2015

Strain Typing Mycobacterium marinum from Outbreaks at Zebrafish Research Facilities

Brooke M. Clemons

Strain typing *Mycobacterium marinum* from outbreaks at zebrafish research facilities

by

Brooke M. Clemons

SUNY College of Environmental Science and Forestry, Department of Environmental and Forest Biology, Syracuse, NY 13210

Candidate for Bachelor of Science

Biotechnology with Honors

May 2015

Thesis Project Advisor:_____
Christopher M. Whipps PhD

Second Reader:_____
Carolyn T. Chang PhD Student

Honors Director:_____
William M. Shields PhD

Date: _____

Abstract

Zebrafish (*Danio rerio*) are used as model organisms for biological research due to their rapid and transparent development and high fecundity amongst other reasons. Research has expanded beyond embryonic studies, with adult fish used for longer-term studies such as human disease and senescence. Zebrafish are often housed at high density in large colonies. As with any similar husbandry situation, diseases can occur, with impacts that range from morbidity to premature mortality costing researchers time and money. Understanding the impact of underlying diseases in zebrafish is crucial, particularly for long-term studies where chronic infections may confound results. One such disease problem is mycobacteriosis, caused by numerous *Mycobacterium* species. Mycobacteria likely spread when fish are exchanged between facilities with no precautions for biosecurity. Bacteria can also persist in the water and live on surface biofilms, making control more difficult. *Mycobacterium marinum* is one species that is of interest because it is highly virulent, contributing to serious outbreaks. It is also a concern for occupational health because it can cause skin infections in humans. To better understand the spread of mycobacteriosis caused specifically by *M. marinum*, strain typing was conducted to categorize isolates from seven different zebrafish facilities throughout the United States. For strain typing a variable number of tandem repeat assay was used. This technique was adapted using fluorescently labeled primers followed by fragment analysis in order to increase throughput and repeatability. These results were then used to determine if any epidemiological linkages exist between facilities experiencing outbreaks.

Table of Contents

Abstract 2
Table of Contents 3
Acknowledgements 4
Introduction 5
    Research Objectives 8
    Specific Objectives 9
Methods and Materials 11
    Cultivation and Identification 11
    Agarose Gel Electrophoresis Analysis of VNTR PCR 11
    Fragment Analysis of VNTR PCR 12
    Data Analysis 12
Results 14
    Cultivation and Identification 14
    Agarose Gel Electrophoresis Analysis of VNTR PCR 14
    Fragment Analysis of VNTR PCR 14
    Data Analysis 15
Tables and Figures 16
    Table 1 16
    Table 2 17
    Table 3 17
    Table 4 18
    Table 5 19
    Figure 1 20
    Figure 2 21
Discussion 22
Work Cited 27
Appendix 30
    Protocol A1 30
    Figure A1 30
    Figure A2 31
    Figure A3 32
    Figure A4 33
    Figure A5 34
    Code A1 35
    Table A1 36

Acknowledgements

Thank you to Dr. Whipps for teaching me the laboratory skills, guiding me through each step and inspiring me as I worked over the past two years on this project. I also would like to thank Carolyn Chang for mentoring me in the lab over the past year and introducing me to zebrafish husbandry, allowing me to work hands on with the organisms we're working to save. Funding was provided in part from the National Institutes of Health (5R24RR01386-07), the SUNY Center for Applied Microbiology, and the SUNY-ESF Honors Program.

Introduction

Throughout the scientific community zebrafish (*Danio rerio*) are utilized as model organisms in vertebrate developmental and genetic analyses in a variety of applications due to their rapid and transparent development as embryos, rapid regenerative abilities, high fecundity, short generation time, and mutant phenotypes which mimic human disorders (Kimmel *et al.*, 2004; Dooley and Zon, 2000; Gemberling *et al*., 2013). Research has expanded far beyond developmental biology however, with zebrafish being used in longer-term studies such as human disease, toxicology, behavior and aging (Gerhard and Cheng, 2002; Phillips and Westerfield, 2014; Dai *et al., 2013*; Gerlai*,* 2014). Understanding the impact of underlying diseases in zebrafish is crucial for all kinds of research, but particularly long-term studies where chronic infections may confound important results of biomedical research (Kent *et al.*, 2012). Zebrafish are often housed at high density in large colonies, at times numbering in the tens of thousands, and can be considered a form of intensive aquaculture. As with any similar husbandry situation (e.g. laboratory mice, rats, *Xenopus* (frogs) and Japanese Medaka), there are also disease problems, with impacts that range from premature fish mortality to morbidity. As a consequence of these epizootic events, control and management of disease outbreak cause zebrafish research facilities significant amounts of both time and money.

One such bacterial disease is mycobacteriosis, caused by several *Mycobacterium* species (Kent *et al.*, 2004; Ostland *et al.*, 2008; Whipps *et al.*, 2012). Mycobacteria likely spread when fish are exchanged between facilities with no precautions for biosecurity, including lack of quarantining new fish, egg surface disinfection and UV sterilization of water (Kent *et al.*, 2009). Further complicating

matters is that the bacteria can persist in the water and live on surface biofilms, making control more difficult (Beran *et al.*, 2006; Falkinham, 2009). *Mycobacterium marinum* is one species that is of particular interest because it is highly virulent, often resulting in serious outbreaks, recognized to infect freshwater and aquaria fish, including zebrafish (Watral and Kent, 2007; Ostland *et al.*, 2008; Yanong *et al.*, 2010). It is also of great concern due to the zoonotic properties it possesses; infecting humans working within close quarters of infected fish leading to fish handler's disease (Aubry *et al.*, 2002; Wu *et al.*, 2012). These facultative, Gram-positive, acid-fast positive bacilli also possess differential antibiotic and chemical disinfection resistance within the *M. marinum* species thus contributing to its overall success in its persistence (Aubry *et al.*, 2000; Aubry *et al.*, 2002; Mainous and Smith, 2005; Whipps *et al.*, 2012; Chang and Whipps, in press).

Mycobacteriosis outbreaks create both acute severe effects and high-level chronic subclinical problems, with complete elimination from large fish research facilities, especially challenging due to their ability to colonize quickly within recirculating water systems and persist on surface biofilms within systems (Whipps *et al.*, 2012). Severe acute outbreaks result in signs such as disequilibrium, hemorrhaging and protuberance at the infection site and lack of appetite with death occurring between five to sixteen days after initial infection (van der Sar *et al.,* 2004). Chronic low-level to subclinical outbreaks result in granuloma in the organs and external signs including loss of scales and skin contusions; allowing the fish to survive at least four to eight weeks (van der Sar *et al.,* 2004). Chronic low-level infection is what is most commonly presented in zebrafish. Current prevention and treatment of established infections includes fish depopulation and re-derivation and tank disinfection, which has proven to be effective, yet extremely time consuming, interruptive, and requires the destruction of

invaluable fish (Whipps *et al.*, 2012).

One tool used to explain epidemiological information about *M. marinum* is DNA fingerprinting. Like a human fingerprint, it is highly unlikely two organisms would possess identical DNA information. This testing of DNA is used to determine relationships between samples, proving beneficial in distinguishing disease-causing agents. In particular, variable number tandem repeat (VNTR) analysis has been used on *M. marinum* isolated from fish (Yanong *et al.*, 2010; Sun *et al.,* 2011; Broutin *et al.,* 2012). VNTRs are locations within the genome that possess a nucleotide sequence arranged in a series of tandem repeats; creating an identical allelic pattern by samples within an individual. Sun *et al.* (2011) performed a study on *M. marinum* isolates looking the ability of previously identified loci and in addition to two newly identified VNTR loci to enhance current *M. marinum* genotyping tools (Ablordey *et al.*, 2005; Stragier *et al.*, 2007). VNTR loci Locus 16, MIRU5, Locus 6, VNTR2067, MIRU2 and VNTR3422 were identified by PCR as having strong discriminatory power, with a calculated Hunter-Gaston diversity index of greater than or equal to 0.362 (Sun *et al.,* 2011). This study also concluded that there was no significant relationship between VNTR cluster and virulence with the isolates examined; despite findings by van der Sar *et al.* (2004) that found genetic variation within two distinct clusters played an important part in determining pathogenicity, supporting virulence is strain dependent (Sun *et al.,* 2011).

More recently, VNTR techniques have been applied to *M. marinum* isolates from humans and fish in France to understand genotypic diversity analyzing 15 VNTR loci (Broutin *et al.,* 2012). Both genetic and genotypic diversity values were higher for fish isolates, and only a limited number of genotypes possess zoonotic properties (Broutin *et al.,* 2012). Broutin *et al.* (2012) also noted that genetic diversity of *M. marinum* was

higher in aquaria fish than in aquacultures; this being attributed to differences in sanitary control and varying levels of fish species biodiversity. Importantly, VNTR fingerprinting provided information about the differing gene pools within this species, implicating the biological and ecological niches, as wells as the epidemiological capabilities of the strains (Broutin *et al.*, 2012). Because little is known regarding the genetic diversity of *M. marinum* isolated from laboratory zebrafish, VNTR fingerprinting may allow insight into potential differences between isolates from different facilities and regions. VNTR fingerprinting can provide insight into zebrafish *M. marinum* outbreaks as genetic polymorphisms could correspond to geographic regions, facilities, practices, and fish strains providing important epidemiological information.

Due to an increased prevalence in the number of mycobacteriosis outbreaks at zebrafish facilities throughout the United States more information is required about the epidemiology of this infection in order to implement control and management. Outbreaks of *M. marinum* occurred at seven zebrafish research facilities in the United States, including Massachusetts, Virginia, Mississippi, Arkansas, Oregon and two in the state of California. To assess epidemiological linkages between individual facilities and other locations 30 samples were strain typed using VNTR polymerase chain reaction (PCR) techniques.

Research Objectives

The main objectives of this study were to identify *M. marinum* strains infecting zebrafish research facilities and assess the epidemiological linkages between individual facilities and other locations within the United States by using previously identified VNTR loci. From this information epidemiological relatedness can be evaluated in the

context of biosecurity and husbandry practices in order to make recommendations for *M. marinum* control.

Specific Objectives

1. Can molecular fingerprinting of *M. marinum* at VNTR loci be used to identify *M. marinum* strains from isolates isolated from zebrafish research facilities throughout the United States?
    a. Based on a previous study by Sun *et al.* (2011) using a VNTR DNA fingerprinting method for strain typing *M. marinum,* I predict that these same methods can be successfully applied to isolates collected from United States zebrafish research facilities. Sun *et al.* (2011) identified seven VNTR loci within the genome possessing strong discriminatory power when isolates were studied, making associations between VNTR cluster and virulence (Sun *et al.,* 2011).
2. Utilizing information from fragment analysis and creating a similarity matrix, are epidemiological linkages of *M. marinum* present between regions within the United States and other facilities as seen in other studies?
    a. I hypothesize if *M. marinum* isolates are endemic, possessing distinctive genotypes in distinct geographic regions as they occupy different biological and ecological niches (Ucko *et al.*, 2002; Broutin *et al.,* 2012), then isolates collected from zebrafish research facilities in the same facility or region of the country will be more genetically similar than those collected from other areas within the United States.

3. Do the findings from this study support previous knowledge of *M. marinum* and can our results impact methods for mycobacteriosis prevention, management and eradication?
    a. Understanding the locations of specific strains at zebrafish research facilities will indicate if breaches in biosecurity have or have not occurred, meaning current protocol when handing disease screening may need to be improved. Strain identification will also provide insight into differential susceptibilities between areas.

Methods and Materials

Cultivation and Identification: *Mycobacterium marinum* isolates (Table 1) were collected from United States zebrafish facilities submitting fish for diagnostic testing or submitted to the Whipps lab by collaborators who isolated *M. marinum*, stored in 30% glycerol. Isolates were then grown on 60uM hemin enriched Difco™ Middlebrook 7H10 agar plates (BD©, 262710). Single colonies were selected for each sample and grown in Difco™ Middlebrook 7H9 broth (BD©, 271310) at 28°C at a 45° angle on a shaker. A cold Kinyoun method acid-fast stain was then completed to confirm *M. marinum* identity (Appendix, Protocol A1). DNA extraction of each broth culture occurred via MO BIO Laboratories, Inc. UltraClean® Microbial DNA Isolation Kit following manufacturer protocol.

Agarose Gel Electrophoresis Analysis of VNTR PCR: Variable number tandem repeat (VNTR) uniplex polymerase chain reaction (PCR) was performed using primers selected from Sun *et al.* (2011). Locus 6, Locus 16, MIRU2 and VNTR2067 (Table 2) were amplified and analyzed by agarose gel electrophoresis to determine approximate number of VNTR for each isolate. Each reaction was completed as a 25.0uL mixture; with 12.5uL 2x concentration of quick load ® Taq 2x Master Mix by New England Biolabs ® Inc., 0.25uL forward primer at a 0.5uM concentration, 0.25uL reverse primer at a 0.5uM concentration and 2uL extracted DNA, from method previously mentioned. PCRs had an initial 3 minute denaturation step at 95.0°C, followed by 34 cycles of denaturation for 20 seconds at 95.0°C, annealing for 30 seconds at 56.0°C and elongation for 1 minute at 68.0°C, with a final elongation for 5 minutes at 68°C. Each PCR product (5uL) was analyzed on a 1% agarose gel with 0.0001% gel red 10,000x in Water (PHENIX Research) in 0.5x TE (20L: 108.0g Tris base, 55.0g boric

acid, 40mL 0.5M EDTA pH 8.0 + water to 20L) buffer at 140 volts. The size standard used to approximate VNTRs was quick load 100bp (basepair) DNA ladder by New England BioLabs® Inc. PCRs were set up as multiplex reactions combining primers VNTR 2067/Locus 16 and MIRU2/Locus 6 using the previously mentioned set up in a 25uL reaction.

Fragment Analysis of VNTR PCR: VNTR multiplex PCR was performed combining MIRU2/Locus 6 (mix 1) and VNTR 2067/Locus 16 (mix 2) with fluorescently labeled forward primers in each amplification (Table 3) (PCR prepared with Hotstart Taq DNA polymerase and followed previously mentioned protocol). PCR purification was performed using a ZYMO RESEARCH DNA Clean & Concentrator™-5 kit using standard protocol. Fragment analysis was prepared using 96 well plates with 1.0uL purified PCR product, 0.5uL MapMarker 1000 Ladder, and 8.5uL PCR grade water. Capillary electrophoresis was performed for fragment analysis by the DNA Analysis facility on Science Hill at Yale University.

Data Analysis: Agarose gel electrophoresis VNTR values were scored by visual approximation. Fragment analysis through capillary electrophoresis of VNTR values were scored by establishing bin intervals at exactly half of each repeat value; with electropherogram analysis performed using Peak Scanner ™ (Life Technologies ©). Fragments were scored with Supply (2005) guidelines and compared to agarose gel electrophoresis images.

The data set was formatted into a data matrix in Microsoft Excel (cite) and converted into a text file that was analyzed using R 3.1.0 (R Core Team, 2013) and R Studio (RStudio, 2012) by a graduate student co-supervising this project. Agglomerative hierarchical clustering of the dataset was carried out using the “cluster” package (cite –

Maechler et al. 2015:) (Appendix, Code A1). Euclidean metrics (distances are root sum-of-squares of difference) and average method ([unweighted pair-]group [arithMetic] average method, aka ‘UPGMA’) were the setting used in this analysis (Appendix, Code A1).

Results

Cultivation and Identification: Of the 30 isolates acid-fast stained, all were positive, presenting red-stained rods under compound light microscopy.

Agarose Gel Electrophoresis Analysis of VNTR PCR: Results from agarose gel electrophoresis are listed in Table 4. Amplification did not occur at Locus 16 for isolates ORA1, ORB, and MA3 (Appendix, Figure A1). PCR products amplifying Locus 16 ranged in size from approximately 445 to 799 base pairs, or one to seven repeats. At loci VNTR2067, PCR products ranged in size from approximately 227 to 451 base pairs, or zero to four repeats. MIRU2 PCR products amplified in the range of >353 to approximately 565 base pairs, or less than zero repeats to four. PCR products amplifying at Locus 6 ranged in size from approximately 462 to 686 base pairs, or one to five repeats. The results from agarose gel electrophoresis were inconsistent between duplicates and did not provide enough resolution of fragment size (100 bp) to determine repeat values, which differ by 53 to 59 bp.

Fragment Analysis of VNTR PCR: Results from fragment analysis are listed in Table 5. Amplification confirmation could not be confirmed at one or both of the loci in mix 1 (Table 3) for isolates Davis1, TG2, and AR#1 via agarose gel electrophoresis (Appendix, Figure A2). For mix two (Table 3), amplification did not occur at one of both of the loci for isolates ORA1, ORB, ORD1, MA3, MA8, MA11, MA2011, AR#1, AR#2, ATCC927, TG2, BC1, SH1, KST214, KST 266, and KST 687 (Appendix, Figure A3). Multiplex reactions that failed for amplify PCR products were completed in uniplex and pooled during the PCR purification step. A dilution of 1:5 (1uL PCR product + 4uL water) was found to provide the most reliable results for fragment analysis, creating a range of intensity peaks approximately half to the same size as the size standard. Purified PCR

concentrations at lower levels produced peaks significantly smaller than the size standard and full PCR concentrations created off scale peaks when analyzing electropherograms. Repeat values for isolates ORD1 at Locus 16 and KST 214 at loci Locus 16 and VNTR2067 were not gathered.

MIRU2 PCR products amplified in the range of approximately 406 to 618 base pairs, one repeats to five. PCR products amplifying at Locus 6 ranged in size from approximately 462 to 686 base pairs, or one to five repeats. PCR products for Locus 16 ranged in size from approximately 386 to 622 base pairs, or zero to four repeats. Loci VNTR2067 PCR products ranged in size from approximately 283 to 451 base pairs, or one to four repeats.

Data Analysis: Following analysis a dendrogram (Figure 1) and banner plot (Figure 2) displaying results of the cluster analysis was produced. This analysis produced an agglomerative coefficient (AC) of 0.8 which is a dimensionless quantity that indicates the degree of structuring found (http://www.unesco.org/webworld/idams/advguide/Chapt7_1_4.htm) . AC values range from 0 to 1 with a value of 1 indicating very clear structuring. The banner plot (Figure 2) is an alternative way to visualize the data compared to a dendrogram. The white area on the left shows unclustered data, whereas areas where white stick into the red indicate heights where clusters are found. At a height of 3.5 4 clusters are observed or at a height of 2.5 6 clusters are observed, past these heights the number of clusters increases dramatically. The resulting dendrogram (Figure 1) shows the 30 isolates used in this study produced four different groups based on four loci. These groups contained eight, six, thirteen, and three isolates.

Tables and Figures

Table 1. Isolate collection location, identification and sources

Location	Sample ID
Arkanas	AR #1
Arkansas	AR #2
ATCC (PA)	ATCC927
California	DAVIS 1
California	KST 214
California	KST 266
California	KST 417
California	KST 458
California	KST 687
California	KST 94
California	SH1
Massachusetts	BC1
Massachusetts	MA11
Massachusetts	MA2011
Massachusetts	MA3
Massachusetts	MA5
Massachusetts	MA8
Massachusetts	MA9
Mississippi	MSS2
Mississippi	MSS4
Oregon	ORA1
Oregon	ORB
Oregon	ORC2
Oregon	ORD1
Oregon	ORD2
Oregon	OR932
Oregon	TG18
Oregon	TG19
Oregon	TG2
Virginia	VIMS9

Table 2. VNTR loci used for *M. marinum* identity (Sun *et al.,* 2011)

VNTR Loci	Locations	PCR Primer Sequences	Product= flank region + tandem repeats +flank region 2 (bp)
Locus 16[a]	592401–592563	F 5' -CAGATCGTCGAACTGGTGGC R 5'-CCGAAAGCGTAGTGGTAGGTG	548=246+59x2.8+140
Locus 6[a]	2053067–2053330	F 5' -GCTCAGCTCGGCTGGGAAG R 5'-ACATCTTCGTCGCGGTGGA	670=283+56x4.7+123
VNTR2067[c]	2067998–2068171	F 5' -GCGCTCGACTCATCAGGCAC R 5'-TCGCACCCGGATTGTCTAACT	401=136+56x3.1+91
MIRU2[b]	2708167–2708277	F 5' -GCCGCGTCGATGGACTCTT R 5'-GAACGGTTGATCCTTGATGTGC	464=149+53x2.1+204

[a] Loci previously studied by Ablordey *et al.* (2005)
[b] Loci previously studied by Stragier *et al.* (2005)
[c] Loci previously studied by Sun *et al.* (2011)

Table 3. Multiplex reactions including locus and primer detail

Multiplex	Locus	Sequence Name	Sequence 5'-3'
Mix 1	MIRU2	MIRU2F-FAM*	[6~FAM]GCCGCGTCGATGGACTCTT
	Locus 6	Locus6F-HEX**	[5HEX]GCTCAGCTCGGCTGGGAAG
Mix 2	VNTR2067	VNTR2067F-FAM*	[6~FAM]GCGCTCGACTCATCAGGCAC
	Locus 16	Locus16F-HEX**	[5HEX]CAGATCGTCGAACTGGTGGC

*FAM blue dye label
**HEX green dye label

Table 4. Agarose gel electrophoresis results of VNTR analysis

Sample ID	Primer			
	Locus 6	Locus 16	MIRU2	VNTR2067
AR #1	2 or 3	4	2	3
AR #2	2 or 3	4	2	3
ATCC927	3	4	1	1 or 3
DAVIS 1	1 or 2	2	3 or 4	2 or 4
KST 214	2	2 or 3	1	0 or 1
KST 266	0 or 1	3 or 4	0 or 1	1
KST 417	0 or 1	2 or 4	0 or 1	1
KST 458	0 or 1	3 or 4	0 or 1	1
KST 687	0 or 1	2 or 4	0 or 1	1
KST 94	0 or 1	3 or 5	0 or 1	1
SH1	0 or 1	2 or 4	0 or 1	1
BC1	2 or 3	3	0 or 1	1
MA11	2	3 or 4	0	2
MA2011	2	4	5	2
MA3	1	-	-1 or -2	1 or 2
MA5	2	3	0 or -1	2
MA8	1 or 2	2 or 3	-1 or -2	1 or 2
MA9	2	3 or 4	0 or -1	2
MSS2	5	4	3 or 4	2 or 4
MSS4	3	4	1	1 or 3
ORA1	3, 4 or 5	-	3	3
ORB	3, 4 or 5	-	3	2 or 3
ORC2	2	3 or 4	2	1 or 2
ORD1	4	7	3	3
ORD2	4	4 or 7	3	3
OR932	3 or 5	4	3 or 4	2 or 4
TG18	3 or 3	3	1 or 3	2
TG19	3 or 5	3	1 or 3	2
TG2	3 or 5	3	1 or 3	2
VIMS9	2 or 3	1	0 or 1	1

Table 5. Capillary electrophoresis results of VNTR fragment analysis

Sample ID	Primer			
	Mix 1		Mix 2	
	Locus 6	MIRU2	Locus 16	VNTR2067
AR #1	2	3	0	3
AR #2	1	4	2	3
ATCC927	1	3	4	2
DAVIS 1	2	3	2	4
KST 214	2	2	2	1
KST 266	2	3	3	1
KST 417	2	3	-	-
KST 458	1	2	1	2
KST 687	2	3	0	1
KST 94	2	2	0	1
SH1	2	2	0	1
BC1	1	2	4	2
MA11	1	2	0	2
MA2011	1	1	0	2
MA3	1	1	0	4
MA5	1	1	0	3
MA8	1	2	4	4
MA9	1	3	0	2
MSS2	1	3	1	3
MSS4	4	2	3	2
ORA1	2	3	0	3
ORB	2	4	0	3
ORC2	2	4	0	3
ORD1	2	5	-	3
ORD2	2	5	4	3
OR932	1	5	4	3
TG18	1	5	4	3
TG19	1	4	4	3
TG2	5	3	4	3
VIMS9	4	2	2	2

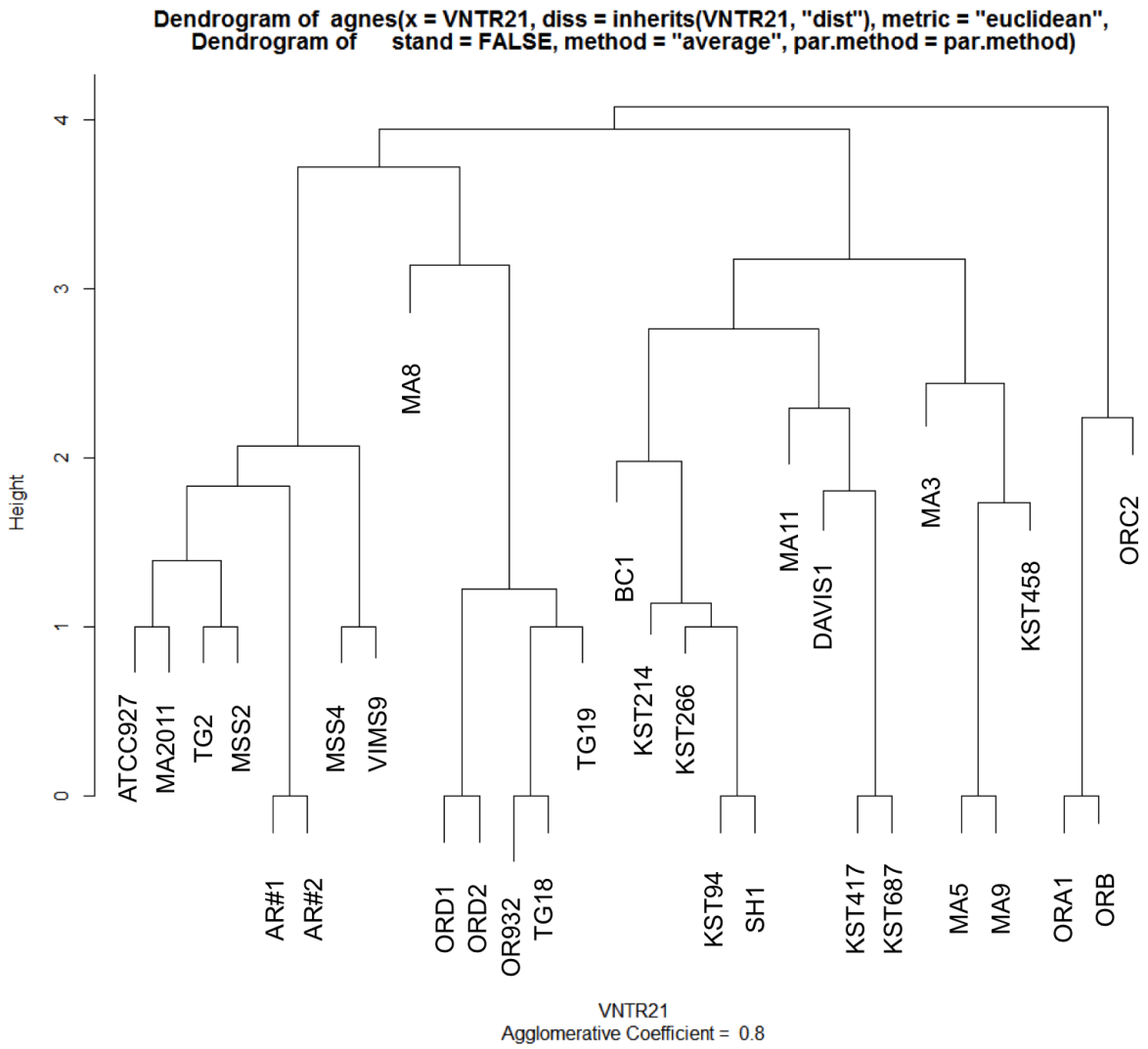

Figure 1. Dendrogram of genetic relationships of *M. marinum* isolates based on four VNTR loci

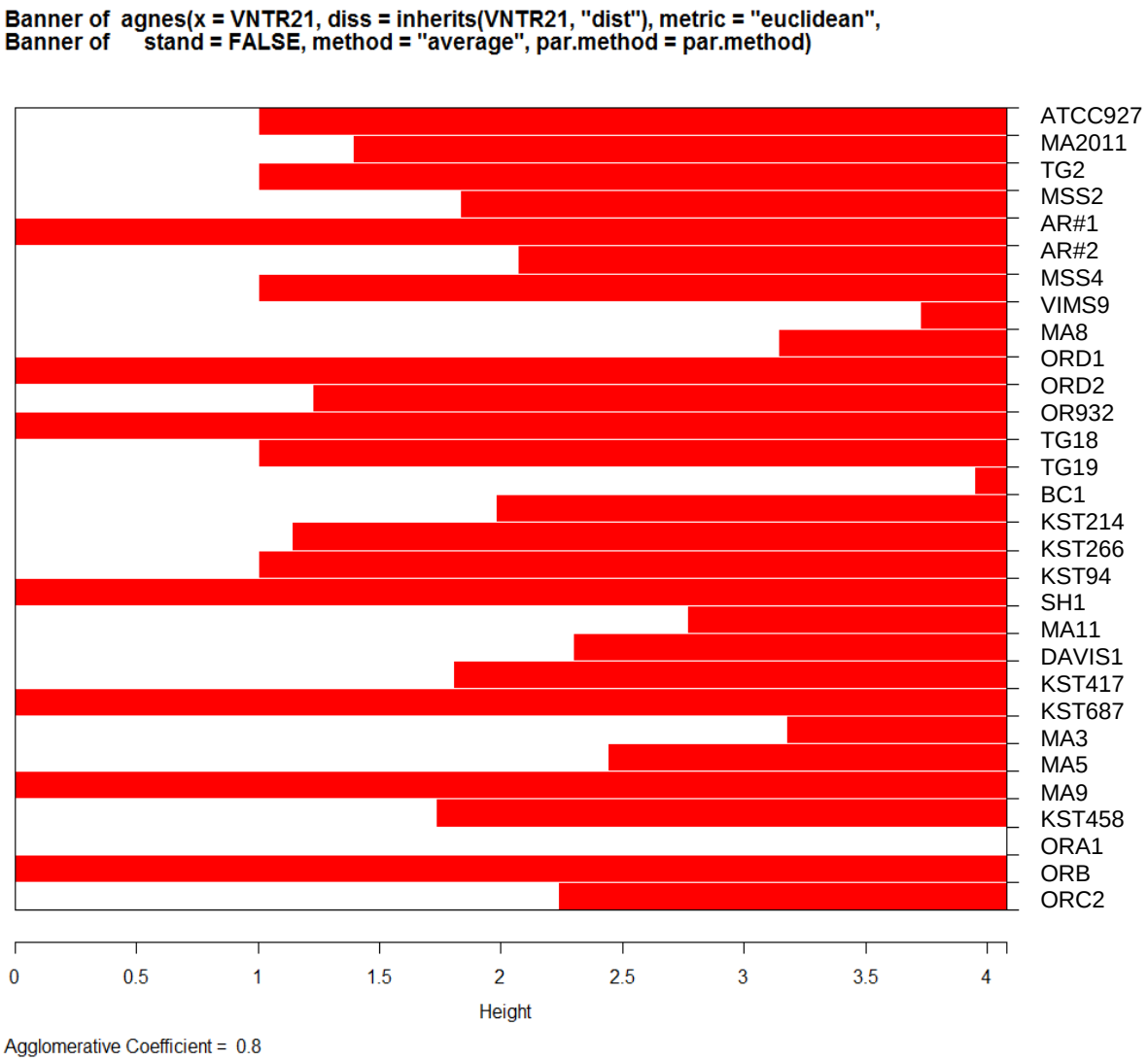

Figure 2. Banner plot genetic relationships of *M. marinum* isolates based on four VNTR loci

Discussion

The purpose of this study was to strain type 30 isolates of *Mycobacterium marinum* collected from mycobacteriosis outbreaks at zebrafish research facilities throughout the United States. Utilizing VNTR molecular fingerprinting techniques we aimed to assess the epidemiological linkages of isolates at individual locations and different regions of the country. We also wanted to make comparisons of our findings to previous knowledge on *M. marinum* in order to make suggestions for improvements on biosecurity during zebrafish husbandry practices.

The first technique looked at in this study used traditional agarose gel electrophoresis in order to determine band location to assign a repeat value at the particular loci. Analysis using this method provided benefits (Appendix, Table A1) including a one-day processing time on site, utilized traditional PCR techniques, was relatively inexpensive and only required a gel rig and gel imaging system. The limitation this method possessed was its specificity. Bands determining repeat values were read using a 100bp standard, which proved difficult to assess on agarose. MIRU2 has a difference of 53bp between each repeat value, Locus 6 has a difference of 56bp, Locus 16 has repeat values with difference of 59bp and VNTR2067 with 56bp increments.

To overcome this limitation we explored using another method of fragment analysis via capillary gel electrophoresis with VNTR amplifying primers labeled with FAM and HEX fluorescent tags. Since the mid 1990's the usage of fluorescent multiplex PCRs has been successful with microsatellite markers studies in order to determine population structure, history and diversity of a species (Findlay and Quirke, 1997; Luikart *et al*., 1999; Fuentes *et al.,* 2008). Though this process takes approximately one week, uses light sensitive primers, is more expensive, and requires outside facilities for

analysis, it has a high specificity; able to identify the exact base pair location where amplification of the loci occurred (Appendix, Table A1). Electropherograms are produced (Appendix, Figure A4) with peaks indicating where amplification of the loci occurred. Peaks in green represented Locus 6 or Locus 16 and blue peaks signified MIRU2 or VNTR2067.

One problem that was encountered when using this fragment analysis method was the presence of multiple peaks from a single fluorescent primer, when it was expected that only one green or blue peak would be present on each electropherogram based on agarose banding patterns (Appendix, Figure A4). Supply *et al.* (2001) reported that stutter peaks are common in MIRU-VNTR loci PCR of mycobacteria. These stutter peaks are most likely caused by artifactual strand slippage of polymerase during PCR and often appear as a ladder of low intensity peaks (Supply *et al.*, 2001; Supply, 2009). To interpret data where stutter peaks are present Supply (2009) established guidelines on how to assign repeat values. The peak with the highest repeat number should be used when assigning values (Supply *et al.,* 2001). The presence of further stutter peaks may occur only if they are uniformly small or have a drastic decrease in height in comparison to the “true” assigned peak (Supply *et al.*, 2001). To correct this issue, isolates could also be reanalyzed in uniplex PCR in order to receive single high intensity peaks (Supply *et al.*, 2001).

This study has found that VNTR loci molecular fingerprinting of *M. marinum* can be used as a tool to strain type isolates collected throughout the United States. Through this experiment we were able to examine four of the seven VNTR loci cited by Sun *et al.* (2011) as an area within the genome possessing discriminatory power. This information was be used to produce a dendrogram (Figure 1). The four loci within the VNTR region

were used to disentangle genetic relationships between isolates, which showed four groups were present. Group 1 contained isolates that varied the most geographically, which included the reference strain (ATCC927) and isolates from Massachusetts, Oregon, Mississippi, Arkansas and Virginia (Appendix, Figure A5). Group 2 presented isolates from Massachusetts and Oregon (Appendix, Figure A5). Isolates from Massachusetts and California were found in group 3 and group 4 possessed isolates collected from Oregon (Appendix, Figure A5). Within these groups, smaller clusters were formed showing isolates collected in Arkansas, Oregon, California and Massachusetts tended to be genetically similar. Clusters were produced with isolates collected from individual states including Arkansas, Oregon, California and Massachusetts (Appendix, Figure A5). This supports Ucko *et al.* (2002), which stated that *M. marinum* has unique genotypes specific to a certain geographic area. Results differed from Broutin *et al.* (2012) in the fact that their study saw both ecological and host specific clustering of the *M. marinum* isolates collected from humans, farm fish aquacultures and ornamental fish aquaria. However in this study, clusters based on more broad U.S. geographical regions, such as east versus west coast, were not observed. This is probably due to all samples being from zebrafish laboratories where facility setup does not differ greatly between locations (with the exception of scale). Also host specific grouping was not observed since laboratory zebrafish are highly inbred. It would be interesting to include aquaria and wild zebrafish in a future study. Further studies incorporating the three other VNTR loci used by Sun *et al.* (2011), MIRU5, Locus 18 and VNTR3422, would be beneficial in further teasing apart the epidemiological linkages of the isolates, providing higher resolution for cluster analysis.

A result found that is of interest is the location of MA isolates found across two of the cluster groups. Samples MA3, MA5, MA8, MA9, and MA11 were collected from a single mycobacteriosis outbreak. I expected that these isolates would be genetically similar in terms of the number of repeats since they were collected from one location, at one outbreak, from infected zebrafish tissue, and thus form a single cluster. One explanation for why this may not have occurred is due to fish exchange. Within the zebrafish research community fish are often traded across the United States with little to no morbidity prescreening. MA isolates came from a large facility that likely acquires many genetic mutants for various research projects. This practice is a serious concern due to breaches in biosecurity, with mycobacteriosis infections able to confound research results (Kent *et al.,* 2012).

Kent *et al.* (2009) and Whipps *et al.* (2012) state in order to avoid introducing pathogens to a fish community, precautions such as receiving fish from pathogen free facilities, quarantining fish for several weeks before system introduction, using pathogen free food, filtering all incoming water with ultraviolet light sterilization or ozonation, and prophylactic therapeutic treatments should be utilized. Regular monitoring protocol is also necessary in assessing aquaria health, analyzing dead fish and utilizing sentinel programs (Kent *et al.,* 2009).

In summary this thesis describes utilizing VNTR strain typing on 30 *M. marinum* isolates collected from infected zebrafish tissue from research from seven zebrafish research facilities throughout the United States. First I showed that molecular fingerprinting using VNTR provides insight into the genetic relatedness and genetic diversity of *M. marinum* isolates from outbreaks at several zebrafish facilities across the United States. I originally hypothesized that, as in previous studies, if isolates collected

from an individual outbreak are genetically similar, then isolates collected from the same facility or region of the country will be more genetically alike than those collected from other regions. The results of the cluster analysis support this hypothesis with geographic clusters occurring; however additional VNTR loci should be added to this analysis to increase the cluster resolution. I also predicted that epidemiological information could be gained from the results of this study and I found that variability of isolate location did occur with related isolates being found in other regions of the United States. These findings support that currently zebrafish exchange protocol is not sufficient; meaning changes in current biosecurity practices are critical in order to prevent the spread of *M. marinum* and other *Mycobacterium* species.

In conclusion, my results show genetic variation exists within isolates collected from seven zebrafish research facilities throughout the United States. VNTR loci finger printing provided a method to understand the epidemiological linkages of *M. marinum.* Though individual states possessed similar *M. marinum* strains, larger connections in regional areas could not be established. Although these outbreaks could not be linked epidemiologically, it is still likely that movement of fish between facilities presents the greatest risk for introduction of *M. marinum*. These facilities surveyed likely represent independent introductions to each facility, highlighting the need for consistent biosecurity protocols to reduce future possibilities of mycobacteriosis outbreaks.

Work Cited

Ablordey, A., Swings, J., Hubans, C., Chemlal, K., Locht, C., Portaels, F., Supply, P., 2005. Multilocus variable-number tandem repeat typing of Mycobacterium ulcerans. Journal of Clinical Microbiology 43, 1546–1551.

Aronson, J.D, 1926, Spontaneous Tuberculosis in Salt Water Fish. Journal of Infectious Disease 39, 315-320.

Aubry, A., Jarlier, V., Escolano, S., Truffot-Pernot, C., Cambau, E., 2000. Antibiotic Susceptibility Pattern of *Mycobacterium marinum.* Antimicrobial Agents and Chemotherapy 44, 3133-3136.

Aubry, A., Chosidow, O., Caumes, E., Robert, J., Cambau, E., 2002. Sixty-three Cases of Mycobacterium marinum Infection. Arch Intern Med 162, 1746-1752.

Beran, V., Matlova, L., Svastova, P., Pavlik, I., 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. Journal of Fish Diseases 29, 383–393.

Broutin, V., Banuls, A.L., Aubry, A., Keck, N., Choisy, M., Bernardet, J.F., Michel, C., Raymond, J.C., Libert, C., Barnaud, A., Stragier, P., Portaels, F., Terru, D., Belon, C., Dereure, O., Gutierrez, C., Boschiroli, M.L., Van De Perre, P., Cambau, E., Godreuil, S., 2012. Genetic Diversity and Population Structure of Mycobacterium marinum: New Insights into Host and Environmental Specificities. Journal of Clinical Microbiology 50, 3627–3634.

Chang, C.T., Whipps, C.M. (In Press) Activity of antibiotics against Mycobacterium species commonly found in laboratory zebrafish. Journal of Aquatic Animal Health.

Dai, Y.-J., Jia, Y.-F., Chen, N., Bian, W.-P., Li, Q.-K., Ma, Y.-B., Chen, Y.-L., Pei, D.-S., 2013. Zebrafish as a model system to study toxicology. Environ Toxicol Chem 33, 11–17.

Dooley, K., Zon, L., 2000. Zebrafish: a model system for the study of human disease. Current Opinion in Genetics & Development 10, 252-256.

Falkinham, J.O., III, 2009. Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. Journal of Applied Microbiology 107, 356–367.

Findlay, I., Quirke, P., 1997. Fluorescent polymerase chain reaction: Part I. A new method allowing genetic diagnosis and DNA fingerprinting of single cells. Human Reproduction Update 2, 137-152.

Fuentes, F.F., Martinez, E.A., Hinrichsen, P.V., Jellen, E.N., Maughan, P.J., 2008. Assessment of genetic diversity patterns in Chilean quinoa (Chenopodium quinoa Willd.) germplasm using multiplex fluorescent microsatellite markers. Conserv

Genet 10, 369–377.

Gemberling, M., Bailey, T.J., Hyde, D.R., Poss, K.D., 2013. The zebrafish as a model for complex tissue regeneration. Trends in Genetics 29, 611–620.

Gerhard, G., Cheng, K., 2002. A call to fins! Zebrafish as a gerontological model. Aging Cell 1, 104–111.

Gerlai, R., 2014. Fish in behavior research: Unique tools with a great promise!. Journal of Neuroscience Methods 234, 54–58.

Kent, M.L., Harper, C., Wolf, J.C., 2012. Documented and Potential Research Impacts of Subclinical Diseases in Zebrafish. ILAR J. 53, 126-134.

Kent, M.L., Feist, S.W., Harper, C., Hoogstraten-Miller, S., Mac Law, J., Sánchez-Morgado, J.M., Tanguay, R.L., Sanders, G.E., Spitsbergen, J.M., Whipps, C.M., 2009. Recommendations for control of pathogens and infectious diseases in fish research facilities. Comparative Biochemistry and Physiology, Part C 149, 240–248.

Kent, M.L., Whipps, C.M., Matthews, J.L., Florio, D., Watral, V., Bishop-Stewart, J.K., Poort, M., Bermudez, L., 2004. Mycobacteriosis in zebrafish (Danio rerio) research facilities. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 138, 383–390.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F, 2004. Stages of Embryonic Development of the Zebrafish. Development Dynamics 203, 253-310.

Luikart, G., Biju-Duval, M.-P., Ertugrul, O., Zagdsuren, Y., Maudet, C., Taberlet, P., 1999. Power of 22 microsatellite markers in fluorescent multiplexes for parentage testing in goats (Capra hircus). Animal Genetics 30, 431-438.

Maechler, M. Rousseeuq, P., Struyf, A., Hubert, M., Hornik, K., Studer, M., Roudier, P. (2015). cluster: Cluster Analysis Extended Rousseeuw et al. R Package version 2.0.1.

Mainous, M.E., Smith, S.A., 2005. Efficacy of Common Disinfectants against Mycobacterium marinum. Journal of Aquatic Animal Health 17, 284–288.

Ostland, V.E., Watral, V., Whipps, C.M., Austin, F.W., St-Hilaire, S., Westerman, M.E., Kent, M.L., 2008. Biochemical, molecular, and virulence characteristics of select Mycobacterium marinum isolates in hybrid striped bass Morone chrysops × M. saxatilis and zebrafish Danio rerio. Dis. Aquat. Org. 79, 107–118.

Phillips, J.B., Westerfield, M., 2014. Zebrafish models in translational research: tipping the scales toward advancements in human health. Disease Models & Mechanisms 7, 739–743.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

RStudio (2012). RStudio: Integrated development environment for R (Version 0.96.122) [Computer software]. Boston, MA. Retrieved May 20, 2012. Available from http://www.rstudio.org/

Stragier, P., Ablordey, A., Durnez, L., Portaels, F., 2007. VNTR analysis differentiates Mycobacterium ulcerans and IS2404 positive mycobacteria. Systematic and Applied Microbiology 30, 525–530.

Sun, G., Chen, C., Li, J., Gui, X., Li, X., Chen, L., Peng, F., Mei, J., Gao, Q., 2011. Discriminatory potential of a novel set of Variable Number of Tandem Repeats for genotyping Mycobacterium marinum. Veterinary Microbiology 152, 200–204.

Supply, P., 2009. Multilocus Variable Number Tandem Repeat Genotyping of *Mycobacterium tuberculosis*. Technical Guide, INSERM U629, 1-73.

Supply, P., Lesjean, S., Savine, E., Kremer, K., van Soolingen, D., Locht, C., 2001. Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. Journal of Clinical Microbiology 39, 3563–3571.

Ucko, M., Colorni, A., Kvitt, H., Diamant, A., Zlotkin, A., Knibb, W.R., 2002. Strain Variation in Mycobacteriummarinum Fish Isolates. Applied and Environmental Microbiology 68, 5281–5287.

van der Sar, A.M., Abdallah, A.M., Sparrius, M., Reinders, E., Vandenbroucke-Grauls, C.M.J.E., Bitter, W., 2004. Mycobacterium marinum Strains Can Be Divided into Two Distinct Types Based on Genetic Diversity and Virulence. Infection and Immunity 72, 6306–6312.

Watral, V., Kent, M.L., 2007. Pathogenesis of Mycobacterium spp. in zebrafish (Danio rerio) from research facilities. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 145, 55–60.

Whipps, C.M., Lieggi, C., Wagner, R., 2012. Mycobacteriosis in Zebrafish Colonies. ILAR J. 53, 95–105.

Wu, T.-S., Chiu, C.-H., Yang, C.-H., Leu, H.-S., Huang, C.-T., Chen, Y.-C., Wu, T.-L., Chang, P.-Y., Su, L.-H., Kuo, A.-J., Chia, J.-H., Lu, C.-C., Lai, H.-C., 2012. Fish Tank Granuloma Caused by Mycobacterium marinum. PLoS ONE 7, e41296.

Yanong, R.P.E., Pouder, D.B., Falkinham, J.O., III, 2010. Association of Mycobacteria in Recirculating Aquaculture Systems and Mycobacterial Disease in Fish. Journal of Aquatic Animal Health 22, 219–223.

Appendix

Protocol A1. Kinyoun method acid-fast stain

From a previously vortexed broth culture, 1.4mL was aliquoted into a 1.5mL centrifuge tube. Approximately 3.0uL of the culture was then placed on a glass side and allowed to evaporate in a flow hood, followed by a heat fixation. Next, the glass slide was covered with Kinyoun's Fuchsin and allowed it to sit for five minutes. A rinse with deionized water occurred followed by a brief rinse with acid alcohol. A counter stain was then performed using methylene blue for approximately one minute. A final rinse with deionized water was performed before analysis under compound light microscope.

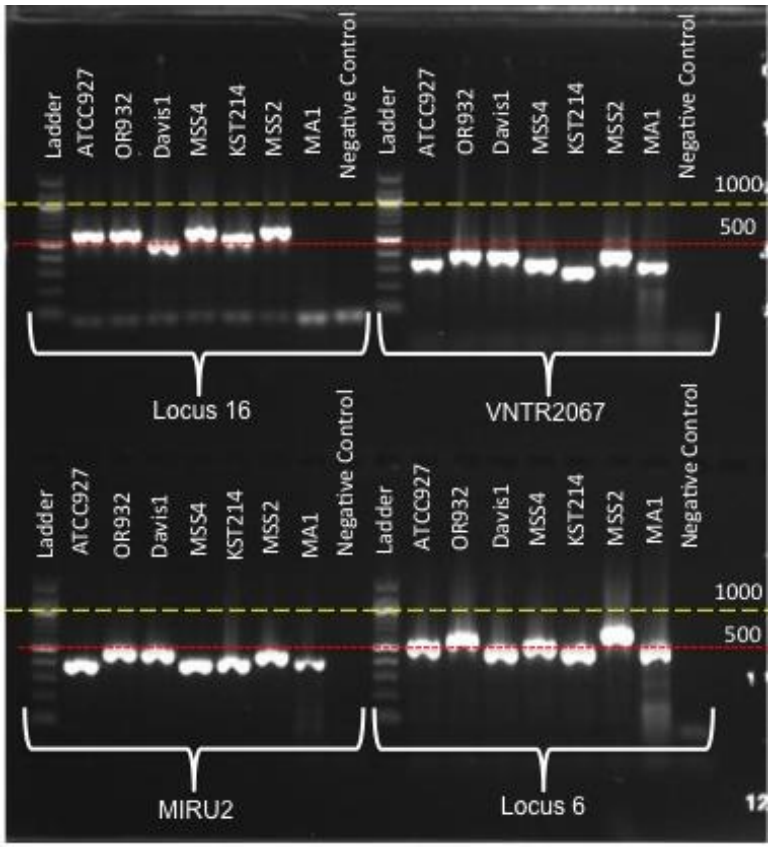

Figure A1. Representative agarose gel of uniplex PCR

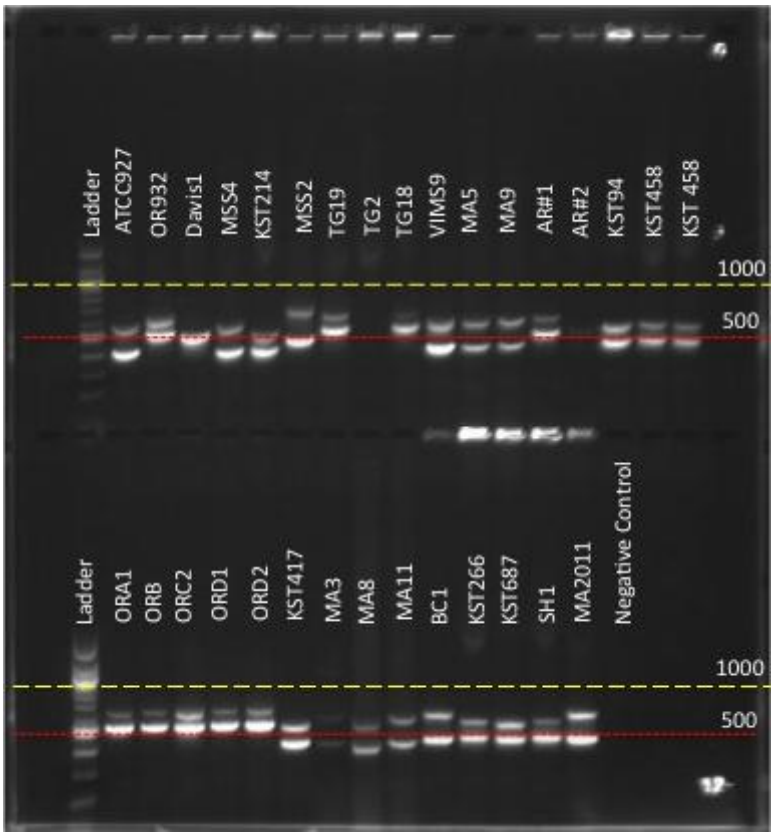

Figure A2. Representative agarose gel of MIRU2 and Locus 6 PCR using Hot Start *Taq* DNA polymerase

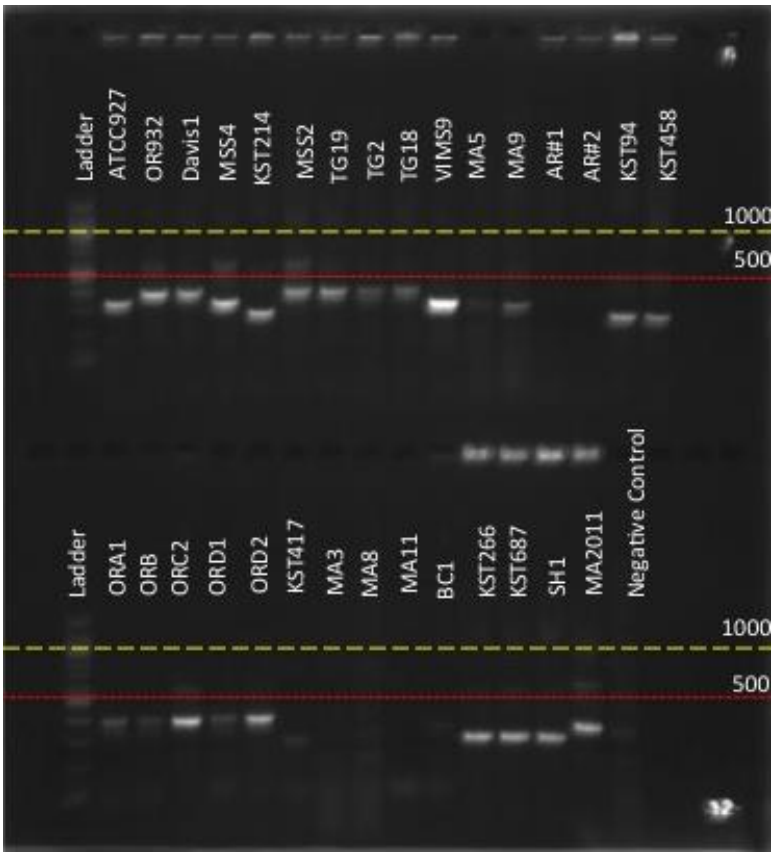

Figure A3. Representative agarose gel of Locus 16 and VNTR 2067 multiplex PCR using Hot Start *Taq* Polymerase

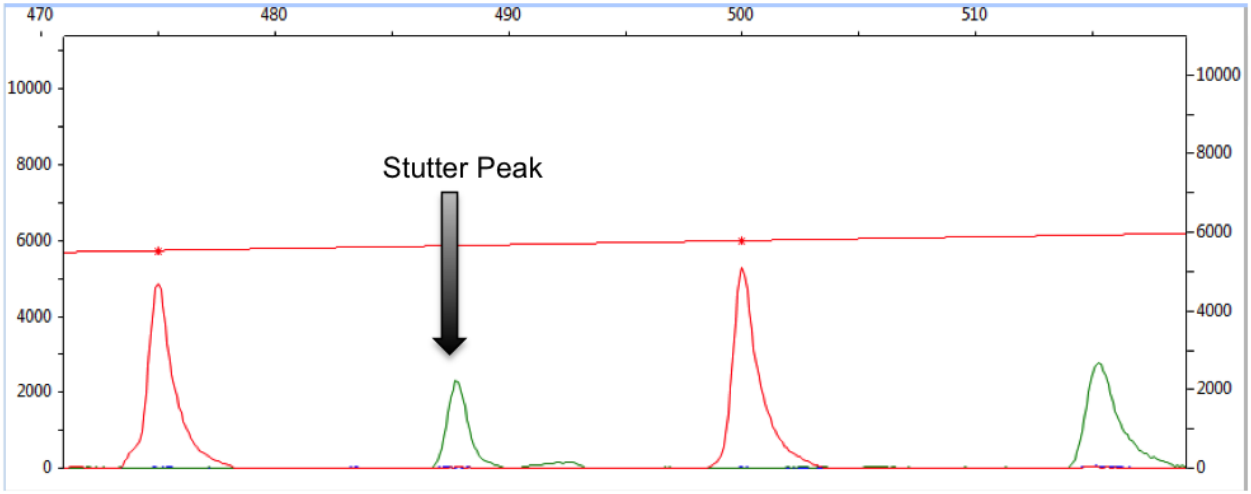

Figure A4. Representative electropherogram with stutter peak for fragment analysis using Peak Scanner ™ (Life Technologies ©)

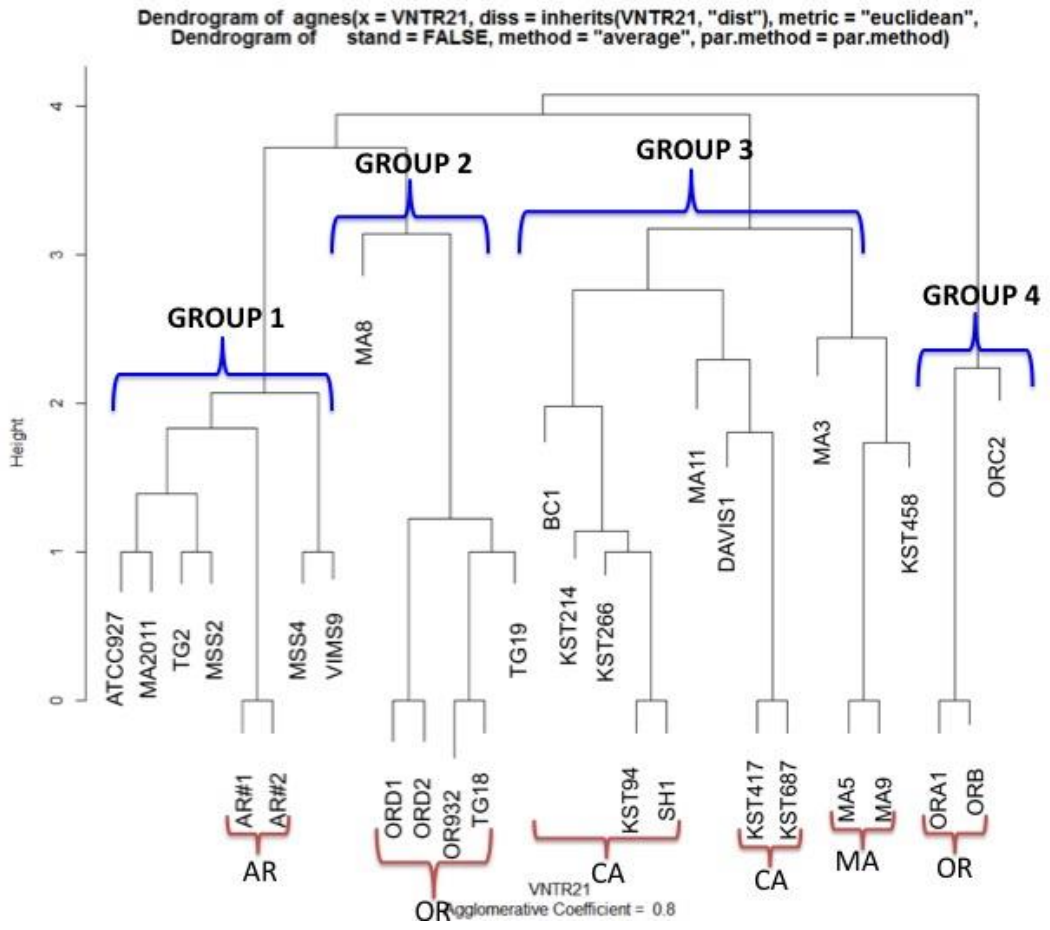

Figure A5. Annotated dendrogram of genetic relationships of *M. marinum* isolates based on four VNTR loci. Clusters for outbreaks at Arkansas, Oregon, California and Massachusetts are denoted by red brackets.

Code:

```
#VNTR Cluster analysis

#Prepared matrix table in excel and saved as csv

#Set working directory

➢setwd("C:/Users/ctchang/Desktop")

#Import data set called "VNTR21"

> VNTR21 <- read.csv("C:/Users/ctchang/Desktop/VNTR21.csv", header=F)
>   View(VNTR21)

#open cluster package from packages library

> library("cluster", lib.loc="C:/Program Files/R/R-3.0.2/library")

ag <- agnes(VNTR21, diss = inherits (VNTR21, "dist"), metric = "euclidean",

     stand = FALSE, method = "average", par.method)

plot(ag)
```

Code A1. VNTR cluster analysis

Table A1. Comparison of agarose electrophoresis and fragment analysis methods

	Agarose Electrophoresis	Fragment Analysis
Time	~1 day	~1 week
Primers	traditional fwd/rev primers, uniplex reactions	light sensitive, fluorescently labeled fwd primer, multiplex reactions
Equipment	agarose electrophoresis rig, gel imaging system	capillary electrophoresis, online software for electropherogram analysis
Analysis Cost	~$0.88/20 samples	$125.28/96 PCR purifications, $76.80/96 well plate analyzed +40.20 size standard/plate
Special Training	process conducted on site	analysis process sent to outside facility for capillary electrophoresis usage
Specificity	nearest 100 bp	exact bp location