THE INCIDENCE OF *TROPHERYMA WHIPPLEI* IN THE POPULATION OF THE BRAZOS VALLEY REGION OF TEXAS

A Thesis

by

ANNA LAVONNE KNOX

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2008

Major Subject: Laboratory Animal Medicine

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Approved by:

Co-Chairs of Committee,	Melanie M. Ihrig
	James Womack
Committee Member,	James E. Samuel
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ABSTRACT

The Incidence of *Tropheryma whipplei* in the Population of

the Brazos Valley Region of Texas. (December 2008)

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Co-Chairs of Advisory Committee: Dr. Melanie Ihrig
Dr. James Womack

An epidemiological study of the bacteria *Tropheryma whipplei* was conducted in the Brazos Valley region of Texas; specifically in the cities of College Station and Bryan. DNA samples from the oral cavities of study participants was extracted and analyzed for the presence of *T. whipplei.* Previously published studies have reported identifying this bacterium in the saliva of healthy individuals with no signs or symptoms of Whipple's disease. These investigations were conducted in Europe and Asia, including London, England and Switzerland, but data of this nature had yet to be obtained within Texas. After analyzing 147 samples obtained from 49 individuals, no indication of *T. whipplei* existing in the oral cavity of Bryan or College Station residents was found. During testing a study was published in May of 2007 indicating that previous investigations of this nature had in fact identified different bacteria resulting in false positives.

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CHAPTER I

INTRODUCTION

The focus of this study was to determine if the bacteria *Tropheryma whipplei* is in the oral cavities of individuals in the Brazos Valley of Texas. Studies of this nature have been done in other parts of the world, but there is no data as to whether *T. whipplei* is present in healthy individuals in this area of the United States. Such a finding would provide the basis for *T. whipplei* and its role in the various forms of the fatal Whipple's disease.

This study attempted to determine if *Tropheryma whipplei* is present in the oral cavity: saliva, tartar, and buccal swab, of Brazos Valley residents.

Is *Tropheryma whipplei* present in the oral cavities of healthy people in this area of the United States? If the bacterium is present, do specific gender, race, age, and/or tobacco habits affect the results?

Multiple studies have concluded that *Tropheryma whipplei* can be a normal part of the flora of the upper gastrointestinal tract, specifically the oral cavity. Can further testing in other geographical areas continue to support the hypothesis that *T. whipplei* is present in the oral cavity of healthy individuals? Although previous studies incorporated multiple races, Caucasians have made up the large majority of individuals with oral samples positive for *T. whipplei*. Is this a trend that accurately demonstrates the

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This thesis follows the style of Clinical Microbiology Reviews.

ratio of Caucasians inhabited with the bacteria to other races? Reportedly males have a much higher likelihood of having Whipple's disease than females. Are males more likely to harbor the bacteria than women? If not, are males more susceptible to the colonization of *T. whipplei* in other areas of the body resulting in Whipple's disease? Does damage to the oral cavity from a history of tobacco use increase the possibility of the bacterium being present in the oral cavity?

It is hypothesized that *Tropheryma whipplei* will be found in the oral cavity of some residents of the Brazos Valley.

CHAPTER II

REVIEW OF LITERATURE

Whipple's Disease

Tropheryma whipplei is the known causative bacterial agent of Whipple's disease. First described by George Whipple in 1907 as a disease of the intestinal tract involving fat metabolism, Whipple's disease has since been associated with nearly every organ system in the human body (1,3,6,13,22). Whipple was a pathologist at Johns Hopkins University when a middle-aged male presented with symptoms that had yet to be described in medical publications. He referred to the new ailment as intestinal lypodystrophy, but the disease name was later changed in his honor. Whipple's disease has proven to be a more fitting description because intestinal lypodystrophy only accounts for a single symptom that infection with *T. whipplei* can produce (2,13). The disease typically begins with polyarthritis occurring at random times throughout the peripheral joints of the body (2,4). Some years later the patient develops other characteristic symptoms and the arthritic attacks usually cease at this point. Characteristic symptoms include arthritis, fever, diarrhea, weight loss, and lymphadenopathy (3,13). Other clinical manifestations may involve the gastrointestinal tract (other than diarrhea), the cardiovascular system, the ocular system, and the circulatory system, and include skin pigmentation changes (2,4).

Symptoms

The gastrointestinal tract, specifically the small intestinal mucosa, of most patients is characterized by a loss of microvilli and the presence of foamy macrophages. The loss of microvilli leads to malabsorption and weight loss and the overall change in structure leads to excess fat in the stool. Malabsorption results in anemia in up to 90% of patients (3). It was the extracellular deposits of lipids that led Whipple to call the disorder “intestinal lypodystrophy” (22). Diarrhea is the most common complaint of Whipple’s disease patients. Overall, the intestinal symptoms are not specific and alone can be confused with other disorders such as Crohn’s disease, celiac disease, amyloidosis, and lymphomas (4).

Arthritic symptoms occur in at least 90% of patients and are the most common non-intestinal manifestations of Whipple’s disease (2,4). Often polyarthritis precedes other symptoms by several years and then dissipates when gastrointestinal symptoms begin. Transient, migratory pain occurring intermittently between the peripheral joints such as knees, elbows, fingers, ankles and shoulders is common (10).

Cardiovascular involvement unaccompanied by other symptoms is considered rare, but does occur. The bacterium can cause pericarditis, myocarditis, systolic murmurs, congestive heart failure, and blood-culture negative valvular endocarditis can occur (6,8).

In the original report, Whipple noted a chronic cough associated with a yellowish expectoration (22). The cough severity varied with climate and weather and the

expectoration was most abundant in the morning. Since that time 30- 40% of patients experience this chronic cough sometimes characterized by chest pain and dyspnea (3).

Central nervous system symptoms rarely occur, but lesions in the brain and spinal cord are found in the majority of patients during postmortem examinations (3,14). Symptoms associated with this aspect of disease are hypersomnia, memory disturbance, facial twitching, and vertical opthalmoplegia (1,14).

The gastrointestinal symptoms accompanied by arthralgias tend to be encompassed as making up the “typical Whipple’s disease” patient. However, there have been several documented incidences of people diagnosed with extra-intestinal Whipple’s disease where the intestines are not affected. All patients should be treated with extended antibiotic regimens, often up to or beyond 1 year of treatment, to rid the body of infection and prevent relapse (15). Even after this vigorous treatment there have been cases where relapse, especially in the CNS, has occurred involving the exact strain of original infection (11).

Caucasian males are predominantly affected with an average onset at fifty years of age (3). The male to female ratio is reportedly eight to one (3). There are several possible explanations for this distribution. One possibility is a genetic susceptibility to the disease which could explain the higher incidence in Caucasians over other races and/or the greater incidence in males versus females. Geographic distribution of the bacteria is another possible explanation. Worldwide healthcare structures may be the most likely explanation due to the years of testing and chronic symptoms associated with Whipple’s disease. Worldwide Caucasian males have more opportunity to visit a doctor

when symptoms begin. A diagnosis of Whipple's disease typically comes after years of ruling out other disorders. It is the Caucasian males with money and health insurance that can spend those years pursuing the diagnosis. Due to the years of testing and the time between original polyarthritic symptoms and gastrointestinal aspects (or other manifestations) of the disease development it may explain why it is not identified until middle-age.

The Bacterium

Although the disease was believed to be of bacterial origin for several decades, this was not confirmed until 1961 with electron microscopy (24). It was another thirty years before the bacterium was named and classified. In 1991 partial 16S rDNA sequencing of an isolate from a single infected patient led investigators to label it as the *Tropheryma whippelii* (23). *T. whipplei* is an actinomycete with a small genome of only 928 kb, grouped between C*ellulomonas* and a rare form actinomycetes with a group B peptidoglycan (15,18).

However, *T. whipplei* is only distantly related to bacteria in either of those classifications further impeding the ability for scientists to make assumptions about the function and physiology of this little understood bacterium. Actinomycete bacteria are often known to exist in environmental soil, but *T. whipplei* has not been identified from soil samples. Some research has shown a possible link between people who work with soil and those who become infected so theories in regards to that have been hypothesized. There is a slightly higher prevalence of the disease in farmers than in person with other occupations (3). The bacterium is gram positive and though originally

thought to be an intracellular obligate, in 2001 it was proven to be a facultative intracellular organism (9). The strain Twist-Marseille was isolated from the cardiac valve of a Whipple's disease patient with endocarditis. Fresh HEL cells were inoculated with the isolated *T. whipplei* and allowed to multiply (12). In the cell cultures, *T. whipplei* appeared in an intracellular form and an extracellular form. The intracellular form was observed within vacuoles of the infected cells and the extracellular form aggregated together and embedded in the extracellular matrix. After analyzing the results found in 2001, *La Scola* et al. corrected *whippelii* to *whipplei* by correctly Latinizing Whipple to *whippleus* (12). *Tropheryma* was derived from *trophe* 'nourishment' and *eryma* 'barrier' because by causing malabsorption the bacterium is a barrier to nourishment.

Identification of the Bacterium

Culture, polymerase chain reaction, and RT-PCR have all successfully been used to test for and identify the presence of *T. whipplei*, but each method has its drawbacks (7). Despite multiple attempts, *T. whipplei* was not successfully cultured until 2000 when it was propagated in a human fibroblast cell line (HEL) (18). The doubling time of this bacterium is extremely slow so even if culture becomes easier to set up and maintain, it is not an ideal candidate for testing. After the successful culture the entire genome of *T. whipplei* was sequenced allowing further characterization of the bacterium. Through this characterization several deficiencies in amino acid biosynthesis and synthetic enzyme pathways were noticed suggesting that *T. whipplei* acquires those missing nutrients from the host cells. This information also allowed investigators to

better tailor culture media to the bacterium's needs. In 2003, *T. whipplei* was cultured in cell free media supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% human non-essential amino acids (20). PCR and RT-PCR are much more straightforward methods of testing. An animal model has yet to be developed and there are a very small number of case studies on animals with Whipple's-like symptoms. Given the difficulty posed by growing the organism, specific and accurate PCR testing is ideal.

Published epidemiological studies in London and Switzerland found *T. whipplei* in the oral cavities of people that were not known or suspected to have Whipple's disease (5,21). These studies analyzed the saliva and plaque of healthy individuals and results led investigators to hypothesize that *T. whipplei* may be part of the normal flora of the oral cavity in some individuals (5,21,25). The reported percentages of the population that may fall in this category were 33%, 35% and 50% (5,21,25).

Other Studies

Based on previous data, the *Zinkernagel* et al. study of 2003 suspected an oral habitat for *T. whipplei* bacteria and they tested saliva, supragingival smooth surface plaque, tongue and cheek samples, and samples from gingival pockets to determine if these are present in the oral cavity (25). Three groups of nine to ten systemically healthy individuals were tested: University Hospital of Zurich staff volunteers, patients with progressive periodontitis, and Chinese subjects that were currently participating in another study. Five positive samples in which the bacteria was identified were harvested

from the gingival sulcus. All samples of saliva, smooth surface supragingival plaque, tongue, and cheek were negative (25).

The data from *Steet* et al. contrasted with the results from the previously discussed study. *Street* et al. detected DNA from *T. whipplei* in 35% of saliva samples taken from healthy volunteers (21). Of the 40 individuals sampled, 10 male and 4 female volunteers were positive for the bacteria. All positive individuals except one Indian woman were Caucasian. The negative group included 3 Africans, 4 Orientals, 2 Indo-Asians, and 17 Caucasians. These researchers suggest that *T. whipplei* is a common environmental organism that is not typically pathogenic (21).

Dutly et al. demonstrated the presence of *T. whipplei* in duodenal biopsies and/or gastric juices of individuals that showed no symptoms of Whipple's disease (5). This study was then followed up by testing the saliva and dental plaques of the 14 individuals who were positive in the first study and 17 controls that were negative in the first study. Six of the 14 previously positive people had positive saliva samples but no positive plaque samples. None of the samples from the control group were positive for the bacteria. These researchers concluded that, based on the results of both studies, *Tropheryma whipplei* can reside in both the lower and upper gastrointestinal tract in patients with no signs or symptoms of Whipple's disease (5).

In May of 2007 Rolain et al. published a study indicating that previous reports of *T. whipplei* in saliva samples were false (19). This study found that the primer sequences used in some previous studies that had a high percentage of positives also amplified other strains of actinomycete bacteria. Among those bacteria in one study that

found 10 of 57 individuals positive, 8 of those samples contained a 231 bp sequence 99.1% identical to *Actinomyces odontolyticus* and 2 yielded a 236 bp sequence 99.6% identical to *Actinomyces turicensis* (19). Three sequences from another study that found 3 of 57 individuals positive for *T. whipplei* yielded a 160 bp sequence that is 98.5% identical to *Capnocytophaga gingivalis* (19). The results of the 2007 study led Rolain et al. to conclude that although asymptomatic carriers may still exist, the prevalence of such individuals is much lower than indicated by previous studies (19).

These previous studies are contradicting, but the 'false positive' study was published after researchers had finalized the results of the study discussed in this thesis. The studies that found *T. whipplei* in oral cavity samples of a high percentage of individuals may have, in fact, been identifying one of the similar bacteria mentioned by Rolain et al. However, without further testing of those samples this cannot be confirmed and those studies could in fact have positively and accurately identified *T. whipplei*.

CHAPTER III

METHOD

As discussed in the literature review, the bacterium *Tropheryma whipplei* has been identified in saliva and other oral cavity samples of healthy individuals (5,21,25). Research has shown that *T. whipplei* may be in up to 40% of people that have not been diagnosed with or shown symptoms of Whipple's disease (5).

This study is modeled after those previously done in hopes of finding *T. whipplei* in the Brazos Valley region of Texas.

Sample Collection

The samples were collected at dental offices in Bryan and College Station, Texas. Two of the three dentists are located in Bryan, Texas. Bryan's ethnic makeup is 50.58% white, 13.84% African American, 21.77% Hispanic or Latino, 0.32% Native American, 1.29% Asian, 0.1% Pacific Islander, and 12.12% from other races. The remaining dental office was located in College Station, Texas. The demographics of College Station, where the third dentist is located, is 82.1% white, 5.6% African American, 10.1% Hispanic or Latino, 0.3% Native American, 7.4% Asian, 0.1% Pacific Islander, and 4.6% from other races.

The dental clinics were chosen based on dentists that were members of the Brazos Valley Dental Association and willing to assist researchers with sample collection and preservation until samples could be picked up by researchers. These dental offices were supplied with all necessary materials for sample collection.

The DNA extraction, polymerase chain reactions, and analyses were performed at Texas A&M University College of Veterinary Medicine Research Building and the Texas A&M Laboratory Animal Resources and Research Building.

Participants were required to be between the ages of 40 and 60 years. Based on previous studies and the reported demographics of Whipple's disease, researchers believed this age group was the most likely oral source of *T. whipplei*. The majority of people who have been diagnosed with Whipple's disease have been middle aged. There are multiple explanations for this, but in keeping with that trend, individuals in this age group were surveyed first. Individuals currently taking antibiotic medications were excluded from participation. The antibiotics would decrease the amount of bacteria present in the oral cavity. *T. whipplei* was expected to be found in extremely low amounts potentially resulting in results that were falsely negative; they would have been positive had the antibiotics not decreased the amount of bacteria in the mouth.

People who met the above requirements were asked by the dentist or dental hygienist if they were interested in giving samples to be used in the study. Interested parties were provided with an informed consent form (Appendix A) supplied by the researchers that included an explanation of Whipple's disease and what participation in the study would involve. This form told the patient what samples they were expected to provide. It was mentioned that there was no risk to the patient other than what is normally encountered at a routine dental visit. Also, the study volunteer would not be compensated or receive direct benefit by participating. This consent form was reviewed and approved by the Texas A&M University Institutional Review Board (IRB) these

were signed by participants and kept on file by the dental office to prevent any knowledge of names of participants from the researchers. Individuals had the option to back out of the study at any time.

Along with the informed consent form the participants were asked to fill out a short questionnaire (Appendix B). Researchers asked the age, gender, race, and tobacco habits of each participant. Based on the demographics served by the dental offices it was expected that a diverse population would be sampled. This would test if the high ratio of Caucasians harboring *T. whipplei* relative to other races was accurate.

Three Brazos Valley dentists volunteered to collect samples for the study. These three offices were provided with pre-assembled, pre-numbered collection kits containing the informed consent form, the questionnaire, directions for collection, needed supplies for sample collection, and numbered 0.2 ml tubes for sample storage. The informed consent form and questionnaire can be found in Appendices A and B.

To collect the samples from the oral cavity each kit contained one sterile brush for the buccal swab (Cyto-Pak CytoSoft Brush, San Marcos, California). Dentists and hygienists were asked to use the probe used in regular cleaning for the tartar scrapings. These probes are autoclaved by the dental office after each use and were deemed the most appropriate instrument for tartar collection by the researchers after discussion with dentists. The saliva was collected in cups designed specifically for this purpose by Oragene (Genotek, Ontario, Canada). The cups contained a buffer solution that allowed for indefinite saliva DNA preservation at room temperature. The cups were filled with two milliliters of saliva directly from the mouth. The lid was then applied to the cup and

screwed tight to release the buffer and the cup was tipped repeatedly to mix the buffer with the saliva.

The dentists were instructed to collect samples in accordance with the directions provided in order to keep all facets of the study as identical as possible decreasing variation. For saliva collection dental personnel were asked to have the cup filled to the 'full' line providing two milliliters of saliva. The buccal swab was to be taken from the midpoint of the right inner cheek by turning the brush 20 times in one direction. Then the brush was to be swirled in the provided cup 20 times in the opposite direction. Using a standard dental probe tartar was taken from the first upper premolar on the left side of the oral cavity then mixed in the provided tube to remove collected tartar from the probe.

Along with the cups in the kit were two color coded 0.2 ml tubes containing lysis buffer: orange for the buccal swab and clear for the tartar scraping. The color coding was to prevent any confusion as to which tube contained which sample. The tubes and the Oragene saliva cups were also pre-numbered in correspondence with the informed consent form and questionnaire so participants were accurately matched with all three samples they provided.

DNA Extraction

The saliva DNA was extracted following the Oragene DNA Purification Protocol that was provided by Genotek with the sample cups. Genotek also provided all necessary reagents except the 95% ethanol, and TE buffer. The protocol gives the option of purifying the total four ml sample or a 500 µl aliquot. Researchers chose to purify the saliva in aliquots to preserve samples in their original form if re-testing or re-extracting

was required. The initial step for any amount of saliva to be purified is incubating the Oragene/saliva sample in the Oragene vial at 50°C in a water bath for a minimum of one hour. The samples in this study were typically incubated at 50°C over night. This step needs only to be performed once so after incubation all vials were marked with an 'X' to prevent repetitive incubation.

After incubation 500 µl of Oragene/saliva sample was transferred to a 1.5 ml microcentrifuge tube and 20 µl of Oragene Purifier (supplied by manufacturer) was added and the tube was inverted at least five times and incubated on ice for 10 minutes. Then the sample was centrifuged for 3 minutes at 15,000 x *g* (13,000 rpm) using a Bio-Rad microcentrifuge (Bio-Rad, Hercules, California). The clear supernatant was transferred via pipet to a clean microcentrifuge tube and the pellet was discarded. To the supernatant, 500 µl, of room temperature 95% ethanol was added and the tube was again inverted five or more times. This new solution stood at room temperature to allow full precipitation of the DNA. At this stage researchers centrifuged tubes for 1 minute at 15,000 x *g* (13,000 rpm), but here the supernatant was discarded and the pellet continues to the next step. All ethanol was removed from the pellet by air drying before 100 µl of TE buffer was added. To fully dissolve the DNA into the solution, the sample was vortexed and left to stand over night at room temperature.

The buccal swab and tartar scraping DNA samples were extracted using the Qiagen QIAmp DNA kit following the manufacturer's procedures as outlined for tissue protocol. The tartar and buccal samples were mixed in 200 µl of lysis buffer and this was transferred to 1.5 ml microcentrifuge tubes to make room for additional solutions to be

added. To this amount 20 µl Proteinase K (provided in kit) was added to this amount and mixed by vortex. The samples were placed in an air incubator at 56°C where they were constantly rotated over night to assure complete lysing. After overnight lysis, 200 µl of Buffer AL (provided in kit) was added followed by 15 seconds of pulse vortexing. Then 200 µl of 95% ethanol was added, the sample was again mixed by pulse vortex, and was left to stand at room temperature for 5 minutes. After 5 minutes the lysate was transferred to a Qiagen MinElute column (provided in kit) and centrifuged at 6,000 x *g* (8,000 rpm) for 1 minute. The collection tube was discarded and the MinElute column was placed in a clean microcentrifuge tube and 500 µl of Buffer AW1 (provided in kit) was added before centrifugation at 6,000 x *g* (8,000 rpm) for 1 minute. The previous step is repeated but 500 µl of Buffer AW2 (provided in kit) is added and the tube is centrifuged at 20,000 x *g* (14,000 rpm) for 3 minutes. The MinElute tube was again placed in a clean microcentrifuge tube and 20 µl of distilled water was added before incubating the sample at room temperature for 5 minutes and then centrifuging the at full speed for 1 minute.

Standard PCR Amplification

As a control universal bacterial primers were used. This set of primers was used to test the success of the PCR reaction and for the presence of bacteria in each sample to demonstrate the success of the DNA extraction.

Two different sets of *Tropheryma whipplei* primers were utilized during this research. The first set included 4 different primers that were chosen based on those used in a previous study by Florence Fenollar (7). They were intergenic spacer primers. The

second set of primers was used when results were not clear enough for a definitive positive or negative analysis. These two primers were heat shock protein primers that were chosen for the more conservative nature of heat shock proteins over intergenic spacer regions.

All samples were prepared with Taq-Pro Complete Red (Denville Scientific, Metuchen, New Jersey) containing 10X PCR 1.5 mM $MgCl_2$, each of the forward and reverse primers, and double distilled water. A Bio-Rad thermal cycler (Bio-Rad, Hercules, California) was used with conditions set at 94°C for 10 minutes followed by 40 cycles of 94°C for 30 seconds, 60°C annealing for 30 seconds, and extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

Every reaction that was set up included the sample of interest, sterile water (GIBCO, Carlsbad, California) as the negative control, and a positive control of *Tropheryma whipplei* that was acquired from a European laboratory.

The forward sequence of the universal bacteria primer was (5’ GTT GGC TTA GAA GCA GC 3’) and the reverse sequence was (5’ CAT TTT GCC GAG TTC CTT 3’).

The first specific *T. whipplei* primers that were used was a set of four different forward and reverse 23S RNA primers labeled SECA, TWT-133, PRO-S, and PRO-184. These primers code for intergenic spacer regions. The forward and reverse sequences for SECA are 5’ TTT GTC ATA GGC ATT TCT GTA G 3’ and 5’ AGA CCT CAC TGT TAT ACG GAT 3’, respectively. The forward and reverse sequences for TWT-133 are 5’ GCT GCG CGA AGT AAT TTG 3’ and 5’ AGA TAC ATG CGG AGA TAC T

3', respectively. The forward and reverse sequences for PRO-S are 5' TCG GAC TAA AAG TGC GAC AC 3' and 5' GCC TTG ACT ATG ACA TAA TCA A 3', respectively. The forward and reverse sequences for PRO-184 are 5' ATA ACA AGA AGC TGG ATA TGC 3' and 5' CGG ATC TTC ACG AAA TGT CC 3', respectively.

After several attempts to identify *T. whipplei* with the intergenic spacer region primers results were inconclusive and a switch was made to use heat shock protein primers that would be more specific (9). These primers were developed for a 620 base pair sequence.

The primer sequences for the heat shock protein were *hsp1* Forward (5' TGA CGG GAC AAC ATC TG 3') and *hsp2* Forward (5' CGC GAA AGA GGT TGA GAC TG 3') with the same reverse sequence; *hsp2* Reverse (5' ACA TCT TCA GCA ATG ATA AGA AGT T 3') (7).

Results were resolved on 2% agarose gels using 1X TBE buffer made up from a pre-mix manufactured by Intermountain Scientific (Intermountain Scientific Corp., Kaysville, Utah). This 10X ready pack TBE Buffer mix was diluted in 10 liters of double-distilled water to make 1X TBE buffer. Every gel was stained with ethidium bromide (AMRESCO, Solon, Ohio) and run in a Bio-Rad electrophoresis chamber for varying amounts of time. The gels were then viewed in a fluorescent imaging chamber (AlphaInnotech, San Leandro, California) and analyzed by researchers.

The sensitivity of the standard PCR was determined for each of the three primers that were used to analyze all samples: universal bacteria primer, heat shock protein primer 1, and heat shock protein primer 2. Serial dilutions were done with the primers

and tested using the same strain of *T. whipplei* bacterium that was used as the positive control in all PCR runs of samples. The sensitivity of the PCR varied between all primers. The universal bacterial primer detected bacteria to 950 *pg/µl.* The first heat shock protein primer, *Hsp1*, detected *Tropheryma whipplei* to 9.5 *pg/µl* and *Hsp2* was sensitive to *T. whipplei* to 0.095 *pg/µl*.

CHAPTER IV

RESULTS

Analysis of the data collected by the epidemiological survey of *Tropheryma whipplei* is discussed in this chapter. Images of gel electrophoresis that were done by researchers were reviewed and data was compiled. All participants were residents of the Brazos Valley region of Texas that were in one of the three dental offices for a routine teeth cleaning and check up. Their office visits and corresponding sample collections took place between November 2005 and August 2006. DNA extraction, PCR amplification, and image analysis for results took place between December 2005 and October 2007. Samples were taken from fifty volunteers that participated in this study.

Of the forty-nine volunteers, forty-two were Caucasian, 5 were Hispanic, 1 was Asian, and 1 was of Middle Eastern descent. Unfortunately this did not provide as broad a spectrum of ethnicity as originally anticipated. The numbers, 85.7% Caucasian, 10.2% Hispanic, 2.05% Asian, and 2.05% Middle Eastern did not coincide closely with the demographics of the area discussed previously. Nevertheless, should the samples collected from these forty-nine volunteers would allow researchers to identify *Tropheryma whipplei* as an oral habitant in the Brazos Valley it would spark further research in this area.

Researchers asked for participants to be between the ages of 40 and 60 years. The minimum age was 42 and the maximum age was 61 with a mean of 49 years and 7 months.

The reported ratio of men to women that suffer from Whipple's disease is eight to one (87%). Although these numbers were first reported in 1987 and there is evidence to suggest that the difference may not be quite as great as originally reported, there was some question whether or not this ratio would prove true if *T. whipplei* was located in Texas (5). The volunteers of this study numbered 15 males and 34 females giving a ratio of three to seven male to female.

Each of the forty-nine participants provided three different samples: the buccal swab, the tartar scraping, and the saliva resulting in a total of 147 samples to be tested. The DNA extraction from all 147 samples was shown to have been successful by positive bands from the universal bacteria primer. When difficulties with reactions arose with several samples, the PCR runs were repeated to assure accurate results were derived.

After multiple PCR amplifications of all samples *Tropheryma whipplei* was not identified in any of the 147 samples from 49 individuals of the Brazos Valley Region of Texas in an amount greater than could be confirmed by the primers. There were occasional faint bands that indicated the possibility of a positive sample, but after those suspicious samples were repeatedly re-extracted and re-run, they were conclusively determined to be negative.

The fact that this study failed to identify *Tropheryma whipplei* in the Brazos Valley is not an indication that the bacterium is not present in this region of the world. Several factors that may have affected the results of this study and the power of the study using the conventional p-value of 0.05 are discussed in detail in Chapter V.

CHAPTER V

DISCUSSION AND CONCLUSIONS

This chapter reviews the study. Included are recommendations for improvement. Future studies would also test samples using RT PCR in an attempt to identify bacteria in amounts below the sensitivity of standard PCR. Another study would also benefit from a more diverse and larger population of volunteers. These and other changes would make for a more complete epidemiological study of the Brazos Valley region of Texas.

The purpose of this study was to identify if the bacteria that causes Whipple's disease, *Tropheryma whipplei*, is present in the oral cavities of healthy residents that are not presenting symptoms of Whipple's disease.

The results of this study were not in accordance with the hypothesis. It was hypothesized that *Tropheryma whipplei* would be identified in the saliva, tartar, or buccal sample of a collection of residents of the Brazos Valley region of Texas.

There are several explanations for why the proposed hypothesis was not supported in this study. The sensitivity of the standard PCR primers that were used may have been too low, the small number of resident volunteers (especially males) may have contributed, or it is possible that previous studies had false positives.

The primers *hsp1* and *hsp2* detected *T. whipplei* to the 9.5 *pg/µl* and 0.095 *pg/µl* respectively. Although the likelihood of the bacterium being identified by *hsp2* was greater than *hsp1* there could still have been bacteria present in amounts less than 0.095 *pg/µl*. If this study were to be extended, the use of RT-PCR or nested PCR would be

necessary. RT-PCR has the capability of identifying smaller amounts of bacteria than standard PCR but this high sensitivity can also result in false positives. As this has possibly been a problem in previous studies it may not lend itself ideally to the identification of *T. whipplei*.

Researchers had planned for at least 100 volunteers, but samples from only 49 individuals were provided. This number may have yielded better results had there been a greater number of males. It has been reported that there is an 8:1 ratio of males: females diagnosed with Whipple's disease. Socioeconomic status and health care situations may play a part in this ratio, but if gender is a determining factor this study with a male: female ratio of 1:2.267 could likely have affected the results.

Whipple's Disease has also heavily affected Caucasians over people of other ethnic backgrounds. This study had a high ratio of Caucasian individuals, but it could be assumed from previous knowledge that the Asian participant would not harbor the bacterium thus lowering the number of possibly positive people to 48 and decreasing the sample size by 3.

Looking back, the survey should have included the profession of those people that gave samples. The survey from 1980 of 700 Europeans indicated that farmers and carpenters were more likely than people of other professions to have Whipple's disease. One theory on this is that being an actinomycete, *T. whipplei* is present in the soil and natural substances that those professionals frequently are frequently in direct contact. Actinomycete bacteria are commonly present in the soil. However, it can be assumed

that the amount of farmers and carpenters in the Brazos Valley is such a low number that very few if any of the participants are involved in those professions.

Given that the bacterium in question was not identified in any of the 49 individuals (147 samples), the *Roulain* et al. study of 2007 claiming the possibility of false positives can not be over looked.

When *Morgenegg* et al. first developed the heat shock protein primer for use in detecting *T. whipplei*, it successfully identified the bacterium in all samples (17) previously known to contain the bacterium[9a]. The 33 negative patient specimens all remained negative with this heat shock protein primer after the first 30 cycles and only 3 of those samples produced weak bands after a further pass of 40 cycles (17). Researchers suggested that 2 of those 3 "negative" patient specimens (1 of the 3 specimens was diagnosed with Whipple's disease) may have been false positives. The faint bands in that study lend support to this study's occasional erratic samples that produced faint bands upon the first amplification, but were thereafter negative on subsequent tests of the same sample.

The Z*inkernagel* et al. study found clear cut positive samples from DNA that was harvested from the gingival sulcus area. All of the samples in that study taken from saliva smooth surface gingival plaque and the cheek were negative. Several reasons have been discussed that could explain the lack of *T. whipplei* in the Brazos Valley, but it could be that the bacterium was not actually present in previous studies therefore it is more rare than presently believed. If that is the case, it was unlikely that we would have located the bacterium in such a small number of samples.

The underlying rate of *T. whipplei* colonization in the oral cavities of people living in the Brazos Valley region of Texas is not known. However we can estimate the maximum prevalence that would be statistically consistent with our result of 0 in 49 trials using a conventional p-value of 0.05. Based on a binomial distribution, an estimated underlying *T. whipplei* prevalence of 1 in 1000 has a 95% chance of giving us this same result at the power of 0.9878.

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APPENDIX A

Texas A&M University

Comparative Medicine Program

Informed Consent Form

Title: Survey of bacterial flora in the oral cavity of peoples ages 40-60 in Brazos Co.

Principal Investigator: Melanie Ihrig

Co-Investigator(s): Robert Rose, Anna Knox

Introduction/Purpose:

You are being asked to volunteer for a research study. The purpose of the study is to determine whether a bacterium called *Tropheryma whipplei* is normally found in the mouths of healthy adults in your age group. The presence of this bacterium in the oral cavity has not been associated with increased risk of disease. The information we hope to gain from this study will be used to support a request for funding further study of this little known bacterium.

We are planning to enroll 500 volunteers between the ages of 40 and 60, from dental offices in Brazos County. The reason you are being asked to participate is because you have a regular dental exam scheduled.

This document is to provide you with information to consider in deciding whether to participate. Please ask questions if there is anything you do not understand. You participation is voluntary and will have no effect on the quality of you dental care if you choose not to participate.

Procedures:

If you agree to participate in the study, saliva, cheek swab, and tartar scrapings will be collected by your dentist or dental hygienist during your visit. These samples will then be given to the research investigators so they can be tested to determine if the *Troperyma whipplei* bacteria is present.

Risks:
There are no known risks associated with collecting saliva samples, cheek swabs, or tartar scrapings other than those you would normally experience during a routine oral exam.

Benefits:

Being in this study may be of no direct benefit to you, but others may benefit from this research.

Alternatives:

If you decide not to participate in this study, you will still receive the standard dental care.

Confidentiality:

We will keep your records private. We will use and ID number rather than your name on study records, including the samples that are collected. The researchers will not have a way of linking the ID back to your name and other facts that might point to you. You will not receive your results from this research study.

Compensation and Cost:

Compensation for lost wages or other direct/indirect costs are not available. You will not receive any money for participating in the study. Your cost will be those incurred based on the routine dental visit. There are no additional costs associated with the study.

Voluntary Participation/Withdrawal:

Your participation in this study is voluntary. You are free to withdraw your consent at any time without the loss of benefits to which you are entitled.

Contact Persons:

If you have any study related questions please contact Melanie Ihrig at 979-845-7433. If you have any questions concerning this research or your rights as a research subject, please contact Angelia Raines, Director of the Office of Research Compliance at 979-847-9362.

Signature:

If you are willing to volunteer for this research, please sign below.

_____ _____
Subject's Signature Date

_____ _____
Person Obtaining Consent Date

APPENDIX B

Survey of Bacterial Flora in the Oral Cavity of Peoples Ages 40-60 in Brazos County
Melanie Ihrig
Robert Rose
Anna Knox

Subject # _____

Age: _____ years

Gender: **□ Male** **□ Female**

Ethnicity: **Check all that apply.**

□ White of non-Hispanic origin
□ African American
□ Hispanic
□ Asian
□ Other _____

Tobacco User: **□ Yes** **□ No**
If yes:
□ Cigarettes/Cigars
□ Smokeless
□ Other_____

APPENDIX C

Sample Collection Instructions

1. Saliva- fill to line, screw on cap until mixing buffer is released
2. Buccal swab- midpoint inner cheek right side, 20 turns with brush, twirl brush in tube with buffer 20 times and secure cap on tube. **Orange tube**
3. Tartar scrape- 1st upper premolar left side, mix in sample tube with buffer to transfer sample from probe, secure cap on tube. **Clear tube**

APPENDIX D

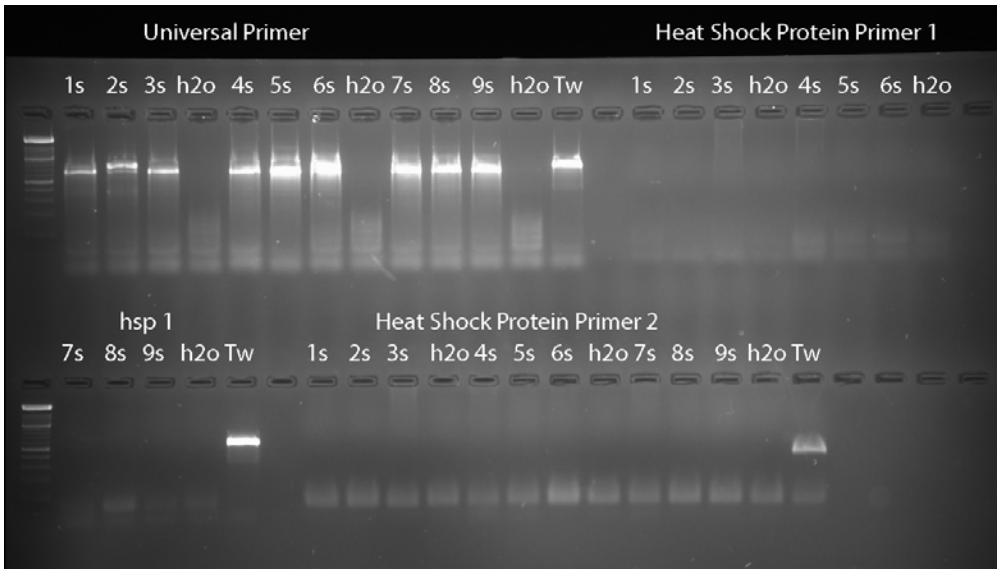
Subject	Age	Gender	Ethnicity	Tobacco Use
1	42	M	W	N
2	48	F	W	N
3	46	F	W	N
4	50	F	W	Y
5	53	F	W	N
6	45	F	W	N
7	47	F	H	N
8	42	F	ME	N
9	58	F	W	Y
10	61	F	W	N
11	42	F	W	N
12	60	M	W	N
13	42	F	W	N
14	50	F	W	N
15	53	F	W	N
16	44	F	W	N
17	58	M	W	N
18	53	F	H	N
19	51	F	W	N
20	47	F	H	N
21	59	F	W	Y
51	49	F	A	N
52	45	F	W	N
53	59	F	W	N
54	53	M	W	Y
55	54	F	W	Y
56	48	F	H	N
57	55	M	W	N
58	48	F	W	Y
81	44	M	W	N
82	58	M	W	N
83	53	F	W	Y
84	50	M	W	N
85	40	F	W	N
86	45	F	H	N
87	49	M	W	N
88	58	F	W	N
89	44	F	W	N
90	53	M	W	N
91	43	F	W	N
92	49	F	W	N

Subject	Age	Gender	Ethnicity	Tobacco Use
93	51	M	W	N
94	51	F	W	Y
95	41	M	W	N
96	43	M	W	Y
97	55	F	W	N
98	40	F	W	N
99	56	M	W	N
100	45	F	W	N

KEY	
M	Male
F	Female
W	Caucasian
H	Hispanic
A	Asian
ME	Middle Eastern
N	No
Y	Yes

APPENDIX E

Figure	Samples Tested Successfully
Figure 1	1s 2s 3s 4s 5s 6s 7s 8s 9s
Figure 2	1t 2t 3t 4t 5t 6t 7t 8t 9t 10t
Figure 3	11s 11b 11t 12s 12b 12t 14s 14b 14t
Figure 4	18b 18t 19b 19t 20s 20b 20t
Figure 5	51s 51t 52s 52t 53s 53t
Figure 6	51b 52b 53b 100s
Figure 7	54s 54b 55s 55b 55t 56s 56b 56t
Figure 8	18s 19s 57s 57b 57t 58s 58b 58t
Figure 9	83s 83b 83t 84s 84b 84t 85s 85b
Figure 10	81s 81b 81t 86s 86b 89s 89b 89t
Figure 11	57s 81s 81t 89s 89b 89t
Figure 12	87s 87b 87t 91s 91b 91t 96s 96b 96t
Figure 13	92s 92b 92t 94s 94b 95s 95t
Figure 14	93s 93b 93t 97s 97b 97t 98b 98t
Figure 15	82s 82b 82t 88s 88b 88t 99s 99b 99t
Figure 16	90s 90b 90t 94s 94b 94t 95s 95b 95t
Figure 17	17b 17t 21b 21t 100s 100b 100t



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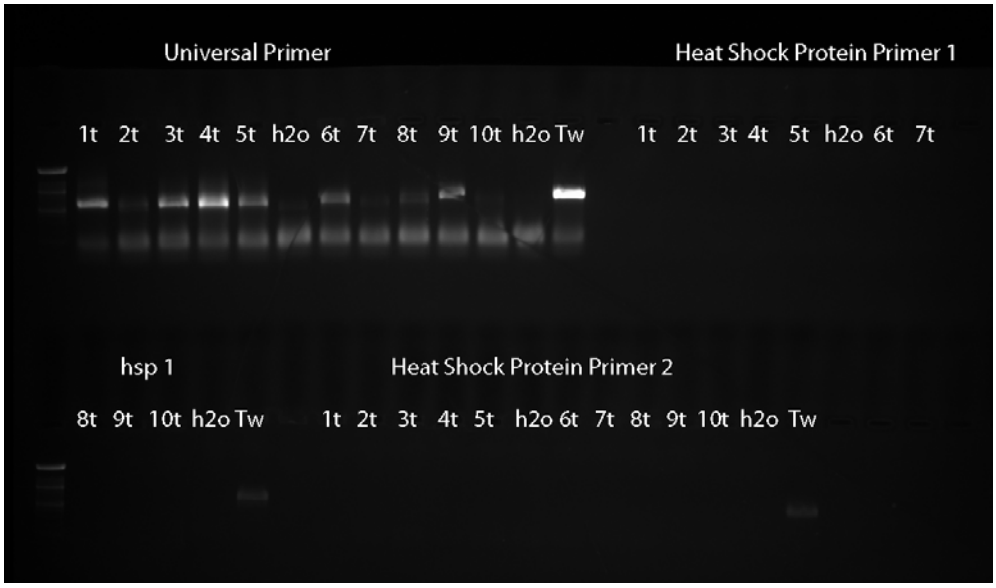

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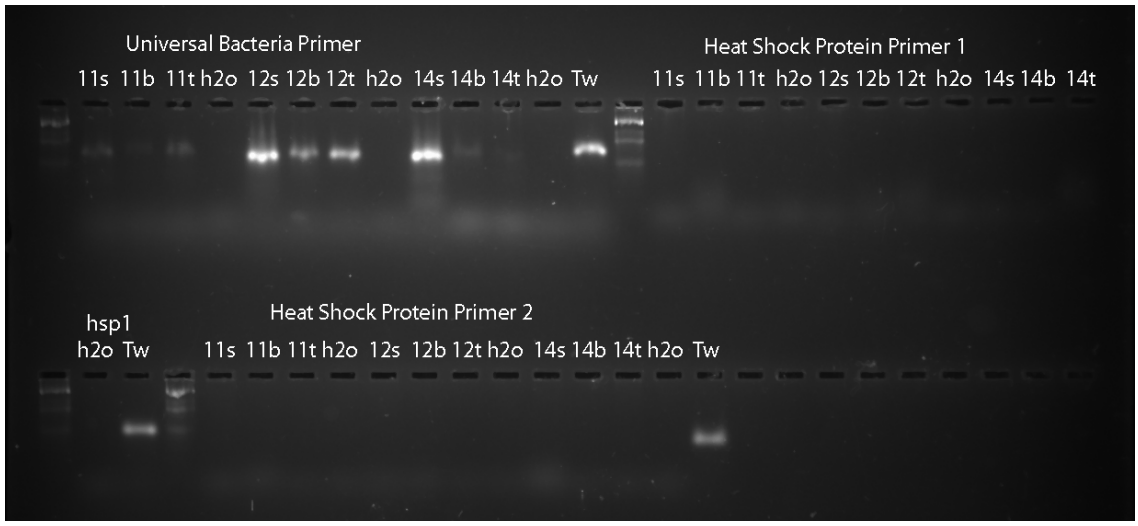

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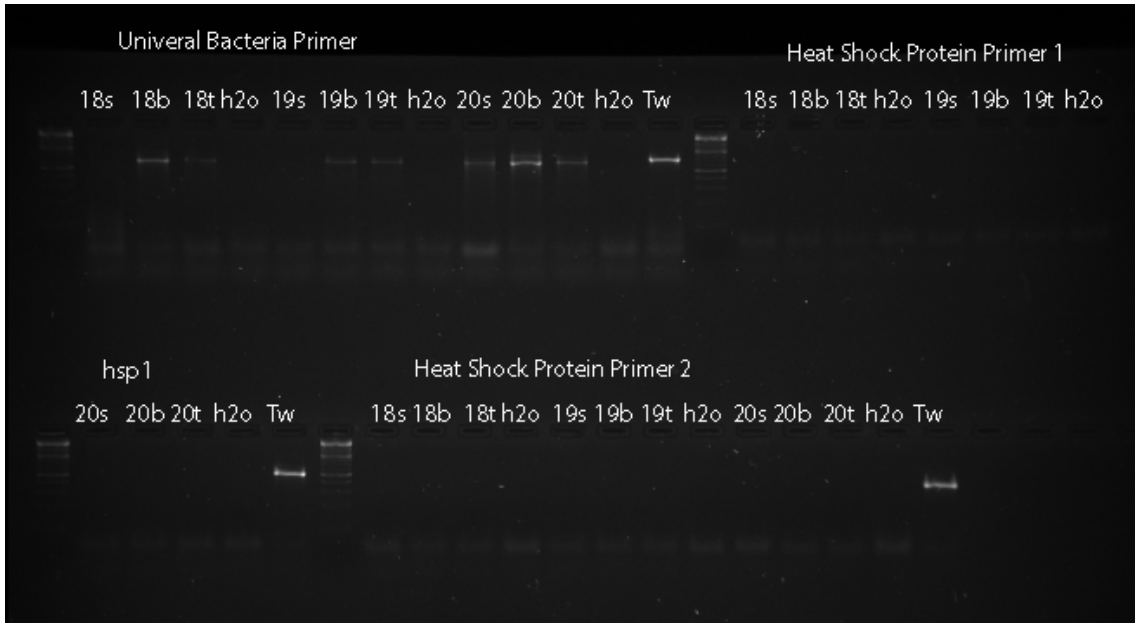

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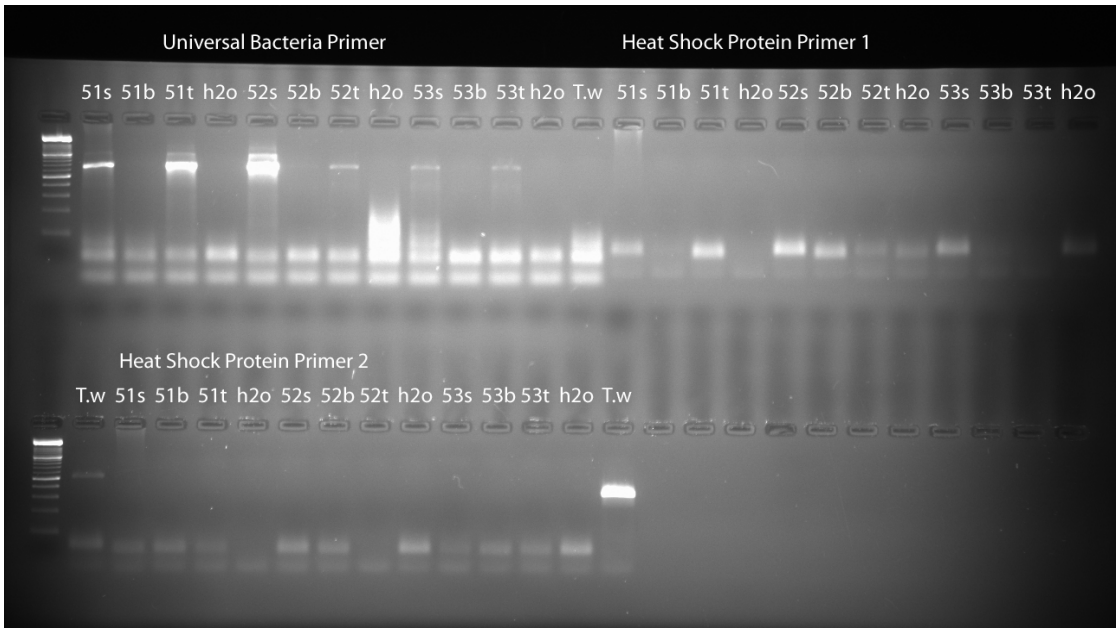

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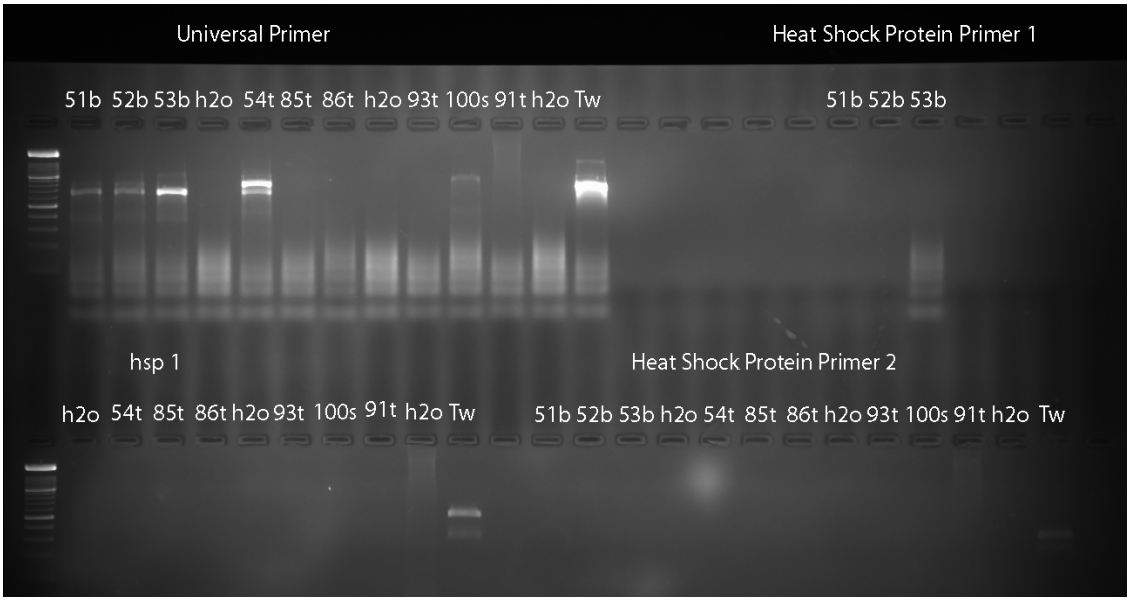

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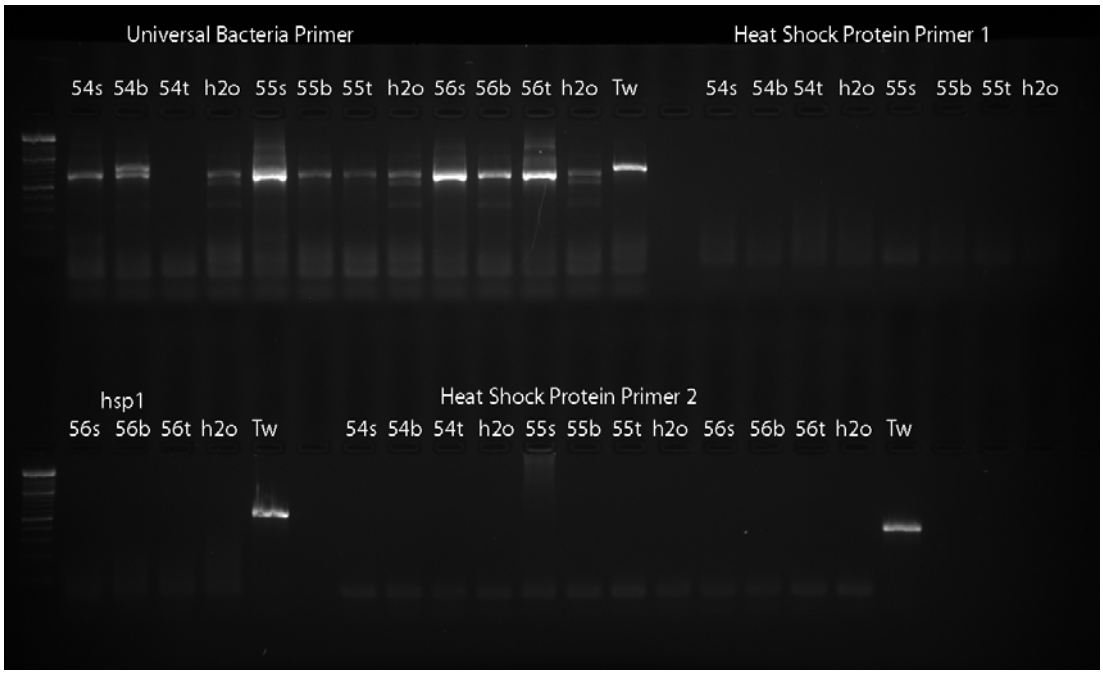

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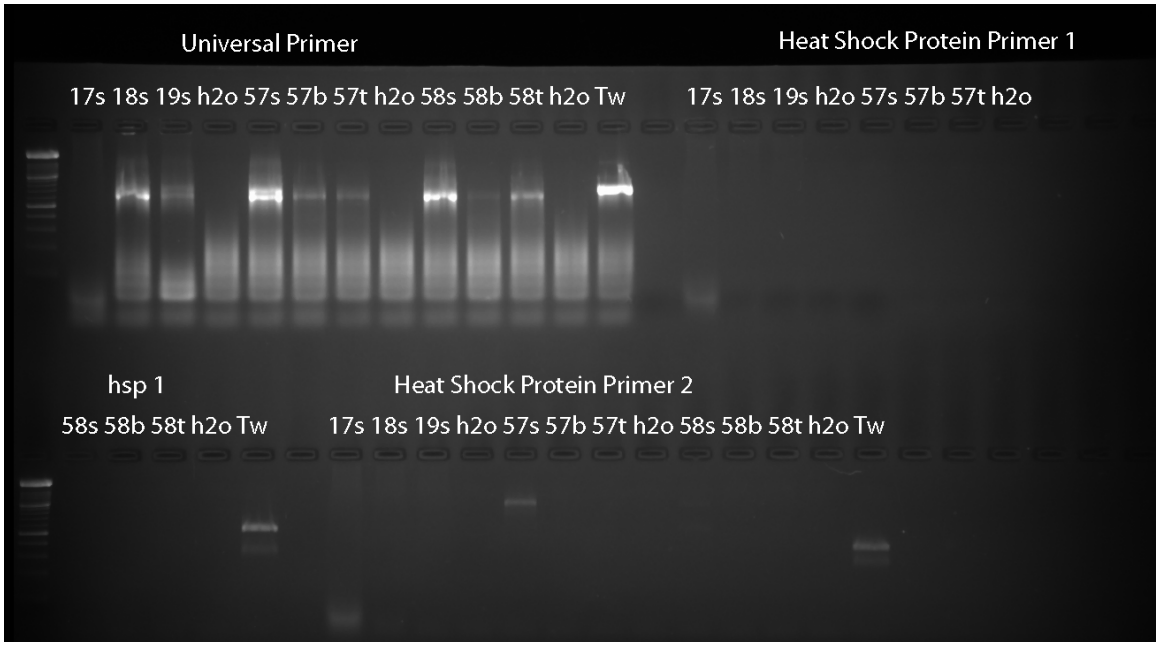

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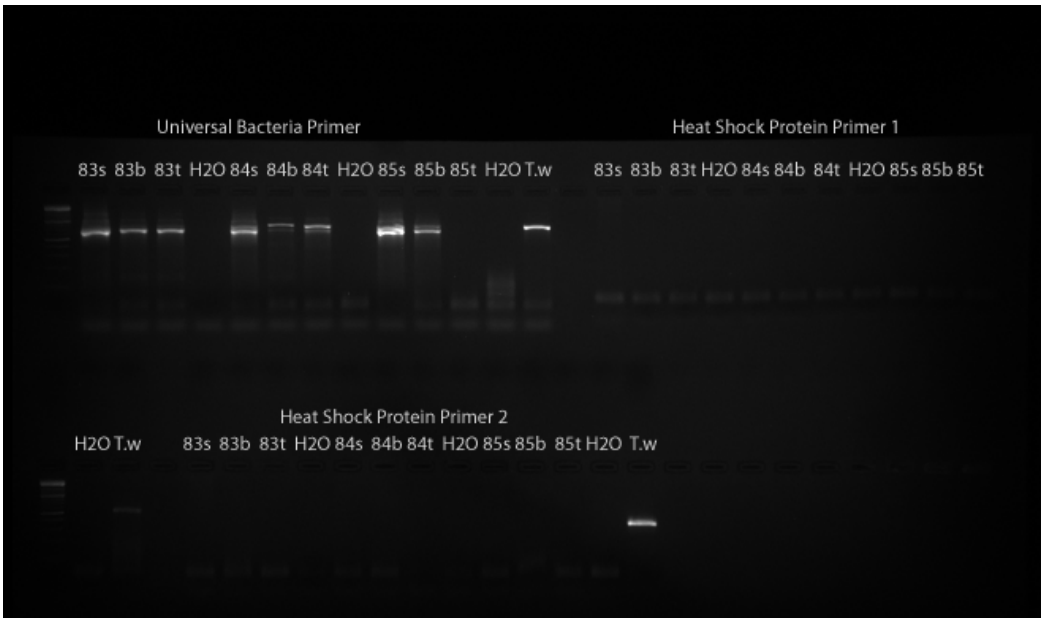

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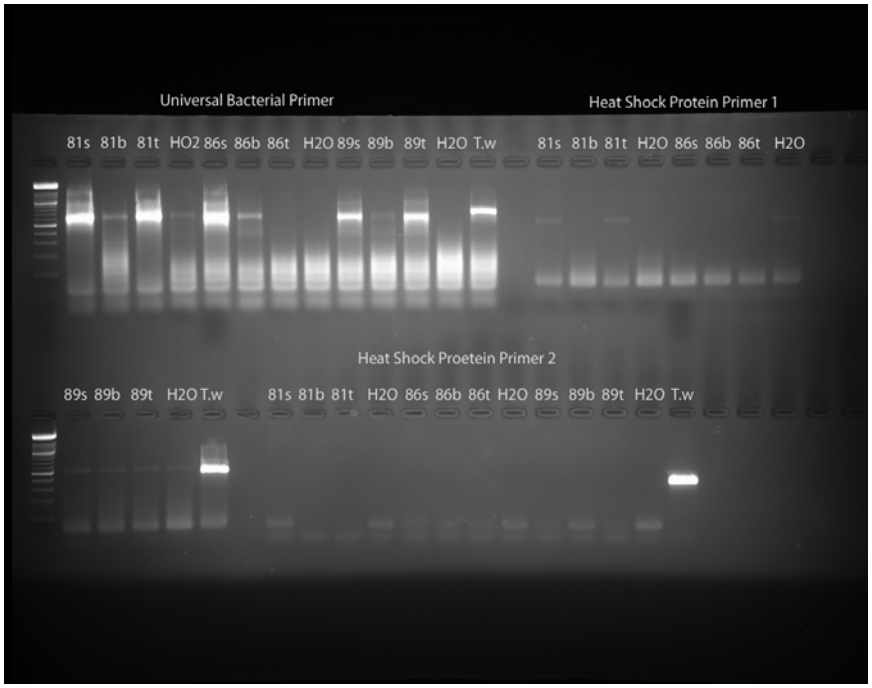

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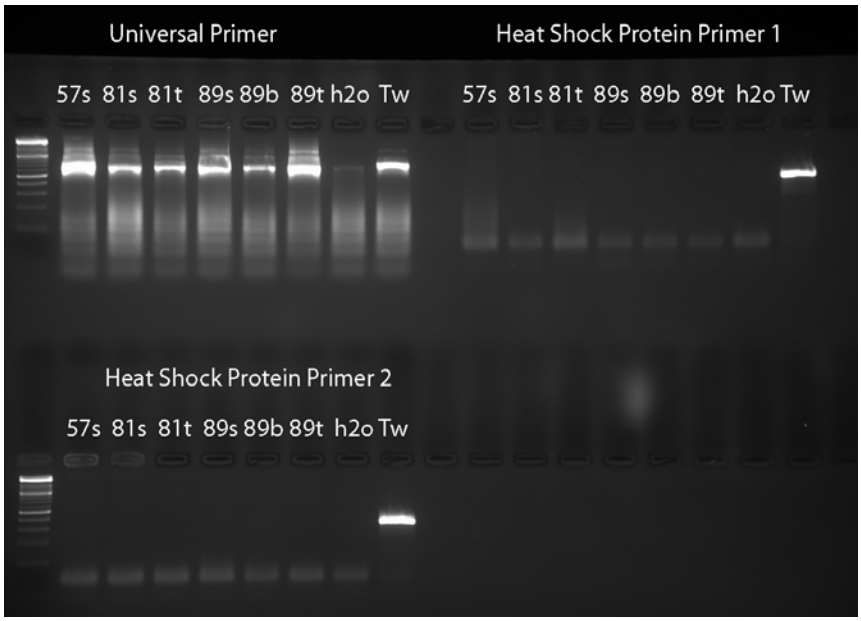

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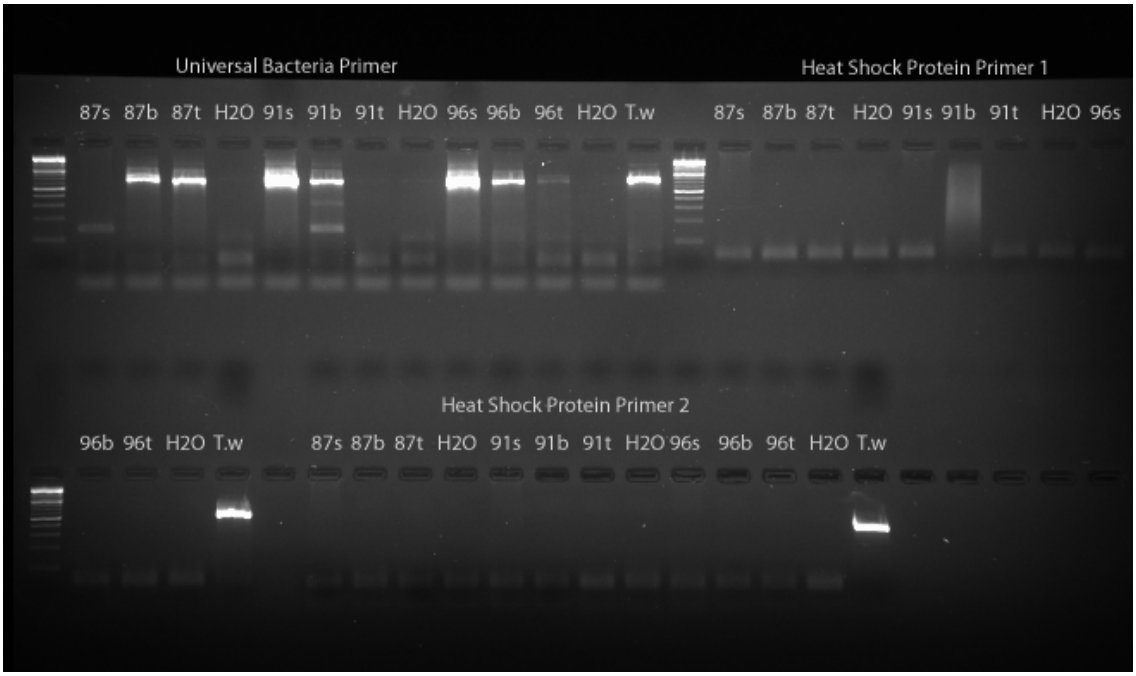

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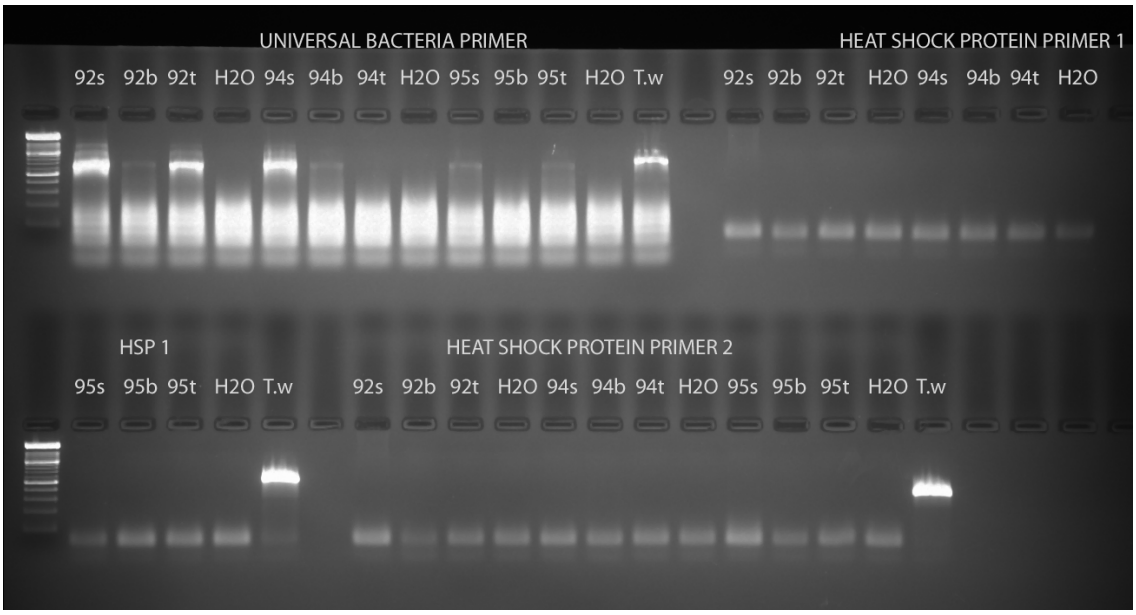

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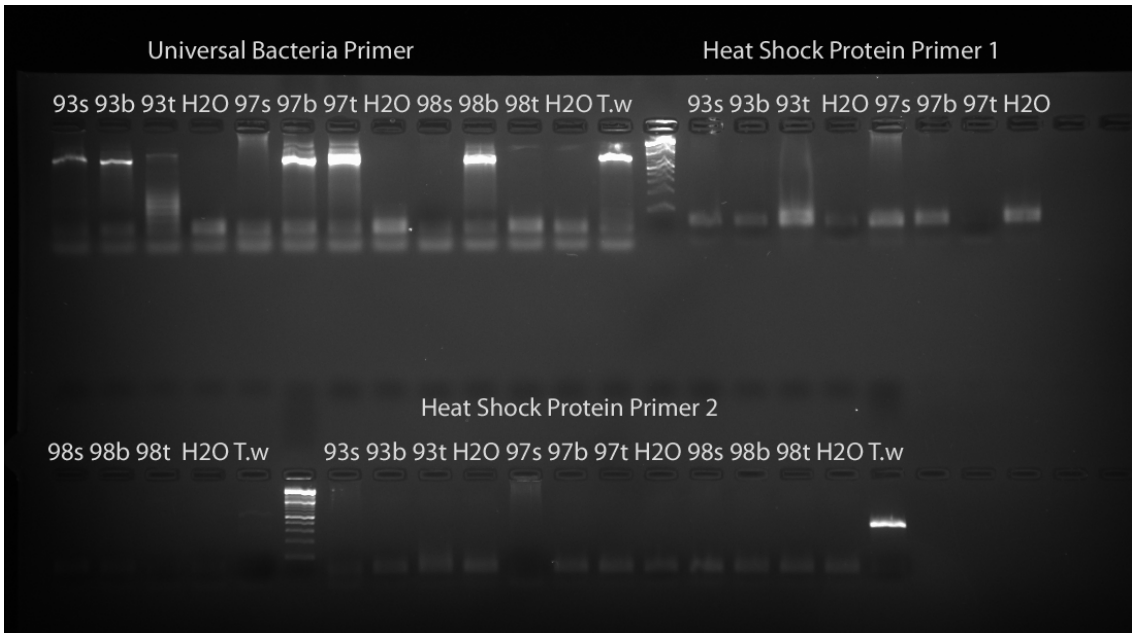

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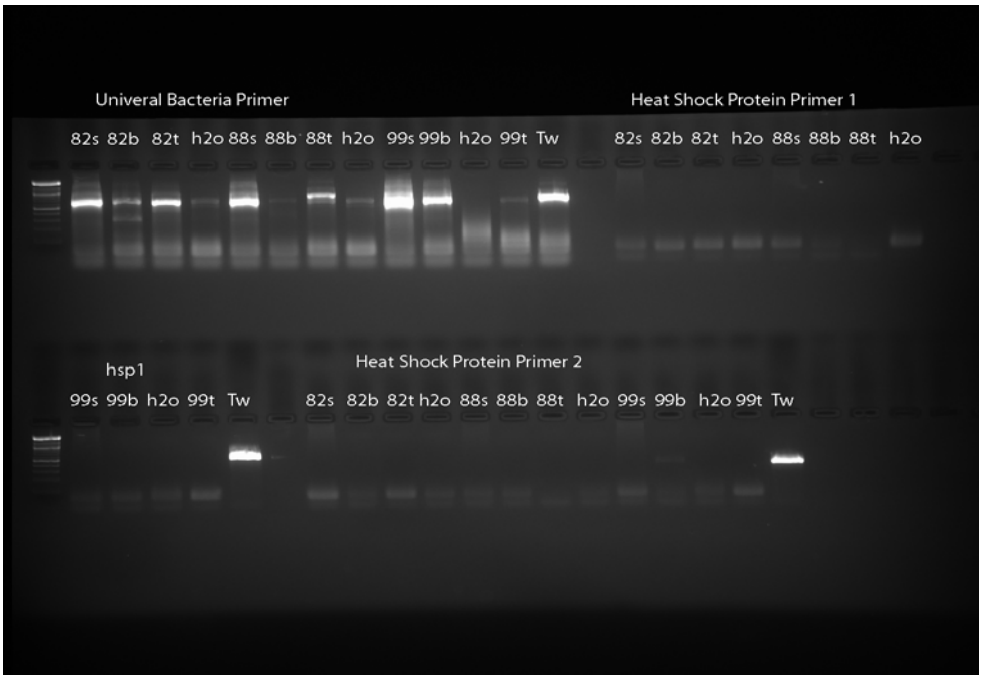

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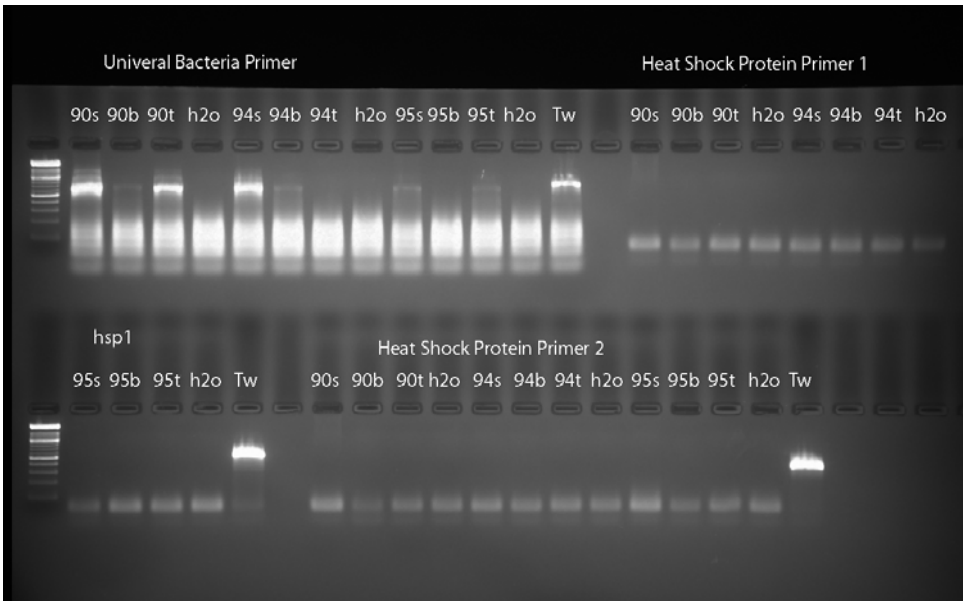

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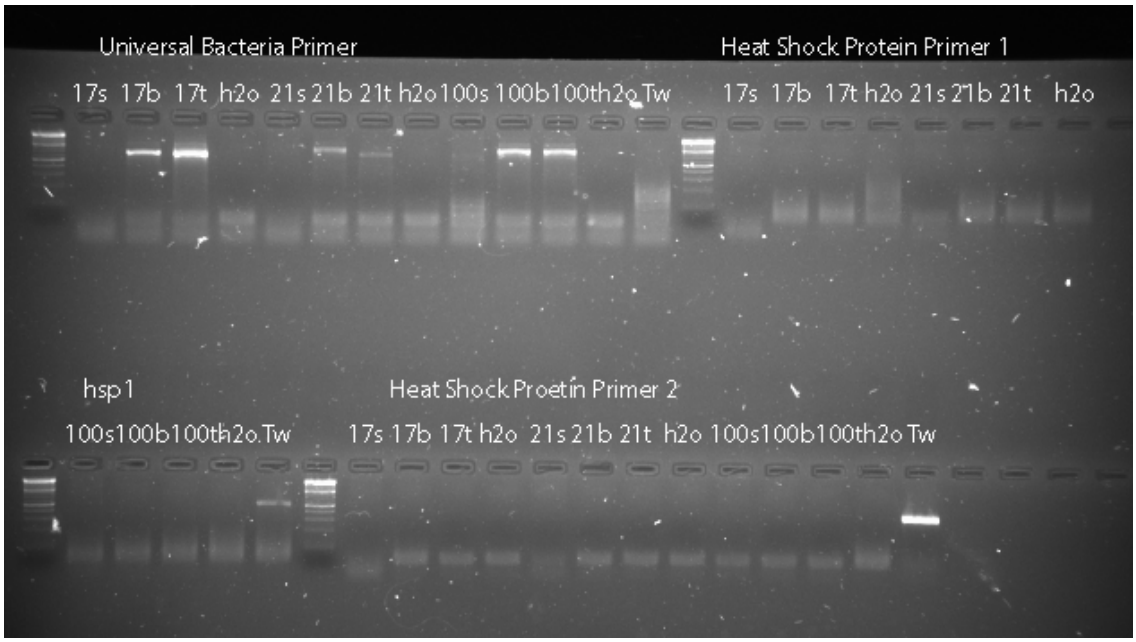
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Heat Shock Protein Primer 1
17s 17b 17t h2o 21s 21b 21t h2o
hsp1
100s100b100th2o Tw
Heat Shock Proetin Primer 2
17s 17b 17t h2o 21s 21b 21t h2o 100s100b100th2o Tw

Figure 17.

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