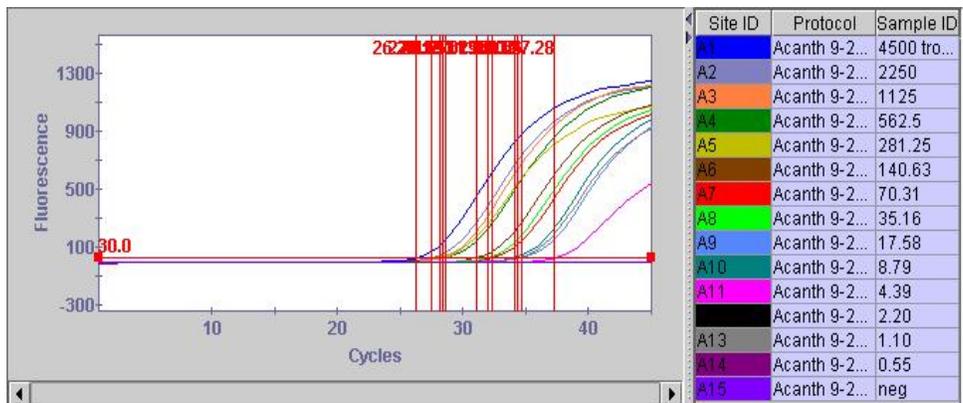
Figure 15 Rivière RT-PCR Trophozoite Limit of Detection
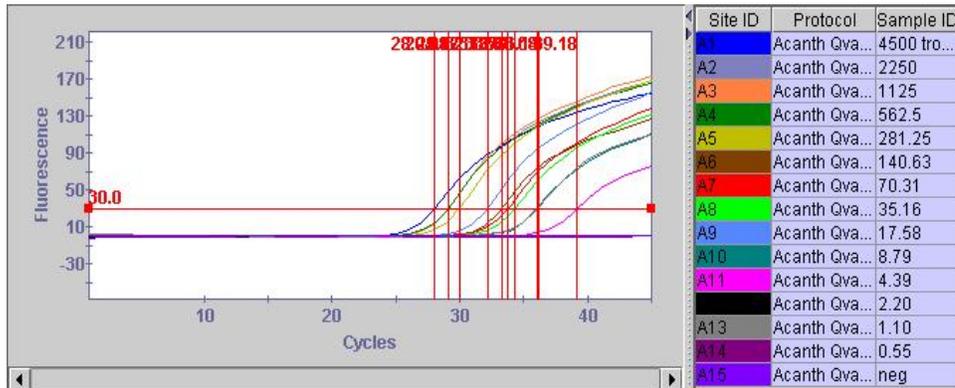
Figure 16 Qvarnstrom RT-PCR Trophozoites Limit of Detection

4.1.4   Rivière and Qvarnstrom RT-PCR: Cyst Limit of Detection

A. polyphaga ATCC 30461 cysts were prepared as per the materials and methods including growth in axenic culture, cyst serial dilution and DNA extraction of 300µl. RT-PCR was then run for both assays. The limit of detection for both assays was determined to be the same at 0.7 ± 0.0 cysts per 300µl (Figures 17 and 18).
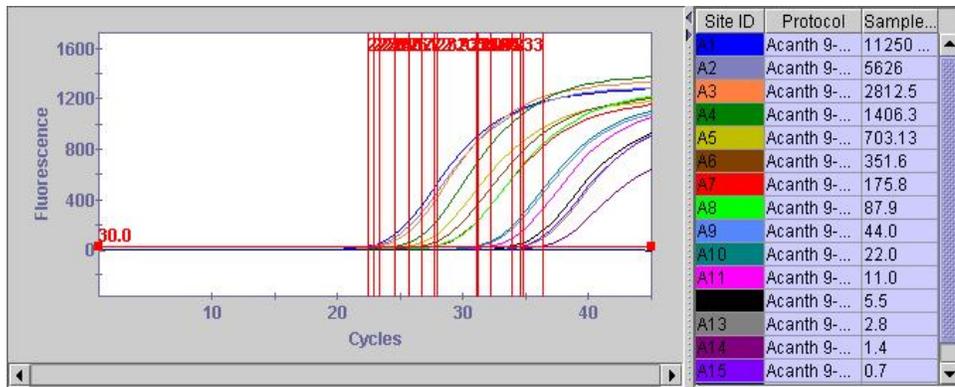
Figure 17 Rivière RT-PCR Cyst Limit of Detection
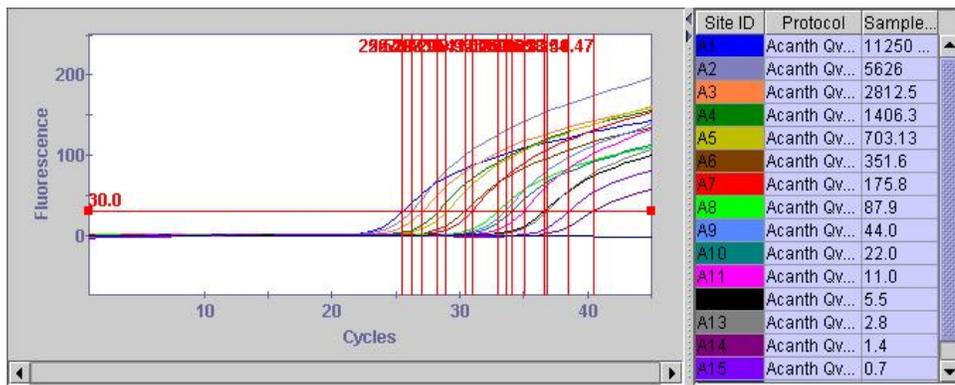
Figure 18 Qvarnstrom RT-PCR Cyst Limit of Detection

4.1.5   Negative Controls

Thirty eight non–*Acanthamoebic* negative controls which consisted of bacteria, virus, fungi and free-living amoeba were prepared as per materials and methods and run using both RT-PCR assays. All isolates (38/38) were negative by both assays indicating no cross-reactivity between the primer-probe sets and the non-*Acanthamoebic* DNA (Table 3).

To illustrate the ability of the RT-PCR assays to detect the *Acanthamoeba* DNA in the midst of non-specific microbial DNA, 15 of the negative strains were spiked with *Acanthamoeba* plasmid DNA from either target. All spiked samples were positive (15/15) for both assays (Table 4).

4.1.6   Positive Controls

The set of in-house positive *Acanthamoeba* controls and the strains purchased from the ATCC were prepared as per the materials and methods and run using both RT-PCR assays. All strains were positive by the Qvarnstrom assay (14/14), however only 13/14 were positive by the Rivière assay. The one strain that was negative was the *A. culbertsoni* A-1 ATCC 30171 (Table 5), a T10 genotype that has not been associated with keratitis.

4.2   THE EVALUATATION OF COMMONLY USED TOPICAL OPHTHALMIC DRUGS ON REAL-TIME PCR PERFORMANCE

4.2.1   Determination of Non-Inhibitory Concentration using RT-PCR

Each of the drugs and the corneal tissue were prepared as per the materials and methods and RT-PCR run targeting the Rivière plasmid DNA. It was ascertained that the RT-PCR was not inhibited by Proparacaine®, Econopred Plus®, Xibrom™, Tobramycin, Amphotericin B and the corneal tissue. Nevanac™, Zymar®, Vigamox®, Cefazolin, Brolene® and Chlorhexidine were

found to have a non-inhibitory concentration of 1/10. Trifluridine® was found to have a 1/40 non-inhibitory concentration and PHMB was found to have a non-inhibitory concentration of 1/2560 (Table 6).

The Trifluridine® was repeated and was found again to have a 1/40 non-inhibitory concentration pre DNA extraction. However after the DNA extraction the Trifluridine® was found not to be inhibitory (1/1).

PHMB has a short shelf life once in solution so fresh compound needed to be prepared (Leiter's Pharmacy, CA). Dilutions were prepared using the new lot of PHMB and the pre extraction RT-PCR run. The non-inhibitory concentration had dropped two-fold from the previous 1/2560 dilution to 1/640. Despite being two-fold less the 1/640 dilution was still considered significant. The DNA extraction was performed but the dilution only improved by one two-fold dilution to 1/320. PHMB therefore could potentially be a problem for RT-PCR testing (Table 7).

4.2.2 Effect of Non-Inhibitory Drug Concentration on RT-PCR Amplification Efficiency

Using the non-inhibitory drug and cornea tissue dilution, the concentrated Rivière plasmid DNA was serially diluted as per the materials and methods and the RT-PCR run. Considering that adequate amplification efficiency is in the range 90% - 105%, the following drugs were shown to have acceptable efficiencies: Proparacaine® (100.1%), Econopred Plus® (91.2%), Xibrom™ (97.4%), Tobramycin (102.9%), Nevanac™ (95.0%), Zymar® (92.6%), Vigamox® (92.0%), Cefazolin (91.6%) and Trifluridine® (94.6%) (Table 6). The cornea tissue (86.9%), Amphotericin B (108.7%), Brolene® (85.7%) and Chlorhexidine (77.0%) had efficiencies outside the

recommended range but considering their low non-inhibitory concentrations in procedure 4.2.1, the efficiency would probably improve with a slight increase in dilution which would occur in vivo during the specimen collection process. These concentrations in vitro are much higher than would be found in the cornea in vivo. The most significant result was that of PHMB. With its very high non-inhibitory dilution, it was shown to inhibit RT-PCR amplification efficiency (72.7%). It is unlikely that the amplification efficiency would improve during the specimen collection process and therefore would be grounds for concern when clinical samples are collected from patients suspected of having *Acanthamoeba* and have started treatment with PHMB.

4.3   THE VALIDATION AND COMPARISON OF REAL-TIME PCR ASSAYS USING OCULAR CLINICAL SAMPLES

One hundred and thirty nine ocular clinical samples were tested for genus-specific *Acanthamoeba* 18S rDNA by the Rivière and Qvarnstrom RT-PCR assays using the Cepheid Smartcycler® II. Among them 93 (66.9%) were cornea samples, 32 (23.0%) were contact lens/cases, 12 (8.6%) were contact lens solution bottles, 1 eyelid (0.7%) and 1 (0.7%) eyelid/conjunctiva. The two assays performed comparatively with 134/139 samples (96.4%) (Tables 8 and 9) showing consensus. Of the 134 results, 5 samples were culture positive and RT-PCR positive for both assays, 4 samples were culture negative and RT-PCR positive for both assays, 2 samples were culture negative and RT-PCR inhibitory for both assays, 1 sample was culture negative and RT-PCR equivocal for both assays (Table 9) and 122 samples were culture negative and RT-PCR negative for both assays.

Of the 5 samples that had results that did not show consensus between the two RT-PCR assays, 1 sample was positive by the Rivière RT-PCR but negative by the Qvarnstrom RT-PCR (sample could not be amplified using the Booton primer set for conventional PCR either and therefore the attempts at sequencing were unsuccessful), 3 samples were equivocal by the Rivière RT-PCR but negative by the Qvarnstrom RT-PCR and 1 sample was inhibitory by the Rivière RT-PCR but negative by the Qvarnstrom RT-PCR (Table 9).

The 4 samples that were culture negative but positive in RT-PCR (by both assays) were amplified using the Booton primers, confirmed by 6% PAGE then sent to the core sequencing facility with the internal sequencing primers. All 4 samples produced sequences that matched with other *Acanthamoeba* sequences in the NCBI GenBank database (Tables 10–13).

5.0   DISCUSSION

The popularity for wearing contact lenses has grown as an alternative to prescription glasses. In 1998 there was an estimated 33 million contact lens wearers (8) in the United States alone. Current incidence rates for *Acanthamoeba* keratitis (AK) of 1-2 cases per million (45) are based on CDC data from a multi-state investigation from the mid 1980s. AK is not a notifiable disease, so the incidence rates are probably higher than twenty years ago, especially in light of reported increases in cases recently in Chicago (25) and Philadelphia (51), and links to *Acanthamoeba* contamination in a popular contact lens solution (1).

Because of the severity of the disease, the best and most efficient diagnostic methods should be utilized on a routine basis. The established methods such as cytology and culture for *Acanthamoeba* do not need to be superseded because they still serve a purpose in the clinical laboratory but can be enhanced by the rapid and sensitive RT-PCR. Together with the clinical examination which may include Heidelberg Retina Tomography II (HRTII) imaging (11) (HRTII is a high resolution digital scanning microscopy which can be used to examine the cornea at 800x magnification), a more definitive and positive outcome is possible.

The basis for this study was to compare the Rivière and Qvarnstrom TaqMan assays to determine if one was more reliable in detecting genus-specific *Acanthamoeba* 18S rDNA from clinical ocular samples. The Rivière assay was designed against *Acanthamoeba* strains in the T4 genotype which has been associated extensively with keratitis and some cases of encephalitis.

43

The Qvarnstrom assay was designed against multiple strains of *Acanthamoeba* from different genotypes. The advantage of designing such a broad based assay is that there is an opportunity to target more *Acanthamoeba*-associated diseases such as Granulomatous Amoebic Encephalitis (GAE), Cutaneous Acanthamoebiasis in addition to AK. The Qvarnstrom assay was also triplexed against two other free-living amoeba which cause encephalitis, *Balamuthia mandrillaris* and *Naegleria fowleri.*

In this study, the two assays were optimized for use on the Cepheid SmartCycler® II RT-PCR system. Both assays performed appropriately in terms of their ability to detect low levels of *Acanthamoeba* DNA (Table 2). The Rivière assay had a lower limit of detection for DNA copies /µl than did the Qvarnstrom assay but this was not considered a problem due to *Acanthamoeba* containing over 600 copies of the target in its ribosomal gene repeat unit per organism. Both assays were shown not to cross-react with other known ocular pathogens. 14 *Acanthamoeba* strains were tested by both assays. Qvarnstrom was able to detect 14/14 however Rivière only detected 13/14. The one strain that it did not detect was the T10 strain *A. culbertsoni* A-1 ATCC 30171 which has not been associated with keratitis. On examination as to why the Rivière assay did not detect this strain, the multi sequence alignment illustrates that the reverse primer (TaqAcR1) shows little homology with the representative GenBank strain of *A. culbertsoni* AF019067 (Figure 7).

During the validation process, 139 ocular clinical samples were tested and 134 results showed consensus overall (96.4%) between the two assays. These included 5 samples that were culture positive and RT-PCR positive, 121 culture negative and RT-PCR negative and 4 samples that were culture negative and RT-PCR positive. All 4 were confirmed by DNA sequencing. This highlights the fact that RT-PCR is an important tool in diagnosing AK and corroborates with

other previous PCR based studies (53, 59). There was an initial concern when running RT-PCR for detection of an organism such as *Acanthamoeba* which is so ubiquitous in nature. One might expect substantial contamination from environmental strains of *Acanthamoeba* especially when testing contact lens paraphernalia. The 4 samples that were RT-PCR positive but negative by culture can not be regarded as false positives because the samples were collected from patients with a differential of microbial keratitis. Future perspectives maybe to look at contact lens paraphernalia from patients with no differential of microbial keratitis (true negatives) to determine *Acanthamoeba* contamination rates.

Results from 5 samples did not agree between the two assays. 1 result (Sample 11) was positive by the Rivière assay but negative by the Qvarnstrom assay, 3 results (Samples 14, 57 and 63) were equivocal by the Rivière assay but negative by the Qvarnstrom assay and 1 result (Sample 93) was inhibitory by the Rivière assay but negative by the Qvarnstrom assay. Sample 11 was rerun a number of times either from the original DNA extract and from the original sample and consistently the result was Rivière assay positive and Qvarnstrom assay negative. The sample was also assayed by conventional PCR using the primer set designed for the larger target amplicon for DNA sequencing. But following 6% PAGE no band was present. An explanation could be a combination of the Rivière assay being slightly more sensitive as shown in the first specific aim in terms of its limit of detection and a lack of reproducibility at the higher Ct values. The Ct value was quite elevated from the Rivière assay which means that there was a minimal amount of target DNA to begin with. Because the Qvarnstrom assay had a slightly higher limit of detection there is a chance that it did not detect the very low level of *Acanthamoeba* DNA present in the sample. Reproducibility issues at the higher Ct could be the reason why Samples 14, 57, 63 and 114 had equivocal results also. Many RT-PCR assays now

employ cut-offs or gray zone values at which a certain Ct value is utilized rather than relying on a positive curve at the end of the programmed run cycles. If the patient sample Ct falls beyond the cut-off point it is deemed negative or if it falls in the gray zone it is deemed indeterminate and may be cause for a repeat sample to be collected. To determine these limitations many positive clinical samples would be required. In doing so, positive culture results could be compared with the respective Ct values. Patients with suspected AK who have negative culture but have positive RT-PCR Ct values would also be needed.

Sample 93 result may be explained simply by the fact that many of the samples were RT-PCR tested with the Rivière assay first. There is a possibility that during the extra freeze-thawing process that the inhibitors were inactivated by the time the sample was run by the Qvarnstrom assay.

In looking at the comparative data thus far and knowing the history and development of both assays, it would be easy to say that the Qvarnstrom assay was the better of the two because it was designed to detect a broader range of *Acanthamoeba* genotypes. This may be the case in laboratories where the need to diagnose systemic *Acanthamoeba* infection is required. However in situations where *Acanthamoeba* keratitis diagnosis is required, the Qvarnstrom assay maybe too broad because it has the potential to detect *Acanthamoeba* environmental genotypes that have not been associated with keratitis especially if contact lens paraphernalia is being tested. Essentially more samples are required to substantiate this point.

PCR inhibitory factors are a major concern in any clinical molecular laboratory as false negative results can be life-threatening. In Ophthalmology, a gamut of topical drugs are used to treat or relieve the symptoms of the infection. Unfortunately, if PCR testing is required to diagnose a specific disease, these same drugs could potentially inhibit the assay. In this study a

46

number of different antimicrobials, steroidal and non-steroidal drugs were evaluated for their effect on the detection of *Acanthamoeba* plasmid DNA using the Rivière assay. Twelve topical drugs were ruled out as being potential exogenous sources of PCR inhibitors. The most noteworthy finding was that PHMB inhibited the RT-PCR at high dilutions despite having an extraction procedure. Interestingly, in the context of this thesis, PHMB is a commonly used drug in the treatment of *Acanthamoeba* infection. This finding provides enough evidence to make recommendations to the clinical staff on when a sample for *Acanthamoeba* RT-PCR should be collected. Ideally the sample should be taken before PHMB therapy is initiated to avoid any complications.

6.0   CONCLUSIONS AND PUBLIC HEALTH RELEVANCE

In conclusion, the Cepheid SmartCycler® II RT-PCR system was successfully used to adapt two published TaqMan methods for detecting genus-specific *Acanthamoeba* 18S rDNA. The assays were successfully optimized and then shown to have appropriate limits of detection in terms of DNA copy number, trophozoites and cysts. A number of topical drugs were tested to determine any possible inhibitory affects on RT-PCR. Out of 13 different drugs tested, PHMB was found to inhibit the RT-PCR in a significant manner with a high dilution of 1/320 post extraction. This is sufficient evidence to make recommendations to the clinical staff that samples need to be collected for *Acanthamoeba* RT-PCR before PHMB treatment is started.

Both assays were found to be comparable for detecting Acanthamoeba 18S rDNA from ocular samples. 139 clinical samples were assayed in the validation process and 96.4% of results were in consensus overall. Both assays detected 4 more positives than culture highlighting again that PCR is more sensitive than culture, as has been shown in previously published papers (59).

The public health relevance of this study was to utilize RT-PCR in diagnosing AK and to study the effects of a group of topical drugs on RT-PCR performance. RT-PCR is a more rapid and reliable tool to diagnose *Acanthamoeba* in the laboratory. By diagnosing AK early in the infection, pain and suffering to the patient can be minimized by allowing the appropriate treatment to be initiated sooner. If diagnosis is delayed, complications of the disease process such as uncontrolled inflammation can occur, resulting in surgical intervention including corneal

transplantation or loss of sight. By understanding which topical drugs interfere with RT-PCR, this study hopes to reduce the potential for false negative results and possible delays in turn around times.

Table 1 PCR Primer and Probe Sequences

Pathogen	Target	Size (bp)	Primers and Probe
Acanthamoeba spp (Rivière)	18S rDNA	65	Forward Primer TaqAcF1: 5' CGA CCA GCG ATT AGG AGA CG 3' Reverse Primer TaqAcR1: 5' CCG ACG CCA AGG ACG AC 3' LNA TaqAcP1: 5' / 56-FAM / A +CA +CCA +CCA TCG GCG C / 3 BHQ_1 / 3'
Acanthamoeba spp (Qvarnstrom)	18S rDNA	180	Forward Primer AcantF900: 5' CCC AGA TCG TTT ACC GTG AA 3' Reverse Primer AcantR1100: 5' TAA ATA TTA ATG CCC CCA ACT ATC C 3' LNA AcantP1000: 5' / 5Cy5 / TG +C CA +C CGA A +TA +CA / 3 BHQ_2 / 3'
VZV (55)	ORF 38	82	Forward Primer: 5' AAG TTC CCC CCG TTC GC 3' Reverse Primer: 5' TGG ACT TGA AGA TGA ACT TAA TGA AGC 3' Probe: 5' FAM-CCG CAA CAA CTG CAG TAT ATA TCG TCT CA-TAM 3'
Acanthamoeba spp (Booton)	18S rDNA	470	Forward Primer JDP1: 5' GGC CCA GAT CGT TTA CCG TGA A 3' Reverse Primer JDP2: 5' TCT CAC AAG CTG CTA GGG GAG TCA 3'
Acanthamoeba spp	18S rDNA		Internal Sequencing Primers 892: 5' CCA AGA ATT TCA CCT CTG AC 3' 892C: 5' GTC AGA GGT GAA ATT CTT GG 3'

+: position of Locked Nucleic Acid

Table 2 Summary of RT-PCR Optimization

	Rivière	Qvarnstrom
Lowest Dilution (Plasmid DNA)	$1x10^{-11}$	$1x10^{-9}$
Limit of Detection (Copies/10μl)	11.3	43.8
Limit of Detection (Mass – attogram)	38.12	157.75
Correlation Coefficient – r^2 (%)	99.8	99.2
Amplification Efficiency (%)	94%	92%
Limit of Detection (Trophozoites/300μl)	2.3 ± 1.7[a]	2.3 ± 1.7[a]
Limit of Detection (Cysts/300μl)	0.7 ± 0.0[b]	0.7 ± 0.0[b]

[a] Results based on two experiments

[b] Results based on two experiments

Table 3 Non-*Acanthamoebic* Negative Control Results

Isolate	Rivière RT-PCR	Qvarnstrom RT-PCR		Isolate	Rivière RT-PCR	Qvarnstrom RT-PCR
S. aureus B1349	Neg	Neg		*E. faecalis* ATCC 29212	Neg	Neg
S. aureus B1348	Neg	Neg		*S. marcescens* K1674	Neg	Neg
S. aureus B1342	Neg	Neg		*E. coli* K1671	Neg	Neg
S. aureus B1340	Neg	Neg		*Bacillus spp* K1593	Neg	Neg
P. aeroginosa B1354	Neg	Neg		*M. chelonae* K1687	Neg	Neg
P. aeroginosa B1312	Neg	Neg		*N. farcinica* K1801	Neg	Neg
P. aeroginosa B1350	Neg	Neg		*Fusarium spp*	Neg	Neg
P. aeroginosa B1306	Neg	Neg		*Alternaria spp*	Neg	Neg
H. influenzae B1345	Neg	Neg		*Penicillium spp*	Neg	Neg
H. influenzae B1346	Neg	Neg		*A. niger*	Neg	Neg
H. influenzae B1343	Neg	Neg		*Hartmannella spp*	Neg	Neg
S. pneumoniae B1351	Neg	Neg		ADV 763	Neg	Neg
S. pneumoniae B1339	Neg	Neg		ADV 761	Neg	Neg
S. pneumoniae B1335	Neg	Neg		ADV 762	Neg	Neg
Nutrionally variant strep K1666	Neg	Neg		HSV 602	Neg	Neg
Nutrionally variant strep K1623	Neg	Neg		HSV 611	Neg	Neg
S. viridans K1654	Neg	Neg		HSV 586	Neg	Neg
S. viridans K1680	Neg	Neg		HSV 575	Neg	Neg
Moraxella spp K1664	Neg	Neg		VZV 626	Neg	Neg

Table 4 Spiked Negative Controls

Isolate	Rivière RT-PCR[†]	Qvarnstrom RT-PCR[‡]
S. aureus B1349	Pos	Pos
P. aeroginosa B1354	Pos	Pos
H. influenzae B1345	Pos	Pos
S. pneumoniae B1351	Pos	Pos
Nutrionally variant strep K1666	Pos	Pos
S. viridans K1654	Pos	Pos
Moraxella spp K1664	Pos	Pos
E. faecalis ATCC 29212	Pos	Pos
M. chelonae K1687	Pos	Pos
S. marcescens K1674	Pos	Pos
Bacillus spp	Pos	Pos
Hartmannella spp	Pos	Pos
ADV 763	Pos	Pos
HSV 602	Pos	Pos
VZV 626	Pos	Pos

[†] 1µl of Rivière *Acanthamoeba* plasmid DNA (1.13×10^5 copies/µl)

   mixed with 20µl of extracted isolate DNA

[‡] 1µl of Qvarnstrom *Acanthamoeba* plasmid DNA (4.38×10^3 copies/µl)

   mixed with 20µl of extracted isolate DNA

Table 5 *Acanthamoeba* Positive Control Results

Isolate	Rivière RT-PCR	Qvarnstrom RT-PCR
A. lugdenensis clone L3A ATCC 24050 (T4)	Pos	Pos
A. polyphaga "eye" ATCC 30461 (T4)	Pos	Pos
A. hatchetti 2HH ATCC PRA-113 (T11)	Pos	Pos
A. castellanii Neff ATCC 30010 (T4)	Pos	Pos
A. rhysodes Haas ATCC 50368 (T4)	Pos	Pos
A. culbertsoni A-1 ATCC 30171 (T10)	**Neg**	Pos
A. griffini TI0:H37 ATCC 50702 (T3)	Pos	Pos
Acanthamoeba spp A1	Pos	Pos
Acanthamoeba spp A2	Pos	Pos
Acanthamoeba spp A3	Pos	Pos
Acanthamoeba spp A4	Pos	Pos
Acanthamoeba spp A5	Pos	Pos
Acanthamoeba spp A6	Pos	Pos
Acanthamoeba spp 'Schuster'	Pos	Pos

Table 6 Non-inhibitory Drug Dilutions and Amplification Efficiency

Drug	Non–Inhibitory Dilution	Amplification Efficiency
Proparacaine[®] (anesthetic)	1/1	100.1
Econopred Plus[®] (steroid)	1/1	91.2
Xibrom[™] (non-steroidal)	1/1	97.4
Nevanac[™] (non-steroidal)	1/10	95.0
Zymar[®] (antibiotic)	1/10	92.6
Vigamox[®] (antibiotic)	1/10	92.0
Tobramycin (antibiotic)	1/1	102.9
Cefazolin (antibiotic)	1/10	91.6
Amphotericin B (antifungal)	1/1	**108.7***
Trifluridine[®] (antiviral)	**1/40***	94.6
Brolene[®] (anti-acanthamoebic)	1/10	**85.7***
PHMB (anti-acanthamoebic)	**1/2560***	**72.7***
Chlorhexidine (anti-acanthamoebic)	1/10	**77.0***
Corneal Tissue	1/1	**86.9***

[*] Potential inhibitor to PCR amplification

Table 7 Pre and Post Extraction Non-Inhibitory Dilutions

Drug	Non-Inhibitory Dilution Pre-Extraction	Non-Inhibitory Dilution Post-Extraction
Trifluridine[®] (antiviral)	1/40	1/1
PHMB[†] (anti-acanthamoebic)	1/640	1/320

[†] Different lot of PHMB from drug used in Table 6

Table 8 Summary Rivière RT-PCR Results

All Samples		Culture		
		+	-	Total
Rivière RT-PCR	+	5[c]	5[d]	10
	-	0	122[e]	122
	Equivocal[a]	0	4[f]	4
	Inhibitory[b]	0	3[g]	3
	Total	5	134	139

[a] Equivocal defined as a sample that tested positive initially but result could not be reproduced
[b] Samples where the internal control tested negative after extraction and re-extraction
[c] 4 Cornea, 1 Contact Lens / Case
[d] 4 Cornea , 1 Contact Lens /Case
[e] 83 Cornea, 25 Contact Lens / Case, 12 Contact Lens Solution Bottle, 1 Eyelid, 1 Conjunctiva / Eyelid
[f] 2 Cornea, 2 Contact Lens / Case
[g] 3 Contact Lens / Case

Table 9 Summary Qvarnstrom RT-PCR Results

All Samples		Culture		
		+	-	Total
Qvarnstrom RT-PCR	+	5[c]	4[d]	9
	-	0	127[e]	127
	Equivocal[a]	0	1[f]	1
	Inhibitory[b]	0	2[g]	2
	Total	5	134	139

[a] Equivocal defined as a sample that tested positive initially but result could not be reproduced
[b] Samples where the internal control tested negative after extraction and re-extraction
[c] 4 Cornea, 1 Contact Lens / Case
[d] 3 Cornea , 1 Contact Lens /Case
[e] 86 Cornea, 27 Contact Lens / Case, 12 Contact Lens Solution Bottle, 1 Eyelid, 1 Conjunctiva / Eyelid
[f] 1 Contact Lens / Case
[g] 2 Contact Lens / Case

Table 10 Summary RT-PCR Non-Negative Results

Sample #	Specimen	Culture Result	RT-PCR Result (Rivière)	Ct Value	RT-PCR Result (Qvarnstrom)	Ct Value	Sequence Confirmation
2	Cornea	Neg	Pos	35.59	Pos	32.28	Yes
4	Cornea	Neg	Pos	37.44	Pos	36.83	Yes
11	Cornea	Neg	Pos	37.98	Neg		No
14	Cornea	Neg	Equivocal		Neg		
16	Cornea	Neg	Pos	32.88	Pos	35.94	Yes
27	Cornea	Pos	Pos	30.32	Pos	33.61	
47	CL / Case	Neg	Inhibitory		Inhibitory		
57	Cornea	Neg	Equivocal		Neg		
63	CL / Case	Neg	Equivocal		Neg		
85	Cornea	Pos	Pos	33.67	Pos	36.31	
93	CL / Case	Neg	Inhibitory		Neg		
98	CL / Case	Pos	Pos	22.69	Pos	25.32	
112	CL / Case	Neg	Pos	42.42	Pos	38.19	Yes
114	CL / Case	Neg	Equivocal		Equivocal		
121	CL / Case	Neg	Inhibitory		Inhibitory		
133	Cornea	Pos	Pos	31.50	Pos	32.29	
139	Cornea	Pos	Pos	29.29	Pos	32.51	

Table 11 NCBI Blast Sample 2

	Sequences producing significant alignments:	Score (Bits)	E Value
gb|DQ087295.1|	Acanthamoeba sp. S29 18S ribosomal RNA gene, part	287	3e-74
gb|S81337.1|	18S rRNA Acanthamoeba griffini, H37, Genomic, 2781	287	3e-74
gb|AF019053.1|	AF019053 Acanthamoeba pearcei 18S ribosomal RNA ge	287	3e-74
gb|AF019052.1|	AF019052 Acanthamoeba polyphaga Panola Mountain...	287	3e-74

Table 12 NCBI Blast Sample 4

	Sequences producing significant alignments:	Score (Bits)	E Value
gb|DQ087295.1|	Acanthamoeba sp. S29 18S ribosomal RNA gene, part	272	3e-70
gb|S81337.1|	18S rRNA [Acanthamoeba griffini, H37, Genomic, 2781	272	3e-70
gb|AF019053.1|	AF019053 Acanthamoeba pearcei 18S ribosomal RNA ge	272	3e-70
gb|AF019052.1|	AF019052 Acanthamoeba polyphaga Panola Mountain...	272	3e-70

Table 13 NCBI Blast Sample 16

	Sequences producing significant alignments:	Score (Bits)	E Value
gb|EF050506.1|	Acanthamoeba sp. MA51 18S ribosomal RNA gene, par	272	6e-70
gb|EF050505.1|	Acanthamoeba sp. MA49 18S ribosomal RNA gene, par	272	6e-70
gb|EF050503.1|	Acanthamoeba sp. MA44 18S ribosomal RNA gene, par	272	6e-70
gb|EF050499.1|	Acanthamoeba sp. MA37 18S ribosomal RNA gene, par	272	6e-70

Table 14 NCBI Blast Sample 112

		Score	E
Sequences producing significant alignments:		(Bits)	Value
gb|DQ087295.1|	Acanthamoeba sp. S29 18S ribosomal RNA gene, part	296	2e-77
gb|S81337.1|	18S rRNA Acanthamoeba griffini, H37, Genomic, 2781	296	2e-77
gb|AF019053.1|	AF019053 Acanthamoeba pearcei 18S ribosomal RNA ge	296	2e-77
gb|AF019052.1|	AF019052 Acanthamoeba polyphaga Panola Mountain...	296	2e-77

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