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Development of a Molecular Method for Detecting the Causative Agent of Swimmer's Itch (Trichobilharzia) in Freshwater Ecosystems

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DEVELOPMENT OF A MOLECULAR METHOD FOR DETECTING THE CAUSATIVE AGENT OF SWIMMER'S ITCH (*TRICHOBILHARZIA*) IN FRESHWATER ECOSYSTEMS

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ABSTRACT

Development of a molecular method of detecting the causative agent of swimmer's itch (*Trichobilharzia*) in freshwater ecosystems

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Cercarial dermatitis, commonly known as swimmer's itch, is caused by penetration of larvae of the schistosome, *Trichobilharzia,* into the dermis and epidermis layers of the skin. Symptoms are characterized by painful swelling and itching at the site of penetration. The normal hosts for the life cycle of the schistosome are aquatic birds and aquatic snails. The most frequently used method of detection for *Trichobilharzia* is microscopy. With increases in the occurrence of cercarial dermatitis outbreaks in freshwater in the Southwestern United States, it is becoming increasingly important to develop and standardize a molecular method for rapid detection that can assist health professionals and those who monitor public freshwater recreation systems. Therefore, the objective of this study was to develop a genus-specific real-time polymerase

chain reaction (PCR) assay for the detection of *Trichobilharzia* parasites in public freshwater systems. A literature search was completed to gather existing published material, and the DNA sequence database was searched to design and test potential primers and probes. The PCR assay primers and probes were developed, tested and optimized and used successfully to detect reference strains of *Trichobilharzia* species. The method was then used to test for the presence of *Trichobilharzia* schistosomes in surface grab samples and aquatic snails collected from Lake Mohave (AZ-NV). None of the water or snail samples tested with the primers and probes resulted in amplification. While the field collected samples did not yield any positive PCR results, it remains important to understand temporal and spatial patterns of *Trichobilharzia* prevalence in order to provide a better understanding of risks for exposure periods during the year.

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CHAPTER 1

INTRODUCTION

Cercarial dermatitis, commonly known as swimmer's itch, is caused by penetration of the epidermal layers by the schistosome larvae, *Trichobilharzia*, into the dermis and epidermis layers of the skin. *Trichobilharzia* is the largest genus in the Family Schistosomatidae and is classified as an avian blood fluke (Dvorák et al., 2002). There are four genera of schistosomes that infect mammals and ten that infect birds with approximately 30 and 67 species, respectively (Brant & Loker, 2013). There are 40 species of the *Trichobilharzia* genus described worldwide, with incomplete taxonomic structure identified due to lack of phylogenetic analysis (Brant & Loker, 2009a; Dvorák et al., 2002).

Trichobilharzia is an avian fluke that affects aquatic and semi-aquatic organisms. Swimmer's itch is non-contagious and will typically heal without medical treatment. However, allergic or hypersensitivity reactions have been known to occur in rare circumstances (Tammaro, 2012; Verbrugge et al., 2004). Schistosomiasis is a similar disease, but is more severe and is caused by a human blood fluke. The organisms that cause swimmer's itch have been isolated from fresh water and brackish waters around the world.

Swimmer's Itch-Cercarial Dermatitis

Swimmer's itch is a non-contagious disease of the skin that creates papules at the site of exposure. After leaving the water, a person may

experience mild tingling or a burning sensation on the skin and then the sensation may recede. Unlike seabather's eruption that occurs in the areas under the garments caused by the larval form of jellyfish, cercarial dermatitis occurs in the exposed areas of skin (CDC, n.d.). Symptoms progress one to several hours after leaving the water, followed by itching and a rash-like skin outbreak about 10 to 15 hours later, and lasting about a week (Verbrugge, Rainey, Reimink, & Blankspoor, 2004). The severity of the disease is dependent on the number of exposures to the schistosomes and the duration of the exposure, as well as the host immune system (Kolářová, Horák, & Skírnisson, 2012). More severe reactions may manifest after repeated exposures, such as fever and swelling of the lymph nodes (Tammaro, 2012). The most common contributor of the disease in humans worldwide is the genus *Trichobilharzia* (Olivier, 1949).

Cort (1928) identified the causative agent of cercarial dermatitis while working around Douglas Lake, Michigan. Swimmer's Itch is not considered to be a reportable disease by the Centers for Disease Control and Prevention (CDC), causing difficulty in determining the number of cases seen in the United States. However, according to the Office of Rare Diseases (ORD) of the National Institutes of Health (NIH), cercarial dermatitis is a "rare disease". A "rare disease" is classified as affecting less than 200,000 people in the U.S. (ORDR-NCATS, n.d). It is a more common occurrence in Michigan, Wisconsin, Minnesota, Indiana, Illinois, Iowa, Nebraska, and North and South Dakota. Other affected states considered less common are Washington, Oregon, New York,

Maine, Nevada, Oklahoma, Alabama, Tennessee, Texas, and Florida (Jarcho & van Burkalow, 1952). According to the National Park Service (NPS) website regarding swimmer's itch, the disease-causing parasite is common in 30 states (http://www.nps.gov/lake/naturescience/swimmeritch.htm, 2013). Brant & Loker's (2009b) survey of schistosomes in the southwest revealed the possibility that infestations are not as uncommon as originally thought.

Symptoms are characterized by painful swelling and itching at the site of penetration. Hypersensitivity to the organism can occur from repeated exposures and infections over time, and can cause exacerbated symptoms (Hunter et al., 1949; Jarcho & van Burkalow, 1952). More traumatic reactions to the parasite include irritable bowel, swelling of the limbs, fever and/or nausea (Kolářová et al., 2012). Typical time from infection to disappearance of symptoms is about 10 days (Kolářová et al., 2012). Children may be at a greater risk of infection because they stay near the shore and more shallow waters where the parasite tends to be concentrated (CDC, n.d.). In addition, laboratory controlled infections on mammals have shown the ability of the cercariae to leave the skin and travel to internal organs (Horák, 2011; Jouet, 2010).

Schistosomiasis

Cercarial dermatitis is similar to the disease known as schistosomiasis in that it is caused by a blood fluke. However, schistosomiasis is a human blood fluke disease that affects approximately 200 million people a year (Hamburger, 1998; Horák, 2011; Schistosomiasis, 2012). According to the CDC, the parasite

that causes this disease is not found in the United States; however, schistosomiasis is the second most devastating parasitic disease next to malaria (Schistosomiasis, 2012). It is important to understand schistosomiasis because the parasite is related to *Trichobilharzia* and has similar life cycle patterns. The Schistosomatidae Family consists of approximately 14 genera and 100 difference species (Brant et al., 2006). The most common species that cause schistosomiasis are *Schistosoma haematobium*, *S. japonicum*, and *S. mansoni* (Hamburger, 1998, Hamburger, 2001, Schistosomiasis, 2012). This information is largely based on morphological descriptions of adult worms, and difficult to interpret due to the potential for duplicates (Brant et al., 2006). The trematodes that cause schistosomiasis are found in 53 countries, primarily in Africa and the Middle East (Hamburger, 2001).

The parasites that cause schistosomiasis have greater pathogenicity in humans than the avian schistosomes that cause cercarial dermatitis because they specifically infect humans. *Schistosoma* spp. have a life cycle similar to other schistosomes. The eggs are excreted in the urine or feces of an infected person. Once the eggs reach a water source, the eggs hatch and release miracidia, where they can survive for about 48 hours (Schistosomiasis, 2012). The miracidia then locate an appropriate aquatic snail, the intermediate host. Once the miracidia infect the snails, there are 2 generations of sporocysts where asexual reproduction occurs. After a period of asexual reproduction, cercariae are release from the intermediate host, back into the water where the larvae search for a primary host (Brant et al., 2010).

Once the cercaria come in contact with a human host, the larvae will burrow into the skin of the host, release the forked tail, and become schistosomulae. Once in the body, the schistosomulae travel through the vascular system (Hamburger, 1998; Hamburger, 2001; Schistosomiasis, 2012). The process of schistosomulae infection to adult worm infection may take several weeks to occur (Schistosomiasis 2012). *S. japonicum* is commonly found in the superior mesenteric veins that drain the small intestine whereas *S. mansoni* is more commonly found in the superior mesenteric veins that drain the large intestine (Schistosomiasis, 2012).

Pathogenicity of schistosomiasis includes: egg granulomas in the liver, brain and spinal cord, hypertension, Katayama fever, scarring and calcification, and squamous cell carcinoma (Schistosomiasis, 2012). The symptoms of the disease are not caused by the worms themselves, but by the eggs and how the body reacts to them. The eggs can become lodged in organ tissues causing inflammation and other closely related symptoms. Chronic infection with schistosoma may eventually lead to seizures, bladder cancer, reduced urination, learning disabilities, and other organ damage. Diagnosing schistosomiasis can be done by examining stool or urine samples microscopically for eggs. However, because eggs are passed intermittently and in small quantities, the most accurate method of diagnosis is through a serological test (Schistosomiasis, 2012).

Trichobilharzia

The avian schistosome, *Trichobilharzia*, has fourteen described species occurring in North America (Brant & Loker, 2009a, Verbrugge et al., 2004). Of those, the most common species determined to cause cercarial dermatitis are: *Trichobilharzia physellae*, *T. stagnicolae*, *T. brantae*, *T. querquedulae*, and *T. ocellata* (Brant & Loker, 2009a). The other nine species are capable of causing disease; however, those are not as prevalent in the environment in North America and may be more prevalent in other countries. In a study conducted by Brant & Loker (2009a), five species of were found of the fourteen *Trichobilharzia* described species. They located *T. physellae* (Alaska, Michigan, New Mexico and Nevada), *T. stagnicolae* (Michigan, Montana, Minnesota and New Mexico), *T. querquedulae* (Florida, Louisiana, Alaska, Nebraska, Canada, New Mexico and California), *T. brantae* (Canada and Colorado), and *T. szidati* (Michigan and Montana). The most common schistosomes in the southwest from Brant & Loker's study (2009a) were *T. physellae* and *T. querquedulae*. During a study conducted by Dvorák et al. (2002), researchers proposed that *T. ocellata* and *T. szidati*, prevalent avian schistosomes in Europe, may be the same or synonymous with each other. In Central Europe, *T. franki* and *T. regenti* are the most common species. Cercariae presence in the water is dependent on temperature. The optimal range of the water is 20-25°C (Tammaro, 2012). Snails may be infected year round, but will only shed when conditions are optimal.

The parasite can be classified into two groups. One group consists of those species that live in the visceral tissues of the host body, such as the intestinal and hepatic systems. The second group of species consists of those who live in the nasal passages of the host (Jouet, 2010). The species that finally reside in the nasal passages travel through the central nervous system of the host, creating neurological complications (Lucie, 2012). The species known for passing through the skin barrier to the nervous system is *T. regenti*, which is a European schistosome, but may be one of many species able to do so (Lucie, 2012).

Life Cycle

The life cycle of the *Trichobilharzia* schistosome is not completely understood (Cort, 1928), but involves the location of a primary waterfowl host. The normal hosts for the life cycle of the schistosome are aquatic birds and aquatic snails. The life cycle of *Trichobilharzia* spp. is similar to that of the human *Schistosoma* spp. Instead of eggs being released in the stool or urine of humans, the eggs are excreted by year-round resident or migratory birds, such as ducks, geese, and shorebirds. Once the eggs reach a water source, or are exposed to optimal conditions, the eggs hatch and become miracidia. The miracidia are cilia covered and “swim” to locate a suitable intermediate host, a mollusk. Inside the intermediate mollusk host, asexual reproduction occurs. The subsequent miracidia develop into free-swimming cercariae and are released when the environmental conditions are optimal (Tammaro, 2012). At the free-

swimming cercariae stage, humans are susceptible to infection (CDC, n.d.). The primary host is another aquatic bird, but the cercariae will sometimes penetrate the skin of humans. Humans are dead-end hosts and cercariae will not develop into adult organisms (Dvorák et al., 2002).

The adult worms infecting the waterfowl host produce eggs that are excreted in feces. If the eggs are deposited in an aquatic environment, the eggs will develop into the motile pre-larvae stage organisms that search for an intermediate host aquatic snail. The snail is critical to completing the life cycle of the organism. In the host snail, the schistosome develops into cercariae (free-swimming larval stage of the parasite) and extricates itself from the snail when environmental conditions are suitable. In the water, the cercaria searches for a suitable avian host to complete its life cycle (Figure 1) (Cort, 1928; Kolářová et al., 2012).

Human infection with *Trichobilharzia* is incidental due to exposure of the skin to the environment where the schistosomes occur naturally. There is a lack of understanding on what triggers each part of the life cycle changes and how the schistosomes choose their hosts. In a paper published by Kolářová et al (2012), evidence was shown that during repeat exposures, the parasite can migrate to other tissues in the body, including the lungs and central nervous system of laboratory infected mammals (Kolářová et al., 2012).

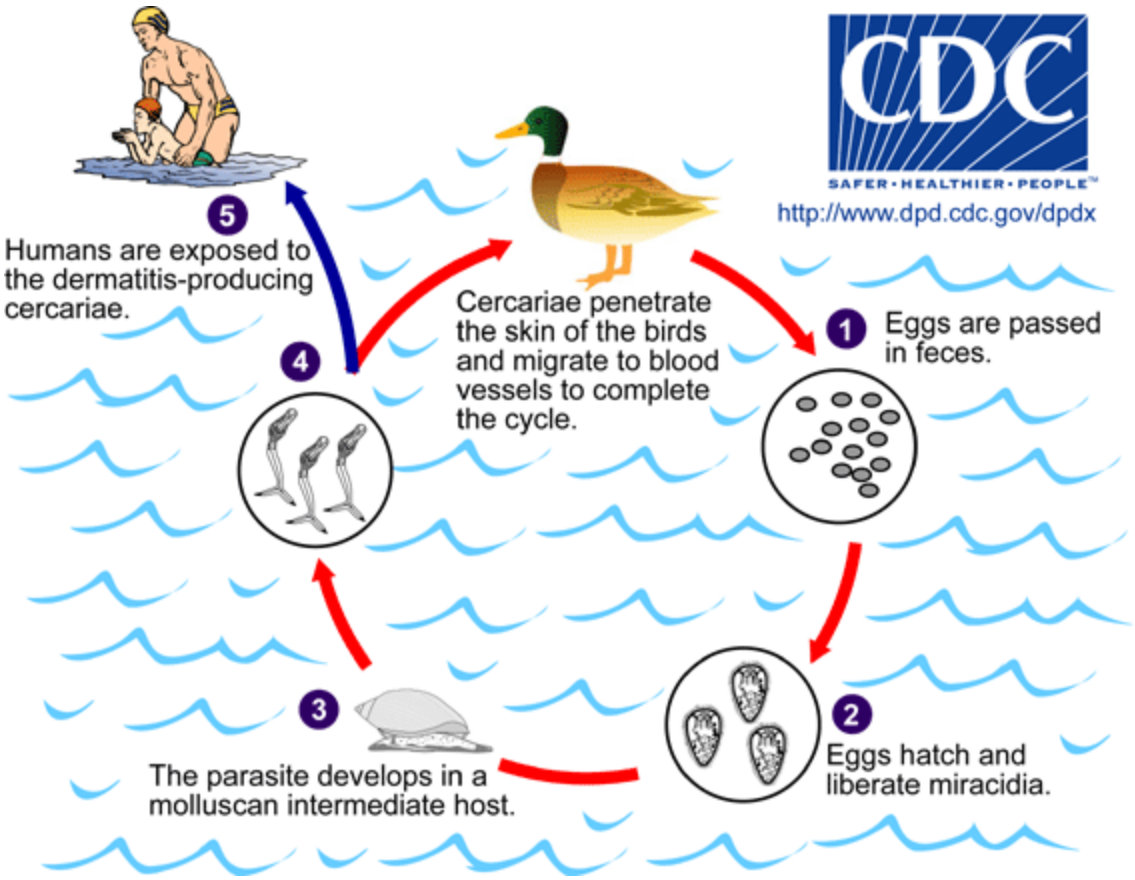

Figure 1. The life cycle of *Trichobilharzia*.

Ecology

The primary pulmonate families of mollusks known to transmit schistosomes are Physidae, Lymnaeidae, and Planorbidae (Brant and Loker, 2013). Different species of *Trichobilharzia* have preferred avian hosts and secondary host mollusks. *T. querquedulae* has been found primarily inhabiting the *Anas* spp., primarily *A. discors, A. cyanoptera, and A. clypeata*. The preferred mollusk host for *T. querquedulae* is *Physa acuta* (Brant & Loker, 2009a) (Figure 2). While avian schistosomes appear to prefer physid snails, no

mammalian schistosomes have been found to infect these snails (Brant and Loker, 2013).

Trichobilharzia, the largest genus in the Schistosomidae family, appears to be found only in ducks and geese. *T. querquedulae* prefers the use of *Anas clypeata* or 'blue-winged duck' as its host. Other species of blue-winged ducks are also common primary hosts for *T. querquedulae*, such as *Anas discors* and *Anas cyanoptera* (Brant and Loker, 2013). *T. physellae* prefers members of diving duck groups such as Aythinae and Merginae. While Brant and Loker (2013) have found these relationships to be true of North American avian schistosomes, Gohardehi et al. found that Lymnaeidae family snails are more frequently used as intermediate hosts in Northern Iran (Gohardehi, Fakhar, and Madjidaei, 2012). They also found the *Anas* spp. as being important primary hosts for *Trichobilharzia* spp. since all the sampled birds in their study with *Trichobilharzia* infections were *Anas* spp. (Gohardehi, Fakhar, and Madjidaei, 2012). The preferred primary and intermediate host behavior could indicate physiological niches between the different genera through competition for host resources.

In the study conducted by Brant & Loker (2009b), aquatic snails and aquatic birds were collected from New Mexico and Colorado to determine the primary host and secondary host of a variety of schistosome species, primarily of those found in the *Trichobilharzia* genus (Brant & Loker, 2009a). The Midwestern states commonly associated with swimmer's itch infections have *Lymnaea* genus snails as the host. They are large, and deposit more

schistosome larvae than the southwest snail genera *Physa* or *Gyraulus* (Brant & Loker, 2009b). *Trichobilharzia* infestations were thought to be restricted by the snails found in the geographic regions where the different *Trichobilharzia* species were isolated. According to Jarcho et al. (1952), water that is slightly more alkaline supports greater populations of snails. The waters of the southwest are alkaline and support large populations of aquatic snails, providing the secondary host for the *Trichobilharzia* life cycle (Jarcho & van Burkalow, 1952).

A 

B 

C 

D 

Figure 2. Common hosts and intermediate hosts for four different species of Trichobilharzia.

A. *Anas discors* and *Physa acuta* – common hosts for *T. querquedulae* (http://www.watsonvillewetlandswatch.org/birds.htm, http://tiee.esa.org/vol/v4/experiments/habitat_shifts/description.html);
B. *Aythya collaris* and *Physa gyrina* – common hosts for *T. physellae* (http://www.allaboutbirds.org/guide/Ring-necked_Duck/id, http://mkohl1.net/Physidae.html);
C. *Mergus merganser* and *Stagnicola emarginata* – common hosts for *T. stagnicolae* (http://en.wikipedia.org/wiki/Common_Merganser, http://mkohl1.net/Lymnaeidae.html);
D. *Branta canadensis* and *Gyraulus parvus* – common hosts for *T. brantae* (http://commons.wikimedia.org/wiki/File:Canada_Goose_(Branta_canadensis)_RWD.jpg, http://www.stitchingnature.com/Snails/Mollusca/Gastropoda/Planorbidae/Planorbidae005.htm).

Detection Methods

Currently, swimmer's itch detection has been limited to traditional methods of Polymerase Chain Reaction (PCR) amplification and/or microscopy. Traditional PCR utilizes agarose gels to detect amplification at the end phases of the PCR assay. Traditional methods of PCR review results at the end phase of the amplification, or plateau. Real-time PCR detects amplification during the exponential phase of the reaction. There are many limitations to the traditional PCR method including low sensitivity and the need to do post-PCR processing that may take days to complete analysis. However, the real-time PCR amplification process takes less than two hours and analysis does not require post-PCR processing. Traditional PCR methods have been reported as complex and not always reliable (Tammaro, 2012). Real-time PCR can detect a two-fold change (10-20 copies) whereas traditional PCR agarose gels are difficult to discern between a five-fold change (10 to 50 copies). Microscopy is another method of detection of swimmer's itch cercariae in fresh water. This method involves taking a subsample and placing it on a slide. The analyst then manually searches for cercariae. This requires the analyst to be trained in identification of microorganisms. Microscopy is time consuming and highly inaccurate at identifying specific species, and may not represent the actual density in the sample. Another form of detection, which is used less often, is through the examination of fecal material of water fowl. This involves the collection of fresh fecal matter and suspension in water to induce hatching (Loken, 1995).

Microscopy of water or fecal material does not provide definitive results and is most useful in presence/absence surveys.

The published scientific literature on swimmers' itch in the U.S. has been minimal, with most research limited to the Great Lakes region, Montana and New Mexico (Brant & Loker, 2009b; Graham, 2003; Jarcho & van Burkalow, 1952). Rizevsky, et al. (2011) developed two genus-specific PCR primers that are considered to be representative of the *Trichobilharzia* genus: T1323-1 or T1323-2 and reverse primer T1323-R to test cercariae recovered from Naroch Lake and Polonevichi Lake in Belarus (Hertel et al, 2002; Rizevsky et al, 2011; Rizevsky et al., 2012). The primers developed for the conventional PCR have been compared to previously published primers in GenBank. The study found that the DNA sequences amplified for the selected species were consistent with those developed by Hertel et al. (2002) in schistosomes from ponds near Höchstadt/Aisch (Germany) and with published sequences in GenBank.

In a study conducted by Schets, et al. (2010), PCR was conducted on cercariae recovered from three recreational lakes in the Netherlands. The primers used were the same as those developed by Hertel et al. (2002) for detection of the ToSau3A sequence. Dvorák et al., (2002), completed a study on three European *Trichobilharzia* species using the internal transcribed spacer regions, ITS1 and ITS2, and 5.8S rRNA genes. It was concluded that the ITS1 region could help in differentiating the species based on the number of base pairs and length of the sequence at the ITS markers (Dvorák et al., 2002). Korsunenko et al. (2010), followed Dvorák et al. methods based on the ITS2

rDNA amplification for species specific identification (Korsunenko, 2010). Brant and Loker (2009a) sequenced *Trichobilharzia* spp. utilizing different primers in a conventional PCR assay and found the ITS2 region to be the ideal gene sequence to use for PCR and species differentiation.

The studies conducted by Brant & Loker (2009a, 2009b), Hertel, et al. (2002) and Rizevsky, et al. (2011) all utilized aquatic snails to isolate the schistosomes for PCR analysis. The only study using water samples to isolate the schistosomes was conducted by Schets, et al. (2010) in Holland. A different study conducted in Egypt on *S. mansoni*, used a filtering method to isolate the cercariae in tissue and urine samples, but not water. However, the study did show that a single cercariae could be amplified in a traditional PCR assay (Hamburger et al., 1998).

In the United States, Brant and Loker (2009b) completed a study to identify cercarial dermatitis causing schistosomes in outbreaks identified in Colorado and New Mexico. They utilized the available mitochondrial *cox1* and the nuclear marker, ITS, to differentiate the species. The sequencing was done using previously published primers (Brant & Loker, 2009b). Another study by Brant and Loker (2009a) did a comprehensive species identification by conducting PCR using previously published primers and then comparing the findings from each sample to published data in GenBank (Brant & Loker, 2009a).

Significance

Many of the waterways in the U.S. are considered public use and are used for recreation. Many of the beach areas around lakes in the southwest bring tourists because the waters are warm and allow for shallow wading. Recreation has become a vital part of society and is also important for local revenues. For water quality monitors and recreational managers, closing water areas to the public means reduced revenues and potentially damaging social value of the area. According to Rizevsky et al. (2012), cases of human cercarial dermatitis have had a significant impact on the local community that depends on the water related recreational activities (Rizevsky et al., 2012).

Lake Mohave is part of the Lake Mead National Recreation Area (Nevada-Arizona) and is popular for recreational activities. According to the NPS, from April through August, 2009, nearly 1 million total visits were recorded for Lake Mohave (National Park Service, 2010). According to the NPS, Lakes Mead and Mohave are suspected of having *Trichobilharzia* infestations (www.nps.gov/lake/naturescience/swimmeritch.htm, 2013.). There have been unpublished reports of swimmer's itch outbreaks in the area; however, no detailed reports or investigations have been completed in the southwest. An outbreak occurred in 2012 at the Boulder Beach area of the Lake Mead National Recreation Area. In a public release by the National Park Service in 2012, six cases of swimmer's itch were reported after swimming at Boulder Beach, Nevada (Vanover, 2012).

A report published by Verbrugge, et al. in 2004 showed an incidence of 6.8 cases per 100 exposure days at Douglas Lake, Michigan, with positive risk factors being associated with shallow water and with onshore winds (Verbrugge et al., 2004). The southwest region of the United States has a longer summer season than Michigan, allowing more possible days of exposure by recreationalists and workers. Conversely, Michigan has more days with below freezing temperatures than the southwest. The warmer temperatures year-round allows for greater numbers of *Trichobilharzia* to survive year to year in overwintering snails and aquatic birds. Backwater ponds of Lake Mohave have accounted for occupational exposures during continuing native fish propagation activities being conducted by the Lower Colorado River Multi-Species Conservation Program (MSCP) (PC, 2013). Lake Mead (AZ-NV) reported (Brant & Loker, 2009b) the latest outbreak in 2012 (www.nps.gov/lake/naturescience/swimmeritch.htm, 2013).

Enhanced detection methods for *Trichobilharzia* parasites are needed to improve the ability of water managers to reduce recreational and occupational exposures and cercarial dermatitis. The DNA sequencing of *Trichobilharzia* and related genera has been conducted in Europe and the Mid-West, but it is lacking in other parts of the United States. The closest samples to Lake Mohave were taken by Brant and Loker (2009a) in Pyramid Lake, Nevada and Imperial Valley, California. Previously developed primers have been used with conventional PCR, which requires post-PCR analysis to confirm the amplification of the target organism. Real-time PCR assays include a fluorescent probe that confirms the

presence of the target sequence as amplification occurs and provides faster results with fewer manipulations than conventional PCR. Therefore, there was a need to develop a real-time PCR assay and to determine the prevalence of *Trichobilharzia* in the Lake Mead National Recreation Area. With increases in the occurrence of cercarial dermatitis outbreaks in freshwater in the Southwest United States, it is becoming increasingly important to develop and standardize a molecular method for rapid detection that can assist health professionals and those who monitor public freshwater recreation systems. There is an increasing concern of the effects of *Trichobilharzia* spp. infections due to the possibility of the cercariae not being trapped in the skin and migrating to other internal organs, as has been seen in laboratory mammals (Jouet, 2010; Lucie, 2012). Understanding temporal and spatial patterns of *Trichobilharzia* prevalence will allow a better understanding of risks for exposure periods during the year.

Objective

It is the objective of this study to develop a PCR assay for the detection of *Trichobilharzia.* The method will be developed in the laboratory, and tested with water samples and snail samples collected in backwater ponds of Lake Mohave.

Research Questions

1. Can a real-time PCR assay be developed to specifically detect *Trichobilharzia* in water samples in the southwest United States?

2. What is the spatial and temporal prevalence of *Trichobilharzia* in backwater fish-rearing ponds of Lake Mohave?
3. Can *Trichobilharzia* be detected with the PCR assay in snails in Lake Mohave backwaters?

CHAPTER 2

MATERIALS AND METHODS

Literature Review

A literature review was completed to gather existing published material on detection and analysis of *Trichobilharzia* spp., including information on closely related genera. Existing PCR primers in the literature were entered into the DNA sequence database, GenBank (http://www.ncbi.nlm.nih.gov/genbank/), to identify target gene sequences. Sections of published sequences were analyzed with the Basic Local Alignment Search Tool algorithm (BLAST, National Center for Biotechnology Information NCBI) feature in GenBank to look at homology of closely related species of *Trichobilharzia* found in North America. The searches of the genome homology consisted of comparing sections of the Internal Transcribed Spacer (ITS) 1, ITS 2, 18S ribosomal DNA (rRNA), 28S, or cox1 (mitochondrial DNA). Each gene sequence was analyzed separately and evaluated for homology. The ITS2 region revealed the greatest homology with the greatest number of *Trichobilharzia spp.* and excluded most of the other related genera. Previously published conventional PCR primers and probes were also reviewed for potential use in a real-time quantitative PCR method (Brant & Loker, 2009a; Hertel et al., 2002; Rizevsky et al., 2012; Schets et al., 2010).

Sampling sites and sampling

Water and snail samples were collected in 2011 at three locations in Lake Mohave AZ-NV. Yuma Cove (AZ), Dandy Cove (NV), and Davis Cove (AZ) were the locations sampled based on the high frequency of fish-rearing work conducted in the area and past incidents of swimmer's itch infections experienced by U.S. Bureau of Reclamation staff (Figure 3) (U.S. Bureau of Reclamation, 1994). A total of 42 replicate water samples were collected from the surface of the water after wading into the pond to approximately waist deep. Sterile, 500 ml bottles were filled with water, stored on ice, and then transported to the Emerging Diseases Laboratory (EDL) at the University of Nevada, Las Vegas (UNLV) where the water samples were stored at -20 °C until further analysis. Snails were collected from the shore line of the ponds by hand and placed into sample collection bags. There were 42 attempted collections between March and September 2011. Of the 42 attempts, 30 tries collected snails.

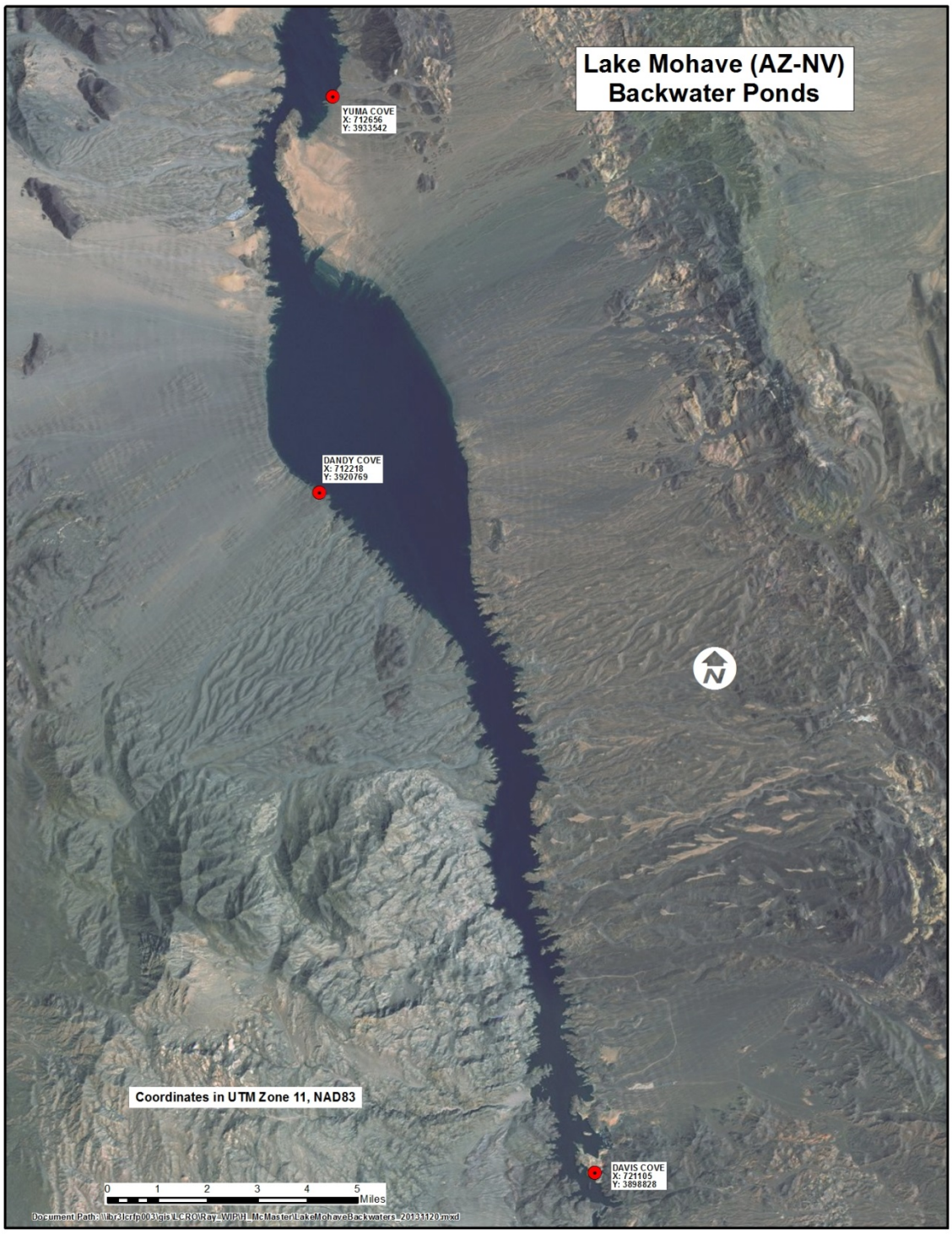

Figure 3. Lake Mohave Sample Sites with UTM coordinates. Yuma Cove, Dandy Cove, and Davis Cove from top to bottom.

Concentration and Processing of water samples

The water samples were allowed to thaw completely. No more than six samples were concentrated at one time to reduce the time the samples were held at room temperature. A vacuum filter assembly was used to filter 500 ml of water onto a 0.64 µm pore size nitrocellulose filter membrane (Millipore Corporation, Billerica, CA). In some instances, the filtering process required multiple filter membranes to filter the entire sample. Each membrane or group of membranes from a sample was placed into a sterile 50 ml centrifuge tube labeled according to the site location, date, and time collected. The samples were then stored at -20ºC until further analysis.

Each sample was removed from the freezer for elution and DNA concentration. To rinse the filters, 10ml of HyPure Molecular Biology Grade, Nuclease-free water (HyClone Laboratories, Inc., Logan UT) was pipetted into the 50ml centrifuge tubes. Each tube was vortexed for two minutes, ensuring the filters remained toward the bottom of the tube. The filters were pushed down to the bottom of the tube and the tubes were placed into a Branson 1200 sonicator (Branson Ultrasonics Corporation, Danbury, CT) and sonicated for five minutes. The samples were re-vortexed for one minute following sonication. With sterile forceps, the filter membranes were removed from the centrifuge tube and discarded. The sample tubes were and centrifuged at 5000g for five minutes (IEC CL31R Multispeed centrifuge, Thermo Electron Industries SAS, France) at a temperature of 4°C. The supernatant was removed without disturbing the pellet,

and discarded. To suspend the pellet, 1ml of nuclease free water was added to the tube and vortexed for 30 seconds. The suspended pellet was then transferred to a 2ml microcentrifuge tube and stored at -20°C.

Prior to the DNA extraction of the water samples, the samples were allowed to thaw completely. Each sample was vortexed for 30 seconds to resuspend anything that settled on the bottom of the tube. After the samples were mixed, 200 µl of the sample was removed and placed into micro bead tubes from the PowerSoil Kit (MOBIO Laboratories, Inc., Carlsbad, CA) for DNA extraction according to the manufacturer's protocol. A volume of 100 µl of purified DNA was eluted and stored at -70 °C.

Snail processing

Snail samples were analyzed approximately 24-48 hours after initial collection. Snails were transferred to sterile petri dishes and placed under a light source to induce shedding of any cercariae according to established protocols (Gohardehi et al., 2013; Brant & Loker, 2009a & 2009b). Any snails that showed possible shedding of cercariae were placed into sterile 1.5 ml microcentrifuge tubes in 100% ethanol. All snail samples were stored at -20°C until further analysis.

For DNA extraction, approximately 30 were taken out of the freezer and were placed into a double-lined stomacher bag. The stomacher bags were placed into a Stomacher (Seward Stomacher® 80 Lab System, United Kingdom) for 1-2 minutes to break the shell of the snails. After the samples were mixed,

200 µl of the sample was removed and placed into micro bead tubes from the PowerSoil Kit (MOBIO Laboratories) for DNA extraction according to the manufacturer's protocol. A volume of 100 µl of purified DNA was eluted and stored at -70°C.

Primer and Probe Design

Candidate primers and probes and reaction conditions were tested in the laboratory for detection of DNA from the target microorganisms. Reference samples were obtained from the Centers for Disease Control and Prevention (CDC) to test the specificity and sensitivity of the PCR primers and probes (Table 1).

The literature was reviewed for potential use of existing PCR primers and probes, and no real-time PCR primers and probes were found. To develop potential primers and probes that would detect *Trichobilharzia* spp*.*, the GenBank accession number HM125959.1 for *T. querquedulae,* a known species found in the southwest United States (Brant & Loker, 2011), was analyzed with a BLAST search. Using the *T. querquedulae* sequence identified, more species of *Trichobilharzia* with higher homologies were formed than searches with other *Trichobilharzia spp*. The ITS2 region of the sequence was identified based on the information provided in the accession number report in GenBank. Comparing the ITS2 region to other sequences in the repository revealed primarily *Trichobilharzia* spp*.* with minimal amounts of unspecified avian schistosomes.

Table 1. CDC obtained reference sample host and locations if known. Adapted from Brant, 2009a.

ID	Name	Snail Host	Avian Host	Locations
W154.2	*Trichobilharzia querquedulae*	*Physa acuta*	*Anas cyanoptera* *Anas clypeata* *Anas discors*	New Mexico California
W222	*T. stagnicolae*	*Lymnaeidae*	*Stagnicola emarginata*	New Mexico
W235	*T. physellae*	*Physa gyrina*	*Aythya collaris*	New Mexico Nevada
W331	*T. brantae*	*Gyraulus parvus*	*Branta canadensis*	Colorado
W514	*T.* spp. A		*Anas americana*	New Mexico California
W403	Avian Schistosome C		*Lophodytes cucullatus*	Pennsylvania
W405	Avian Schistosome D		*Stagnicola* spp.	Canada
W409	Avian Schistosome B		*Anas americana*	Alaska
W246	*Allobilharzia visceralis*		*Cygnus columbianus*	U.S.A
W324	California cercariae	Marine estuary snails		California
W357.3	*Austrobilharzia varglandis*		*Larus delawarensis*	U.S.A.
W499	*Dentritobilharzia pulverulenta*		*Mergus*	U.S.A.

The ITS2 region was found to have one-hundred percent (100%) homology with six different identified species of *Trichobilharzia* (*T. querquedulae, T. physellae, T. stagnicolae, T. franki, T. regenti,* and *T. szidati*) and a number of unidentified *Trichobilharzia* species with an initial reporting of 100 matches (Figure 4). The ITS2 region was entered into the Primer Express software version 3 (Applied Biosystems, Foster City, CA) to find the optimal primer and probes within the sequence. Default factors were used for primer length, melting temperature (Tm), G-C ratio and other parameters. Two different primer and probe sets were designed with the software. The FAM/TAMRA™ quencher

probe utilizes a fluorescent dye to show amplification when the probe is cleaved. The selected probe was labeled at the 5'-end with the reporter dye 6-carboxyfluorescein (FAM) and the 3'-end with the reporter dye tetramethyl-6-carboxyrhodamine (TAMRA). The sequence obtained for the FAM/TAMRA designed probe (Tque-P2) was, 5'-AGTGCCTGCCGGCGTGTATACCC-3' (23bp). The forward primer sequence (TqF2) was, 5'-TGCACTTTAAGTCGTGGATTGG-3' (22bp). The reverse primer sequence (TqR2) was, 5'- CAGCAACCCGCGTTGATATA-3' (20bp) (Figure 5). The amplicon length was 69 base pairs (bp).

The other probe designed utilizes a minor groove binder (MGB) quencher and is non-fluorescent. The sequence for the MGB designed probe (Tque-P3) was 5'-AGTGCCTGCCGGCGT-3' (15bp). The forward primer sequence (TqF2) was the same as that used for the FAM/TAMRA probe, 5'-TGCACTTTAAGTCGTGGATTGG-3' (22bp). The reverse primer sequence (TqR3) was 5'-CCCGCGTTGATATACGGGTAT-3' (21bp) (Figure 6). The amplicon length was 63. All designed primers were obtained from Eurofins MWG Operon (Huntsville, AL); and probes were obtained from Applied Biosystems, Inc.

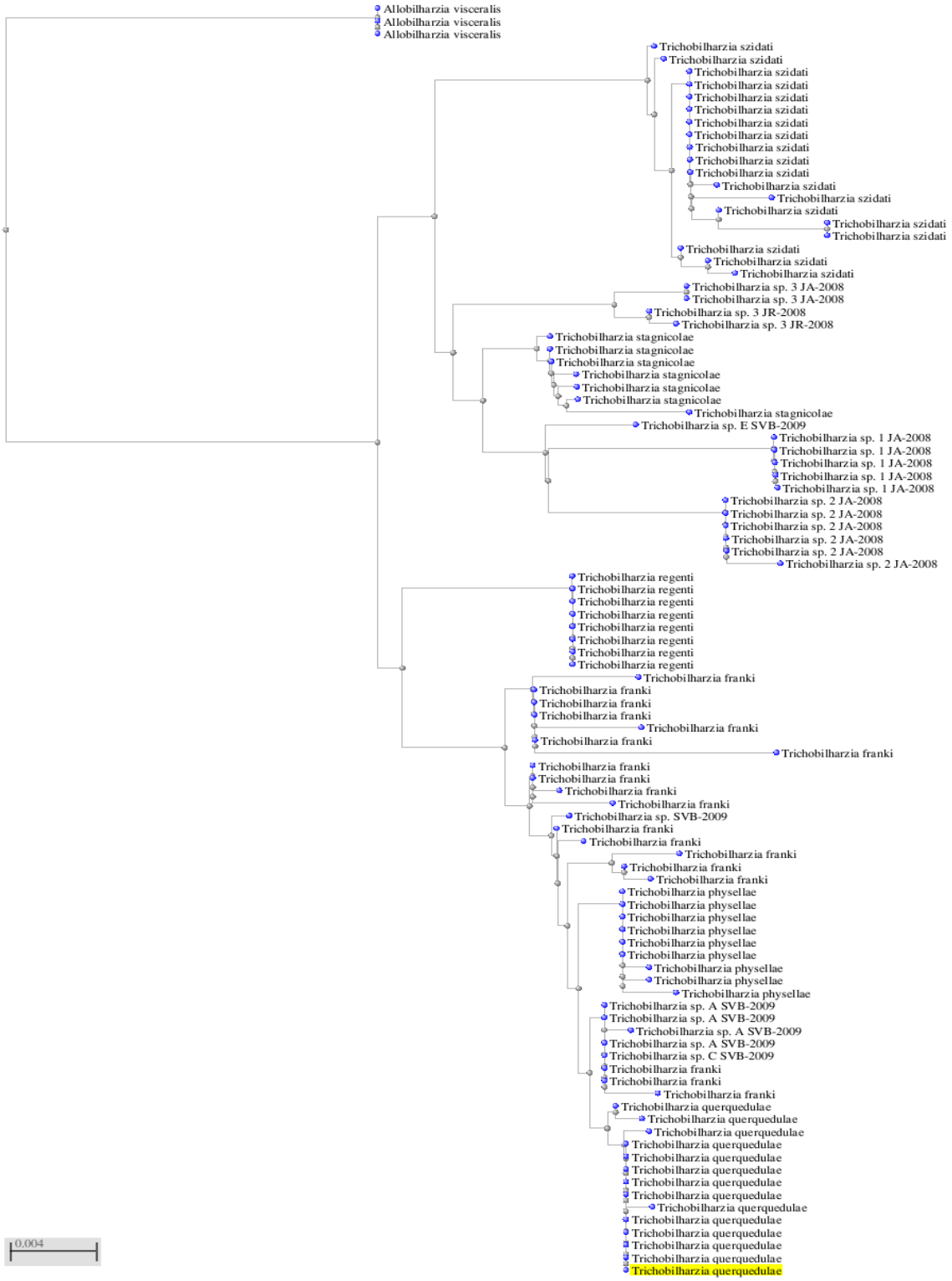

Figure 4. Phylogenic tree highlighting *T. querquedulae* and other related species. Accession# 125959.1.

cggcttt ccatctatca cgatgcactt taagtcgtgg attgggcg**ag tgcctgccgg cgtgtatacc cg**tatatcaa cgcgggttgc tggtctaagg ctctgtccga atttgtccgg ccacagccta gtctggtgta gaacttctga ttgagtcgcc acggtgggtt gtgctcgagt cgtagcttaa tataaat atatataaac gctcgggagt acatgaccta tcgtgaatac cgttatatat taataaacgg ttgattgga ggtctatgca tagtctatgg tttaaccgag aatgtgttat gcacattata aattttac

Figure 5. ITS2 region of *T. querquedulae* from accession number HM125959.1. The underlined sequence areas are the forward and reverse primers. The sequence in bold is the FAM/TAMRA probe.

cggcttt ccatctatca cgatgcactt taagtcgtgg attgggcg**ag tgcctgccgg cgt**gtatacc cgtatatcaa cgcgggttgc tggtctaagg ctctgtccga atttgtccgg ccacagccta gtctggtgta gaacttctga ttgagtcgcc acggtgggtt gtgctcgagt cgtagcttaa tataaat atatataaac gctcgggagt acatgaccta tcgtgaatac cgttatatat taataaacgg ttgattgga ggtctatgca tagtctatgg tttaaccgag aatgtgttat gcacattata aattttac

Figure 6. ITS2 region of *T. querquedulae* from accession number HM125959.1. The underlined sequence areas are the forward and reverse primers. The sequence in bold is the MGB probe.

PCR amplification

PCR analysis was performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems), in the EDL at UNLV. The PCR was performed in Standard Mode and cycling parameters were as follows: initial incubation step of 50°C for 2 min, denaturation of the template DNA at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The PCR Master Mix final concentrations were: 1X TaqMan universal PCR master mix containing AmpErase® UNG (uracil-N-glycosylase); 0.9 µM of the forward primer; 0.9µM of the reverse primer; 0.2µM of the FAM/TAMRA probe; 1X 10X Internal Positive

Control (IPC) Mix; and 1X 50X IPC DNA (Applied Biosystems, Foster City, CA). A total of 5µl of sample DNA was added to each reaction. Sterile, Nuclease Free water (Promega, Madison, WI) was used to adjust the volume of each reaction to 25µl. A negative control was used, containing 5µl of Nuclease Free water instead of DNA. An IPC was used during each PCR amplification to test for the presence of PCR inhibitors.

Each PCR assay reaction was performed in duplicate. After completion of the amplification cycles, the data were analyzed and plotted (fluorescence vs. cycle number) using the software provided with the 7900HT PCR instrument. The amplification level was reported by the software as the mean Ct value of replicate samples. The Ct value refers to the PCR cycle at the point where fluorescence is first detected. This value is inversely proportional to the initial DNA template concentration.

CHAPTER 3

RESULTS

Reference Samples

The reference samples of *Trichobilharzia* and other related organism samples obtained from the CDC were tested with the designed FAM/TAMRA primers (TqF2 and TqR2) and probe (Tque-P2) and MGB primers (TqF2 and TqR3) and probe (TqP3). Four of the five species of *Trichobilharzia* resulted in a positive amplification. In addition, 3 unidentified avian schistosomes thought to be *Trichobilharzia* spp., and *Allobilharzia visceralis,* tested positive (Table 2). The reference samples of closely related genera did not amplify.

Table 2. PCR results for reference samples.

Reference ID	Name	FAM/TAMRA Results	MGB Results
W154.2	*Trichobilharzia querquedulae*	+	+
W222	*Trichobilharzia stagnicolae*	+	+
W235	*Trichobilharzia physellae*	+	+
W331	*Trichobilharzia brantae*	-	-
W514	*Trichobilharzia spp.* A	+	+
W403	Avian Schistosome C	-	+
W405	Avian Schistosome D	+	+
W409	Avian Schistosome B	+	+
W246	*Allobilharzia visceralis*	+	+
W324	California cercariae	-	-
W357.3	*Austrobilharzia varglandis*	-	-
W499	*Dentritobilharzia pulverulenta*	-	-

Water

A total of 41 water samples were tested in duplicate with the FAM/TAMRA primers and probe set. To determine the presence of inhibition, a TaqMan® Exogenous Internal Positive Control (VIC™ Probe) (Applied Biosystems) was added to each reaction. A positive control (reference strain, W154.2, *T. querquedulae*) was used in each PCR. The first PCR with selected samples showed mild to complete inhibition. Therefore, subsequent runs were completed with 1:10 dilutions of sample DNA diluted in Tris-EDTA buffer(TE) (Teknova, Hollister, CA). Inhibition was not observed with 1:10 DNA dilutions. The calculated limit of detection of the assay for water samples is between 2 to 20 *Trichobilharzia* DNA copies per milliliter based on a PCR sensitivity range of 1 to 10 copies. No field collected water samples tested positive for *Trichobilharzia* spp. (Table 3).

Snails

A total of 18 snail samples were tested in duplicate with the FAM/TAMRA primers and probe set. For each sample, 5µl of template DNA was used for PCR analysis. To test for inhibition, a TaqMan® Exogenous Internal Positive Control (VIC™ Probe) (Applied Biosystems) was added to each reaction. A positive control (reference strain, W154.2, *T. querquedulae*) was used in each PCR. The initial PCR analysis of selected samples showed mild to complete inhibition. Therefore, subsequent analyses were performed with 1:10 dilutions of sample DNA in TE. Inhibition was not observed with 1:10 DNA dilutions. The calculated

limit of detection of the assay is 6.7 to 66.7 *Trichobilharzia* DNA copies per snail based on a PCR sensitivity range of 1 to 10 copies. No field collected snail samples tested positive for *Trichobilharzia* spp. (Table 3).

Table 3. Field collection PCR results using FAM/TAMRA probe for field samples (grey areas represent sample that were not analyzed).

Collection Date	Site	Water Sample Positive (Y/N)	Snail Sample Positive (Y/N)
3/14/2011	Davis	N	
3/15/2011	Dandy	N	
3/16/2011	Yuma	N	N
3/29/2011	Davis	N	
4/1/2011	Yuma	N	
4/1/2011	Dandy	N	N
4/12/2011	Davis	N	
4/12/2011	Dandy	N	
4/15/2011	Yuma	N	N
4/29/2011	Yuma	N	N
4/29/2011	Dandy	N	N
5/2/2011	Davis	N	
5/13/2011	Davis	N	N
5/24/2011	Yuma	N	N
5/24/2011	Dandy	N	N
5/27/2011	Davis	N	N
5/31/2011	Dandy	N	N
5/31/2011	Yuma	N	N
6/14/2011	Dandy	N	N
6/14/2011	Davis		N
6/14/2011	Yuma	N	N
6/27/2011	Yuma	N	
6/27/2011	Dandy	N	
6/28/2011	Davis	N	
7/7/2011	Davis	N	
7/12/2011	Yuma	N	
7/12/2011	Dandy	N	
7/29/2011	Yuma	N	
7/29/2011	Dandy	N	
7/29/2011	Davis	N	
8/16/2011	Davis	N	
8/16/2011	Yuma	N	
8/17/2011	Dandy	N	
8/25/2011	Yuma	N	N
8/26/2011	Dandy	N	N
8/30/2011	Davis	N	
9/15/2011	Yuma	N	
9/15/2011	Dandy	N	
9/19/2011	Davis	N	
9/29/2011	Dandy	N	
9/30/2011	Yuma	N	N
9/30/2011	Davis	N	

CHAPTER 4

DISCUSSION

Reference Samples

The primary objective of this study was to develop a PCR assay for the detection of *Trichobilharzia*. The first research question proposed was whether a real-time PCR assay could be developed. Reference strains of *Trichobilharzia* and related genera were obtained from the CDC to test the developed primers and probes as well as provide positive and negative controls. The results of the reference sample amplification were compared between the FAM/TAMRA and MGB primers and probes (Table 2). Both primers and probe sets produced comparable results. Inhibition was observed in the reference strains; however, dilution of the samples removed the inhibition. PCR results were unchanged by dilution of the reference strain DNA.

In theory, the FAM/TAMRA probe is more specific than the MGB probe, meaning that fewer base pair mismatches in the target DNA sequence will allow the probe to bind to the target DNA sequence and result in amplification. The MGB probe has less specificity, allowing more sequence variation. The advantage of using the MGB probe is the potential to detect more species in the genus. The risk in using the MGB probe is cross reactivity with another genus. Only one difference in amplification of reference strains was observed between the two primer and probe combinations developed in this study. The reference sample *T. brantae* was not detected with either primer and probe set. *T. brantae*

has less homology to *T. querquedulae*. It is also a species not commonly found in the southwest United States; the object of our target for this assay. The avian hosts for *T. brantae* are Canadian geese (*Branta canadensis)* (Table 1) (Brant 2009a). Canadian geese are known to winter in the southwest United States. However, during the summer months when swimmer's itch is most prevalent, Canadian geese are not present. Therefore, the negative result obtained with *T. brantae* was determined to be a minor limitation in the overall success of the FAM/TAMRA assay as well as the MGB assay.

Out of the 12 reference samples obtained from the CDC, 5 were previously identified as being present in the southwest U.S. However, one of the samples, California cercariae, is found in coastal, estuary zones and is not thought to be closely related to *Trichobilharzia*. The negative PCR result for this sample was expected. The positive result for *Allobilharzia visceralis* was unexpected, but *Allobilharzia visceralis* is more closely related to *T. querquedulae* than *T. brantae* (Figure 7). In addition, *A. visceralis* can also cause cercarial dermatitis. Therefore, detecting this additional schistosome falls within the objective of this study.

The genera of Avian Schistosomes B, C, and D are unknown and they were not identified as being present in the southwest U.S (Table 1). During the PCR analysis of these three samples using the FAM/TAMRA assay, the results were positive for Avian Schistosome B and D but not for Avian Schistosome C (Table 2). However, the MGB primers and probe detected amplification in all three Avian Schistosomes. This may indicate more base pair differences in this

strain than *Allobilharzia,* but less than *T. brantae*. Because some of the reference samples do not have a complete life history, it is difficult to determine whether some of the closely related samples should have been amplified during PCR.

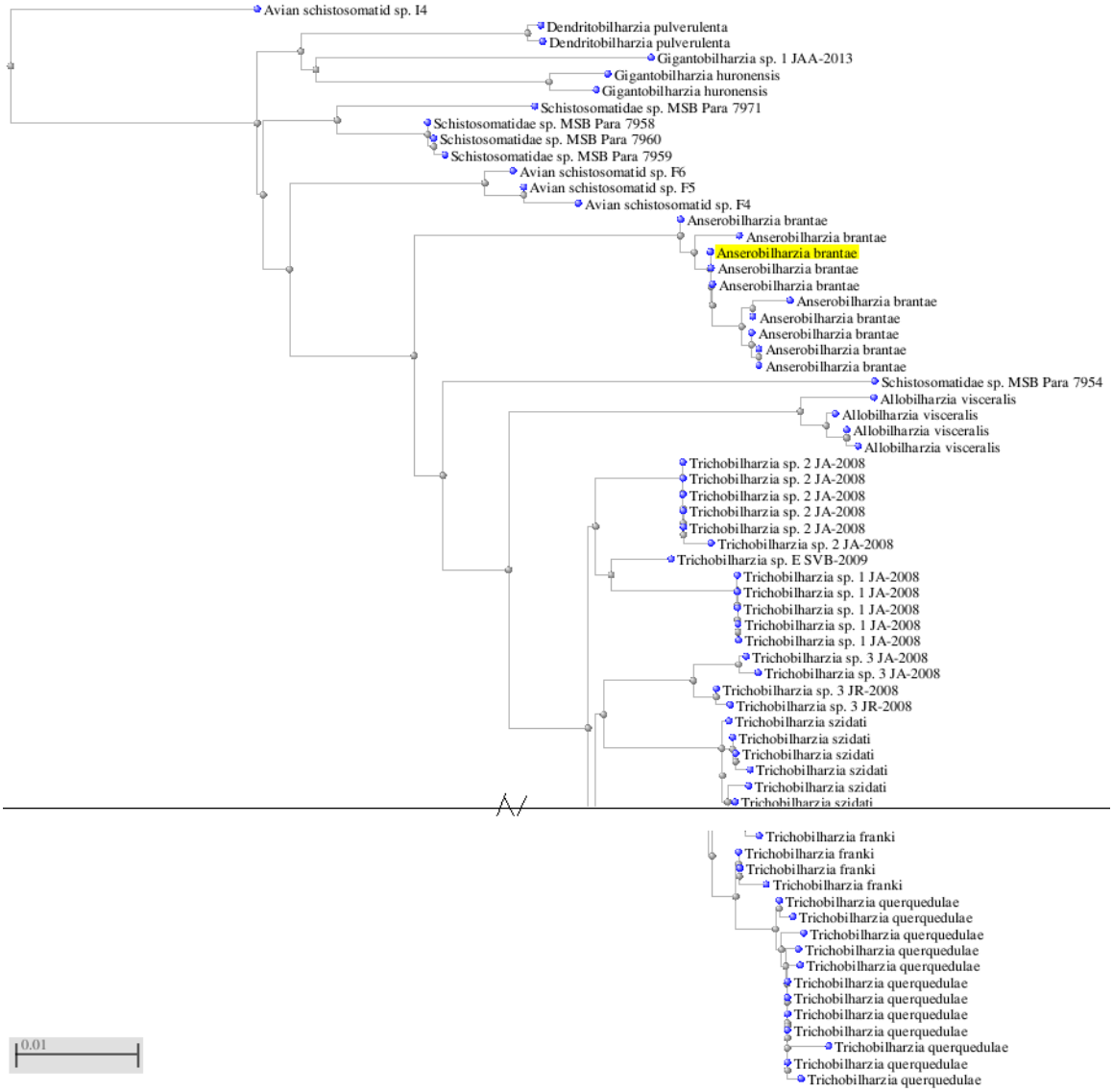

Figure 7. Phylogenic tree highlighting *Anserobilharzia brantae* (*T. brantae*) in relation to *Allobilharzia visceralis*. Accession# FJ175341

Water and Snails

The second and third research questions that were asked in this study involved the analysis of water and snail samples collected at Lake Mohave (AZ-NV). The second question involved analyzing spatial and temporal prevalence of *Trichobilharzia* in backwater ponds of Lake Mohave (AZ-NV). The third question was whether *Trichobilharzia* could be detected in water and snail samples. The FAM/TAMRA assay was used to test the water and snail samples rather than the MGB assay due to the theoretical potential for cross-reactivity outside the *Trichobilharzia* genus. To verify this assumption, two water samples (D502 and D527) and two snail samples (D513S and D527S) were tested with both the MGB and FAM/TAMRA assays in a follow-up PCR analysis. These samples were chosen based on the observation of possible cercariae shedding in the snails. Both assays resulted in negative amplification. No further analysis on remaining samples was performed with the MGB primers and probe.

There are a number of factors that may influence the susceptibility of the secondary host snails to infection, including age, size and the ability of the larvae to infect the snail (Horák, 2011). In Europe, studies have shown that prevalence rates of snail infections may range from 0.05 to 52.4 percent (Horák, 2011). Examination of snails has a low percentage of actual shedding of cercariae (Horák, 2011; Loken, 1995). Identification of cercariae infection in snails is unreliable using induced shedding through the use of a light source (Brant et al., 2010). When the Lake Mohave (AZ-NV) snails were exposed to light to induce

shedding, three (3) instances of possible cercariae shedding were observed. However, the species or genus of the possible cercariae could not be determined. The snails were analyzed by PCR, but results were negative.

None of the water samples or snail samples tested positive for *Trichobilharzia* spp. with FAM/TAMRA or MGB assays. It is possible that *Trichobilharzia* was not present in these samples, and the small sample size is a limitation of this study. Inhibition was detected with undiluted samples; however, after a 10-fold dilution of the sample, inhibition was no longer detected. In addition, the process of filtering the water samples, then eluting the samples, and completing the DNA extraction process, could have resulted in a detection limit that was too high to detect low concentrations of *Trichobilharzia* spp. Because the water and snail sample results were negative for amplification with the PCR assay, spatial and temporal prevalence of *Trichobilharzia* could not be determined.

Advantages and Limitations

One limitation of this study is the small sample size that was tested with the new assay, which may have contributed to the lack of detection of *Trichobilharzia*. Increasing the sample size, sampling after a reported outbreak, and sampling recreational beach areas may increase the chances of collecting cercariae. In addition, sampling larger volumes of water may capture larger quantities of cercariae. Incidences of swimmer's itch have been identified in U.S. Bureau of Reclamation employees at the sample collection locations. The

incidence is low and does not occur year to year. It is possible that in 2011 when the samples were collected, cercariae numbers were lower than in previous years or the samples collected may not have had any cercariae present. The backwater ponds at Lake Mohave (AZ-NV) reached water temperatures as high as 31.5°C during sampling events, and snail death was observed in the ponds beginning in June 2011. Out of the 43 sampling events, no living snails were observed during 13 of those. An assumed snail die-off event occurring as a result of low water levels or elevated water temperatures may explain the lack of positive water or snail samples. If there are no snails present during certain times of the year, a new snail population must establish and be present in order for *Trichobilharzia* to complete its life cycle. No previous sampling and testing of *Trichobilharzia* has been completed at these locations. Therefore, the concentrations of cercariae are unknown at these sites. Sampling areas with steady water elevation and lower average water temperatures may improve the chances of capturing a cercariae during a sampling event. In addition, sampling multiple locations along the beach area instead of collecting a single sample may also improve the chances of acquiring cercariae.

The PCR assay also had potential limitations in sensitivity of detection. A volume of 500ml of water was collected in duplicate. The water was filtered, eluted, and concentrated further. The concentrated pellet obtained at the end of the elution procedure was resuspended in 1ml of sterile water. Out of the 1ml of concentrated sample, only 200µl of sample was utilized in the PCR analysis. This is one-fifth of the sample, causing a five-fold loss in the detection limit. By

resuspending the pellet at the end of the DNA elution process in only 200µl of sterile water, the detection limit could be increased five-fold. The small pore size of the filter membrane captured large quantities of debris. Increasing the pore size of the filter membrane to a size that will still capture cercariae but filter out sands and other small particulates, may reduce the potential for inhibition and the need for dilution.

With the rise of global climate shift, migratory bird patterns could be changing. Areas that were previously free of avian schistosomes have the potential to become newly infected areas (Horák, 2011). This poses a potential challenge to recreational managers. Dr. Brant and her colleagues have said that "The better we understand the spectrum of schistosome species present and their host preferences, the better we can identify and respond to such challenges." (2013). Since spatial and temporal information for *Trichobilharzia* is not known for the southwest United States, and particularly in Lower Colorado River region, further research is needed to understand the prevalence of swimmer's itch causing schistosomes.

Of concern is the possibility that *Trichobilharzia* may be able to broaden its primary and secondary host range due to the regular introduction of exotic species into aquatic environments in the southwest United States (Brant et al., 2010). Species such as the New Zealand Mudsnail (*Potamopyrgus antipodarum*) and quagga mussels (*Dreissena bugensis*) have been introduced to many of the waterways of the southwest United States (http://www.azgfd.net/wildlife/conservation-news/10-most-unwanted-arizona-

invasive-species-plants-animals/2009/05/27/, 2009). *Trichobilharzia* may be able to adapt to a new secondary host, providing the potential for more cercariae to be present in the water. While the schistosome may prefer certain hosts, with the southwest United States having less standing water and ideal habitat as compared to the Midwest and Europe, snail infection densities may increase due to less dilution capacity in the ecosystem (Brant et al., 2013). One study proposed the 'concentrated transmission' hypothesis to explain this phenomenon (Brant et al., 2013). Other researchers have proposed similar hypotheses regarding the increased snail infection phenomenon (Rizevsky et al., 2012). Other hypotheses have also been proposed to explain some of the differences in the southwest United States compared to the Midwest (Brant et al., 2013; Rizevsky et al., 2012).

Results Comparison

Previous research related to *Trichobilharzia* has focused on the sequencing of DNA and identifying specific hosts for each species. A study on *Schistosoma mansoni* showed that PCR amplification was possible when a single cercariae was in the sample (Hamburger et al., 1998). However, the specific gene sequence the researchers used was not identified. The research that has been conducted in the southwest United States has been focused mainly on sequencing DNA of individual species of *Trichobilharzia*. In addition, only traditional PCR methods have been used. Other studies in Europe focused on identification of specific species of *Trichobilharzia* in selected lakes (Schets et

al., 2002; Korsunenko et al., 2010; Rizevsky et al., 2011). Our pilot study focused on identification of *Trichobilharzia* spp. found in the southwest United States. Another objective of this study was to be able to provide "presence/absence" information of this parasite in water and snail samples for the purposes of recreation and occupational management. No other studies have been found with a similar objective.

This study is unique in that it developed a real-time PCR assay that can detect an assortment of *Trichobilharzia* spp. There have been no studies that have developed or tested a real-time PCR assay for the detection of *Trichobilharzia*. In addition, no studies have been completed in relation to spatial and temporal prevalence of swimmer's itch causing cercariae in Lake Mohave (AZ-NV) or other reservoirs on the Colorado River.

CHAPTER 5

CONCLUSIONS

A real-time PCR assay was developed for the detection of *Trichobilharzia*. This pilot study successfully detected *Trichobilharzia* species in reference samples from parasites typically found in the southwest United States. Further testing is advised to improve the sensitivity of the assay. Future studies should include additional sampling of Lake Mohave (AZ-NV) recreational beach areas, specifically, beaches with shallow wading areas known for having the greatest prevalence of cercariae (Verbrugge et al., 2004). Lake Mead has had reports of outbreaks of swimmer's itch in the last three years. Sampling of locations where outbreaks have occurred may provide information on spatial and temporal prevalence of the schistosome in the southwest United States. In addition, further analysis of the remaining samples collected for the purposes of this study may be completed. Improving the detection limits may provide positive results where none were detected previously. For recreational managers to have advanced warning or to be able to issue safety concerns in high risk areas, a tracking database of outbreaks and their locations may be useful. This would allow early warning signs to be placed in areas prone to outbreaks, therefore protecting the public and staff in these areas.

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