SALMONELLAE IN THE INTESTINE OF *HYPOSTOMUS PLECOSTOMUS* IN THE SAN MARCOS RIVER

by

Anna Y. Gates, B.S.

A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biology
August 2016

Committee Members:

Dittmar Hahn, chair

Robert JC McLean

Thom Hardy

FAIR USE AND AUTHOR'S PERMISSION STATEMENT

Fair Use

Duplication Permission

DEDICATION

I dedicate this thesis to the memory of my father, Brian. He taught me the value of hard work and perseverance and how to do this with a sense of humor.

ACKNOWLEDGEMENTS

I would like to thank my committee chair and advisor, Dr. Dittmar Hahn, for giving me the opportunity to conduct this research at Texas State University and for all his support and guidance during my time here.

I would also like to thank my committee members, Dr. Thom Hardy and Dr. Robert McLean for all their encouragement and assistance provided towards the completion of this project. I would also like to thank Dr. Michael R. J. Forstner, Dr. David Rodriguez, and Dr. Hong-Gu Kang for also aiding in the completion of this project by allowing me into their labs and the use of their equipment. I would like to thank Nicholas Menchaca for providing the fish needed for this project, Rachel Erin Bryne for providing the sonde data, and to Fritzina Morrison for aiding in this project as an undergraduate research assistant. A very special thank you to Trina Guerra, who was instrumental in overcoming the hurdles thrown at us when it came to preforming the MLST part of this project.

Finally, I would like to thank my husband, Frank, who continuously encouraged me, even when I doubted myself.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

Heavy rainfall events have been associated with outbreaks of many waterborne diseases including salmonellosis. Salmonellosis is caused by members of the genus *Salmonella* that can enter water systems through sewage contamination, runoff after heavy rainfalls, or flow-through channels through manure fields after heavy rains or flooding. Currently, salmonellae are not closely monitored in regards to water quality. In this study, *Hypostomus plecostomus*, an invasive, algae consuming fish, was sampled from the San Marcos River (San Marcos, TX), the intestines analyzed for the presence of salmonellae by quantitative real-time polymerase chain reaction (qPCR) after semi-selective enrichment, and results related to precipitation and other ecological factors affecting the river area. Salmonellae were detected in the intestines of *H. plecostomus* in 40-100% of the fish following most precipitation events, but were not consistently detected in environmental samples (i.e. water and sediments). Other ecological factors affecting the river do not appear to play a significant role in the prevalence of salmonellae in the intestines of *H. plecostomus*, other than turbidity. This leads us to believe that *H. plecostomus* is ingesting salmonellae through their food sources and that the amount of salmonellae present in those food sources may be increasing after large rainfall events, but may not be dependent on these events.

Further studies included characterization of *Salmonella* isolates from positive samples by repetitive polymerase chain reaction (rep-PCR). Unique isolates were then serotyped using Multi-locus Sequence Typing (MLST). Several sampled *H. plecostomus* were observed to be infected by multiple serotypes of *Salmonella*, whereas other positive fish were observed to be infected by one serotype only. Some serotypes were observed to be common across multiple sampling dates, which leads us to believe that there may be a common environmental serotype

residing in the intestines of infected *H. plecostomus*. Furthermore, detection of multiple serotypes in the intestines of *H. plecostomus* was an unexpected observation.

I. INTRODUCTION

Background

Salmonella is a gram negative bacterial genus found to reside in the intestines of many vertebrate animals. This opportunistic pathogen can be shed into the environment with feces of these animals (Lemarchand 2002) and contaminate terrestrial and aquatic systems through manure or sewage discharge and runoff (Cherry 1972, Polo 1998). It can cause severe gastroenteritis if ingestion of contaminated foods, undercooked meats, and contaminated water occurs (Glynn 1992).

Salmonella spp. have also been detected in pristine aquatic systems, seemingly devoid of fecal pollution (Thomason 1975, Gaertner 2011). One such example is the spring fed headwaters of the San Marcos River in San Marcos, Texas (Gaertner 2011). Previous studies have shown an increase in salmonellae detection in this river system after strong rainfall events (Gaertner 2011), which may be associated with runoff from fecal contamination from livestock (Claudon 1971), wildlife droppings (Polo 1998, Arvanitidou 2005), or by association from other river systems (Negrel 2013). Many studies have illustrated salmonellosis as a result of sewage contamination in aquaculture (Hendricks 1971, Cherry 1972, Polo 1998), but there are fewer studies relating the spread of salmonellae to natural river and water systems (Gaertner 2008a, Sha 2013b).

Heavy rainfall events are associated with outbreaks of waterborne pathogens. Directional flow, creation of flow-through channels, and surface water turbidity are few examples that can occur after a heavy rainfall. Flow-through channels can pick up fecal contaminants in runoff from fields and river banks. Increased turbidity can increase the incidence of infection due to pathogens no longer resting or residing in the sediment.

This increase in turbidity may also be linked to increased nutrients, therefore regrowth of pathogens (Hunter 2003). A previous study found that 51% of waterborne outbreaks occurred after a heavy rainfall event (Curriero 2001). Salmonellae outbreaks seem to have a distinct summertime pattern, although this may be linked to human behavioral patterns (i.e. summertime swimming) (Greer 2008, Centers for Disease Control and Prevention 2013).

Salmonellae have also been detected in environmental biofilms sampled from the San Marcos River (Sha 2013c). Environmental biofilms can contain an assemblage of diverse microbial groups (bacteria, fungi, or protozoa) that have adhered to biotic or abiotic surfaces and are encased by an exopolysaccharide matrix (Tianzhi 2014). Biofilms have been found to be the predominant form of growth in aquatic ecosystems (Costerton 1995). These organisms form communities, produce a matrix consisting of extracellular polymeric substances (EPS), and proliferate within the matrix until nutrient deprivation. Some microorganisms will then detach from the biofilm and disperse in a planktonic form to start formation of a new biofilm elsewhere (O'Toole 2000). Salmonellae are capable of exhibiting biofilm growth and therefore are adapted for long-term survival in non-enteric habitats (Sha 2011). Natural aquatic systems that contain sufficient nutrients can promote rapid biofilm formation, especially if the adherent surface is a nutrient itself (Costerton 1995). The green algae *Cladophora* (Byappanahalli 2009), heterogeneous biofilms (Sha 2013c), as well as the turtle carapace (Gaertner 2008b), have been identified as reservoirs for salmonellae. Once salmonellae are introduced into an aquatic ecosystem, environmental biofilms seem to provide suitable habitats for their long term survival (Sha 2011). Salmonellae concentrations in water

systems might increase during the dispersal phase of biofilm growth due to overcrowding, nutrient deprivation, or competition from other microbes (Parsek 2005), thus increasing the number of reported infections or creating more reservoir hosts during this release of planktonic cells (Sha 2013b). Salmonellae have a high survival rate within aquatic environments (Murray 1991) and can withstand a wide variety of stress brought on by environmental factors (Winfield 2003), making the study of this pathogen's ecology of vital importance.

Salmonellae have been isolated from the intestines of several species of fish, including the suckermouth catfish *Hypostomus plecostomus* (Gaertner 2008a, Sha 2013a). Although salmonellae have been detected in fish, the pathogen does not seem to be part of the natural flora of fish, but its presence might rather be related to food sources and the surrounding water (Cahill 1990). Fish exposed to salmonellae by ingesting the pathogen can become asymptomatic carriers (Novotny 2004). Salmonellae can multiply within the intestines of the fish (Geldreich 1966) and have been found to be in higher concentrations within the intestines than in the surrounding water from which it was ingested (Cahill 1990). Thus, fish might play an important role in the dissemination of salmonellae in aquatic environments. Fish are commonly used as sentinels and bio-indicators for chemical contamination (Andrade 2004) so they may serve as better indicators for pathogen contamination to aquatic systems.

H. plecostomus is an invasive fish species and is thought to have first been introduced to the San Marcos River in the 1990s through illegal release of aquarium fish. This suckermouth catfish is estimated to account for 25-50% of the total fish community in the San Marcos River, with its food sources consisting primarily of algae (Pound

2011). Due to these high population numbers and its food source that include biofilms, *H. plecostomus* could be an important vector for the dissemination of salmonellae along the San Marcos River.

The current standard for monitoring water quality in the Unites States is to test for enterococci, *Escherichia coli* and fecal coliforms. In Europe, it is standard to monitor for salmonellae as well (Polo 1998). Salmonellae are adapted to survive longer than most coliforms outside of a host and it can survive in soil, water, and on a variety of surfaces (Murray 1991, Winfield 2003). *E. coli*, a common fecal coliform tested for fecal contamination in water systems has a low survival rate outside of a host (Winfield 2003). Thus, there is a need to monitor salmonellae contamination of natural river systems and to determine the impact of environmental conditions such as rainfall, temperature or abundance of biofilms on the presence of this pathogen in fish, such as *H. plecostomus.* The San Marcos River can be a useful model for salmonellae contamination in a natural, pristine river system and the effects of environmental conditions on the presence of pathogens in that system.

The purpose of this study was to investigate the prevalence of salmonellae in *H. plecostomus* in the San Marcos River at repeated samplings during an entire year. *H. plecostomus*, sediment, and water samples were collected from the San Marcos River and analyzed for salmonellae using traditional culture methods in conjunction with quantitative polymerase chain reaction (*q*PCR). Data on prevalence were analyzed as function of rainfall events, as well as other ecological factors affecting the river. Additional studies focused on the characterization of isolates of salmonellae from infected fish using repetitive PCR (rep-PCR) followed by multi-locus sequence typing

(MLST), in order to retrieve information on the potential presence of environmental strains (i.e. those present long-term, and thus detected at different times during the year).

Objective

- To study the relationship between selected environmental conditions (rainfall, temperature, periphyton) and the prevalence of salmonellae in the intestines of *H. plecostomus* present in the San Marcos River, Texas, at different sampling times during an entire year
- To identify potential environmental strains of salmonellae
- Are there any patterns in the strains (overall prevalence, specific for a time of year, transient, etc.)

II. MATERIALS AND METHODS

Sampling

Fish and environmental samples were retrieved from the San Marcos River, San Marcos, Texas between Rio Vista Park and I-35 (29.878633, -97.933024), as part of an ongoing study by the City of San Marcos on the effectiveness of spear fishing for the potential removal of the invasive *H. plecostomus* (Figure 1). Five to ten individuals of *H. plecostomus* and environmental samples (surface water and river sediment) were collected at different sampling times (n=15) during an entire year from February 2014 to January 2015, and delivered to our laboratory on ice within one hour of sampling. Directly after delivery, fish were cleaned with 70% ethanol, and dissected. The entire intestines were removed (from anus to pyloric stomach) and homogenized by vigorous shaking and vortexing in sterile ddH_2O to dislodge intestinal contents from their prospective mucosal lining. Any fish observed to have a spear laceration to the abdominal cavity were not evaluated and discarded. Homogenized intestinal contents were decanted to separate contents from intestinal mucosa. 100mL of surface water and 30mL of river sediments were collected in sterile 50mL centrifuge tubes at the time and site of fish collection. Surface water samples were concentrated by centrifugation at 3,000 x g for 15 minutes, and resuspended in sterile ddH_2O to a volume of 1mL. River sediments were pelleted by centrifugation at 3,000 x g for 15 min. Residual water was decanted and remaining sediments were mixed prior to use.

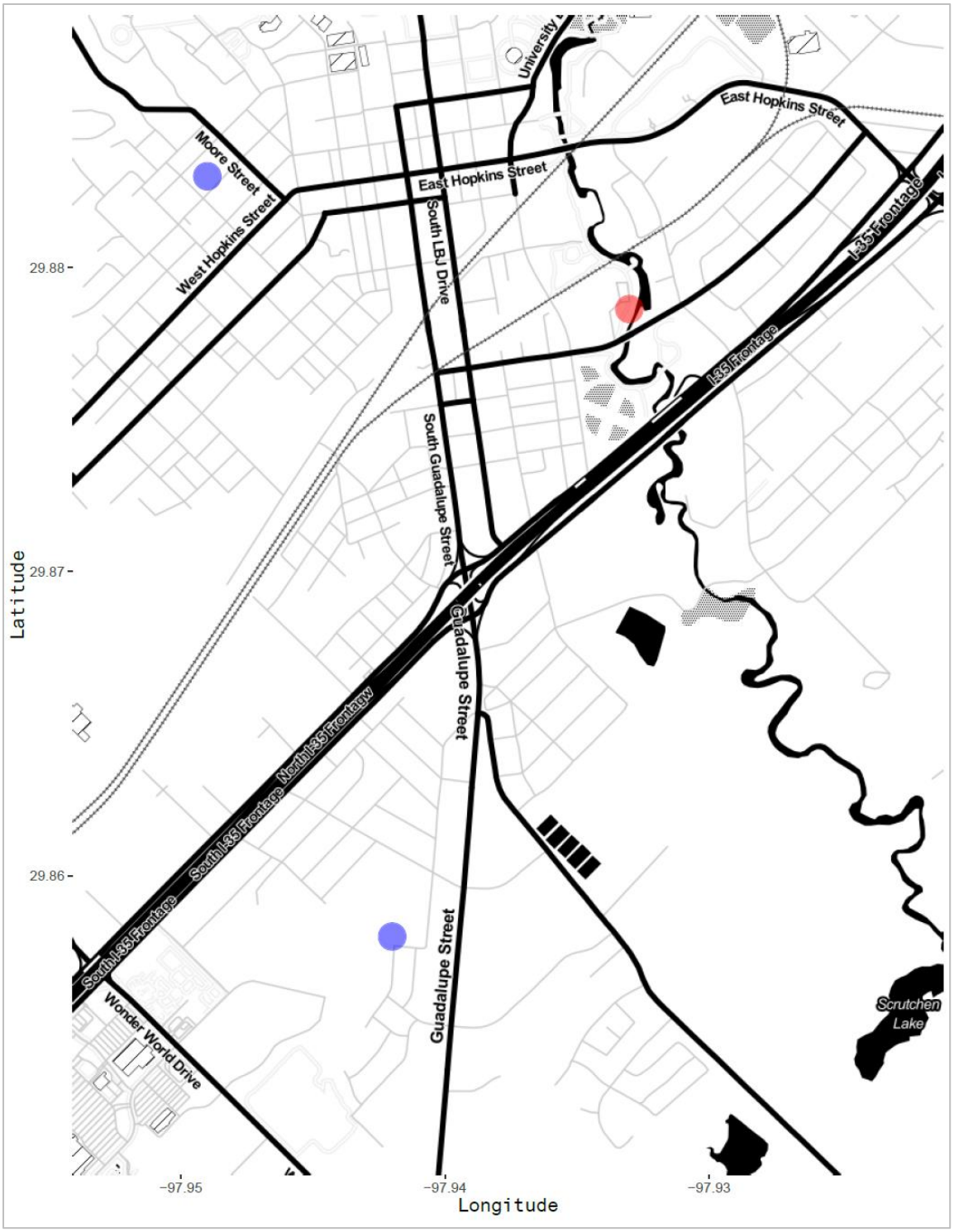

Figure 1: Map of San Marcos, TX displaying sampling area (red) and weather stations (blue) from which precipitation data was obtained. Map was created using ggplot2 package in RStudio.

Preliminary studies

The effectiveness of different detection methods for salmonellae was assessed initially to select for the most sensitive detection method for salmonellae. For this purpose, *H. plecostomus* intestinal contents that had been shown to be free of salmonellae after semi-selective enrichment and *q*PCR negative for the *inv*A gene, were dried at 45°C for 16h. Dried intestinal contents were aliquoted into sterile screw cap tubes and weights recorded. Aliquots of an overnight culture of *Salmonella enterica* serovar Give in Luria-Bertani (LB) broth were fixed for 16h at 4°C in 4% paraformaldehyde (PFA) and cell concentration was determined by epifluorescence microscopy after hybridization with Sal3-Cy3 and DAPI-staining (Table 1) (Amann 1990, Nordentoft 1997). Serial dilutions of living cells of this culture that had been stored at 4°C overnight were inoculated into the aliquoted intestinal contents at densities covering 10-fold serial dilutions between 3 and 3 x 10^6 cells/g intestinal content. Inoculated intestinal contents were extracted using the SurePrep™ Soil DNA Isolation Kit (Fischer Scientific, Waltham, MA, USA) or E.Z.N.A.® Stool DNA Kit (OMEGA Bio-Tek, Norcross, GA, USA), underwent alkaline lysis using 50mM NaOH at 25°C or 65°C, or were subjected to semi-selective enrichment using Buffered Peptone Water (BPW) and subsequent inoculation into Rappaport-Vassiliadis selection (RVS) broth. DNA extracts and lysates were analyzed by *q*PCR.

Semi-selective enrichment

Intestinal content and environmental samples were subsampled for the detection of salmonellae using semi-selective enrichment and analyzed via *q*PCR. 100µL of homogenized intestinal contents, 100 µL of concentrated surface water and 100mg of

mixed river sediment samples were pre-enriched with 1mL aliquots of Buffered Peptone Water (BPW) (10g/L peptone, 5g/L NaCl, 9g/L Na_2HPO_4, 1.5g/L KH_2PO_4, pH 7.2) and incubated at 37°C for 24hr. 100µL of these samples were then transferred to 1ml aliquots of Rappaport-Vassiliadis selection (RVS) broth (4.5g/L peptone, 29g/L $MgCL_2 \cdot 7H_2O$, 8g/L NaCl, 0.4g/L K_2HPO_4, 0.6g/L KH_2PO_4, 0.036g/L Malachite Green) and incubated at 37°C for 24hr. 100µL of these samples were then transferred to 1ml aliquots of RVS broth for a second semi-selection as described above (Gaertner 2008a, Sha 2013a, Sha 2013b). 100µL of both RVS cultures from each sample were transferred to sterile 1.2-mL microcentrifuge tubes and cells were pelleted via centrifugation at 14,000 x g for 3 min. Cell pellets were then washed with sterile nuclease-free water and then underwent alkaline lysis in 100µL 50mM NaOH and incubated at 65°C for 30 min. Lysates were stored at -20°C until analyzed (Sha 2013a).

*q*PCR

Quantitative polymerase chain reaction (*q*PCR) was performed targeting the 284-bp *inv*A gene for the detection of all *Salmonella* spp (Malorny 2003, Malorny 2004, Barbau-Piednoir 2013) using primer set 139 and 141 (Table 1) (Malorny 2003, Sha 2013b). SYBR Green based *q*PCR was performed in a total volume of 10µL containing 5µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 100ng each of primers 139 and 141 (Table 1), and 1µL DNA template in an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) using an initial denaturation at 95°C for 3 min, 40 cycles of 95°C denaturation, 64°C annealing, and 72°C extension for 30 s each, followed by a melting curve analysis (Sha 2013b). A standard curve was generated using 10-fold serial dilutions of *S. enterica* serovar Give cells quantified by *in*

situ hybridization (Amann 1990, Nordentoft 1997). *q*PCR was performed in triplicate on the intestinal samples, semi-selective enrichments and environmental samples (Gaertner 2008a, Sha 2013a, Sha 2013b).

Isolation

Second RVS samples positive for *Salmonella* were then plated onto Bismuth Sulphite agar (Himedia, Nashik, MH, India) to obtain isolates (Thomason 1975). Individual colonies were transferred to 5mL sterile Luria Broth (LB) (Difco, Detroit, MI, USA) using sterile toothpicks and incubated at 37°C for 24 hrs with agitation. Cultures were screened for the presence of *inv*A after alkaline lysis using *q*PCR as described above. Cultures positive for *Salmonella* spp. were recultured in LB and stored in sterile 20% glycerol solution at -80°C. Culture lysates for *Salmonella* spp. were stored at -20°C until analysis using Repetitive PCR (Rep-PCR). A minimum of ten isolates per positive sample was attempted for analysis.

Rep-PCR

Rep-PCR was done for characterization of *Salmonella* spp. positive isolates using the BoxA1R primer (Table 1). Rep-PCR reactions were performed in an Eppendorf Mastercycler (Eppendorf, Hauppauge, NY, USA) in a total volume of 25µL containing 5µL culture lysate, 390ng BoxA1R primer, 5µL of 5x Gitschier buffer (83mM $(NH_4)_2SO_4$, 33.5mM $MgCl_2$, 335mM Tris/HCl, 33.5µM EDTA, 150mM β-mercaptoethanol, pH 8.8), 10% di-methyl-sulfoxide, 3µg bovine serum albumin (BSA), 5mM each dNTPs, 2U Taq polymerase (Genscript, Piscataway, NJ, USA) using an initial denaturation of 95°C for 2min, 30 cycles of 94°C for 3s, 92°C for 30s, 50°C for 1 min, 65°C for 8min, followed by a final incubation at 65°C for 8min (Versalovic 1994,

Gaertner 2011). 2µL PCR products were visually examined using gel electrophoresis on a 2% agarose gel in TAE buffer followed by subsequent post-electrophoresis staining with ethidium bromide (Versalovic 1994). Representative unique isolates were then further analyzed using the Agilent 2100 Bioanalyzer and the DNA 7500 (Agilent, Santa Clara, CA, USA) for a cleaner banding pattern and comparative analyses (Panaro 2000, Gaertner 2008a, Sha 2013a, Sha 2013b).

MLST

Multi-Locus Sequence Typing (MLST) was the technique chosen for serotype identification of unique salmonellae isolates. Isolates with seemingly unique Rep-PCR banding patterns were analyzed using MLST of seven housekeeping genes (*thr*A, *dna*N, *aro*C, *pur*E, *his*D, *hem*D, and *suc*A) and given allele assignments designated by The University of Warwick, UK (Kidgell 2002, Noda 2011, Achtman 2012, Dione 2012). Primary PCR products for each gene were amplified using an Eppendorf Mastercycler in a total volume of 25µL containing 1µL culture lysate, 2.5µL 10x PCR buffer (Genscript), 6.25pmol of each primer (Table 2), 0.2mM dNTPs, 1.25U Taq polymerase (Genscript), 60µg BSA using an initial denaturation of 94°C for 5min, 30 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 1min, followed by a final extension of 72°C for 10min (Dione 2012). 5µL PCR products were loaded with GelRed (Biotium, Hayward, CA, USA) in 10x DNA loading dye and visually examined by electrophoresis on a 2% agarose gel in TAE buffer. PCR products that failed to amplify were then amplified in a total volume of 25µL using 1µL culture lysate, 12.5µL GoTaq® Green Master Mix 2x (Promega, Madison, WI, USA), and 6.25pmol of each primer, using the thermal profile described above. PCR products that again failed to amplify were amplified in a total volume of

100µL using 2µL culture lysates as described above. These PCR products were then cleaned and condensed using the UltraClean® 15 DNA Purification Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's guidelines. These products were then used as the template for a nested PCR reaction, in the same manner described first using the sequencing primers in place of the primary PCR primers (Table 2).

Sequencing

PCR products were purified by adding 5µL product to 1.5µL sterile nuclease-free water, 0.4 unit Shrimp Alkaline Phosphatase (Affymetrix®, Santa Clara, CA, USA), and 1 unit Exonuclease-1 (Affymetrix®) (Werle 1994). Cycle sequencing was performed on both forward and reverse strands of each gene using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations, and sequences analyzed on an ABI 3500XL Genetic Analyzer (Applied Biosystems) (Noda 2011, Dione 2012). Sequences were aligned and edited using Geneious version 8.1 (Biomatters Ltd, Auckland, New Zealand) (Kearse 2012). Consensus sequences were submitted to the University of Warwick MLST database for assignment of allele type numbers and strain type identification (Aanensen 2005).

Impact of environmental conditions

The impact of environmental conditions such as precipitation was compared to the presence of salmonellae in *H. plecostomus* intestinal samples and the environmental samples. Precipitation patterns throughout the year were obtained from NOAA National Centers for Environmental Information (http://www.ncdc.noaa.gov/oa/ncdc.html for

US1TXHYS074 and USC00417983) (Table 2). Data on other environmental conditions affecting the San Marcos River, including temperature and turbidity were obtained at the sampling site as part of the ongoing study by the City of San Marcos, and kindly provided by the PI of that project, Dr. Thom Hardy. Water quality measurements were taken every 15 min using a 6920 V2-2 Multi-Parameter Water Quality Sonde (Yellow Springs Instruments, Yellow Springs, OH, USA) placed at 29.8800, -97.9333. River discharge and gage height were obtained from USGS National Water Information System (http://waterdata.usgs.gov/tx/nwis/uv?site_no=08170500).

Statistical analysis

A 95% confidence interval was determined for the prevalence for each sampling date. Correlations, principle component analysis, and linear regressions were determined using SigmaPlot (Systat Software, San Jose, CA).

Table 1: Sequences of qPCR primers, Rep-PCR primer, and FISH probes used in this study

		Sequence	Target
***q*PCR**	139	5'GTGAAATTATCGCCACGTTCGGGCAA3'	*inv*A gene
	141	5'TCATCGCACCGTCAAAGGAACA3'	*inv*A gene
Rep-PCR	BoxA1R	5'CTACGGCAAGGCGACGCTGACG3'	BOX element
FISH	DAPI	AT rich regions	All cells
	*Sal*3	5'AATCAC TTCACCTACGTG3'	23S rRNA

Table 2: Multi-locus Sequencing Typing (MLST) Primers employed for this study

Gene		Primary PCR Primers	Sequencing Primers
***thr*A**	Forward	5'GTCACGGTGATCGATCCGGT3'	5'ATCCCGGCCGATCACATGAT3'
	Reverse	5'CACGATATTGATATTAGCCCG3'	5'ACCGCCAGCGGCTCCAGCA3'
***pur*E**	Forward	5'GACACCTCAAAAGCAGCGT3'	5'ACAGGAGTTTTAAGACGCATG3'
	Reverse	5'AGACGGCGATACCCAGCGG3'	5'GCAAACTTGCTTCATAGCG3'
***suc*A**	Forward	5'CGCGCTCAAACAGACCTAC3'	5'CCGAAGAGAAACGCTGGATC3'
	Reverse	5'GACGTGGAAAATCGGCGCC3'	5'GGTTGTTGATAACGATACGTAC3'
***his*D**	Forward	5'GAAACGTTCCATTCCGCGC3'	5'GTCGGTCTGTATATTCCCGG3'
	Reverse	5'GCGGATTCCGGCGACCAG3'	5'GGTAATCGCATCCACCAAATC3'
***aro*C**	Forward	5'CCTGGCACCTCGCGCTATAC3'	5'GGCGTGACGACCGGCAC3'
	Reverse	5'CCACACACGGATCGTGGCG3'	5'AGCGCCATATGCGCCAC3'
***hem*D**	Forward	5'GAAGCGTTAGTGAGCCGTCTGCG3'	5'GCCTGGAGTTTTCCACTG3'
	Reverse	5'ATCAGCGACCTTAATATCTTGCCA3'	5'GACCAATAGCCGACAGCGTAG3'
d*na*N	Forward	5'ATGAAATTTACCGTTGAACGTGA3'	5'CCGATTCTCGGTAACCTGCT3'
	Reverse	5'CCGCGGAATTTCTCATTCGAG3'	5'ACGCGACGGTAATCCGGG3'

III. RESULTS AND DISCUSSION

Preliminary Studies

Extraction methods were evaluated for fish intestinal sample inoculated with known *Salmonella* concentrations (Table 3). *Inv*A gene fragments could only be detected by *q*PCR in DNA from intestinal samples after semi-selective enrichment, but not in DNA from non-enriched samples when extracted using the SurePrep™ Soil DNA Isolation Kit (Fischer Scientific). This is probably due to the detection limit associated with *q*PCR and *Salmonella* spp. One study testing this limit of detection found that original cell concentration, sample matrix being tested and other microorganisms present in the sample all influence this limit of detection (Malorny 2004).

Table 3: Employed methods for *Salmonella* recovery efficiency

	***Salmonella* cells inoculated (cells/g)**					
DNA Extraction Method	**300000**	**30000**	**3000**	**300**	**30**	**3**
SurePrep Soil Kit	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Omega E.Z.N.A. Stool Kit	69.4%	69.4%	67.9%	0.00%	0.00%	0.00%
NaOH (50mM) @ 65°C	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
NaOH (50mM) @ 25°C	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
BPW Pre-enrichment	N/A	$5.3x10^5$%	$7.1x10^6$%	$2.5x10^5$%	$4x10^5$%	0.00%
RVS broth	N/A	165.00%	172.00%	337.00%	$2.2x10^4$%	0.00%

Alkaline cell lysis and extraction using the SurePrep™ Soil DNA Isolation Kit extraction kit did not yield detectible *inv*A gene fragments at cell concentrations up to $3x10^6$ cells/g intestinal content. The Omega™ E.Z.N.A. stool DNA Kit exhibited a 69.4% recovery rate for cell concentrations up to $3x10^4$ cells/g intestinal content. After this cell concentration, *inv*A gene fragments were no longer detected. Random samples of

salmonellae positive and negative intestinal contents were extracted using the Omega stool kit and analyzed by *q*PCR. All samples tested were unquantifiable. As a consequence of failed or limited detection, the method of choice for the detection salmonellae in intestine samples of *H. plecostomus* was *q*PCR-based quantification after semi-selective enrichment. Pre-enrichment of samples can significantly improve detection limits prior to employment of molecular detection techniques (Feder 2001), thus making salmonellae detection qualitative. As a result, a true quantification of bacterial cells could not be achieved, only whether salmonellae were present or absent in the samples analyzed.

Analyses of intestines of *H. plecostomus*

H. plecostomus and environmental samples were collected at different times during an entire year (N=15) starting in February 2014 and ending January 2015. During this time, 119 *H. plecostomus'* intestinal samples and 15 of each environmental sample were analyzed using semi-selective enrichment and subsequent *q*PCR. Using semi-selective enrichment, 53 (44.5%) intestinal samples were positive for the presence of the *inv*A gene, thus indicating that these samples contain *Salmonella* cells. At least one catfish analyzed from each sample date was salmonellae positive. Salmonellae has been previously detected in fish residing in the San Marcos River, with prevalence ranging from 17-33% at different sampling sites. Salmonellae was detected in 17% of *H. plecostomus* sampled, however, only the upper intestinal tract was analyzed as opposed to the entire intestinal tract as in this study (Gaertner 2008a). Previous studies evaluating salmonellae in fish have found a much lower occurrence, with 24% occurrence in Ouagadougou, Burkina Faso (Traore 2015), 17% tilapia sampled in Kenya (Awour

2011), 10% of fish sampled in Iran (Rahimi 2013) and 10% of fresh fish sampled in Indonesia (Kusumaningrum 2012). The intestinal bacterial microflora of fish is heavily influenced by its surrounding environment and some bacteria are found in much larger numbers within the intestinal tract than in this environment, which includes salmonellae (Cahill 1990). This can be reflective of contamination as well as possible resuscitation of non-replicating or "hibernating" bacteria. A study involving fish in a sewage-fed pond found that the overall load of bacteria found in the intestines was reduced by 78% when transferred to freshwater after 20 days of maintenance (Balasubramanian 1992). Another study involving *H. plecostomus* fed *Salmonella* Thompson biofilms found that the amount of *Salmonella* cells detected in shed feces increased for 36 hours, at which point the number of cells decreased until it reached a seemingly consistent level (Sha 2013a).

Salmonellae were not consistently detected in environmental samples obtained from the sampling site at the time of sampling (Table 4). Only two (13.3%) water samples and one (6.7%) sediment sample were positive for the presence of the *inv*A gene (Table 4). Salmonellae were detected in water samples for the sampling dates (4/7/14 and 12/20/14), while sediment was found to be positive for only one sampling date (10/3/14). The surface water sampled on December 20, 2014 was the only positive water sample preceded by heavy rainfall. Heavy rainfall can create turbidity and upwelling, resulting in the dispersal of cells of *Salmonella* spp. captured in sediments which subsequently can become detectable in water again (Hunter 2003). Previous studies comparing salmonellae detection in the head waters of the San Marcos River to precipitation found that salmonellae was detected in 1.4% (Gaertner 2011), 21.4% (Gaertner 2009), and 0% (Sha 2011) of surface water samples and could only be detected

after rainfall events. One study found that salmonellae was detected in 31.8% of surface water samples, however, this study was not compared to rainfall and employed filtering water samples rather than concentrating the samples (Sha 2013c). Previous studies comparing the detection of *Salmonella* spp. in surface water samples versus sediment samples have found more detections in sediment samples (Hendricks 1971, Moore 2003, Winfield 2003). This is likely a consequence of sorption of cells, potentially better nutritional conditions that keep the cells alive longer (Moore 2003), and stable, more suitable environments promoting proliferation (Murray 1991). As a result, bottom dwelling aquatic fauna, such as *H. plecostomus*, could be exposed to more salmonellae and at a higher risk of colonization by these bacterial cells. In this study, surface water samples were taken after fish samples were acquired. As a consequence, an increase in turbidity from the disruption of the underlying sediments may have occurred, resulting in more water samples testing positive for salmonellae than sediments. It is also possible that increasing the amount of surface water collected and filtering the sample might impact these results. The limit of detection for surface water samples could be reduced to 2 bacterial cells/ml by utilizing this method (Sha 2013c).

Table 4: *H. plecostomus* intestinal samples positive for salmonellae as detected after semi-selective enrichment and qPCR analyses

Date Collected	Positive	No. Sampled	Prevalence (95% CI)	Positive Environmental Samples
09 Feb 2014	1	5	0.200 (0.036-0.624	
18 Feb 2014	4	11	0.364 (0.152-0.646)	
07 Apr 2014	1	6	0.167 (0.030-0.562)	Surface water
15 Apr 2014	3	7	0.429 (0.158-0.749)	
03 Jun 2014	3	5	0.600 (0.231-0.882)	

Table 4 continued:

15 Jul 2014	4	10	0.400 (0.168-0.687)	
24 Jul 2014	5	8	0.625 (0.306-0.863)	
08 Sep 2014	10	10	1.000 (0.722-1.00)	
03 Oct 2014	3	7	0.429 (0.158-0.749)	Sediment
21 Oct 2014	5	9	0.556 (0.267-0.811)	
31 Oct 2014	6	10	0.600 (0.313-0.832)	
10 Nov 2014	1	5	0.200 (0.036-0.624)	
20 Dec 2014	1	8	0.125 (0.022-0,471)	Surface water
11 Jan 2015	4	8	0.500 (0.215-0.785)	
18 Jan 2015	2	10	0.200 (0.057-0.510)	

Salmonellae were also detected in *H. plecostomus* following rainfall events, though not necessarily in larger percentages. Figure 2 compares the average precipitation of two weather stations located less than 5 km from the sampling site and the percentage of *H. plecostomus* positive for salmonellae after semi-selective enrichment and *q*PCR detection. During April and July, this increase in positive *H. plecostomus* was observed with pre-rain percentages lower than after-rain percentages. Rain does not seem to be the only factor contributing to the high percentage of positive *H. plecostomus* for salmonellae, as seen in the month of September. Despite the low amounts of rain received by the area, 100% of *H. plecostomus* tested positive for the *inv*A gene. However, during this particular sampling date, there was a sharp increase in turbidity in that area of the river. Upwelling of river bottom sediments and an increase in turbidity can allow latent bacterial organisms to become robust and increase proliferation, thus allowing more

opportunity for salmonellae infection in *H. plecostomus* (Hunter 2003). Freshwater systems can be contaminated by salmonellae by either run-off or infiltration of water through contaminated soils. Run-off can occur when the rate of rainfall exceeds the infiltration rate into the soil or if the soil is overly saturated (Jacobsen 2012). It is possible that smaller amounts of rainfall could create runoff and thereby contaminating the San Marcos River with salmonellae if the rate of rainfall was high or this runoff increasing turbidity. Other chemical and physical factors affecting the San Marcos River are relatively constant or do not seem have a pattern of impact on salmonellae prevalence in *H. plecostomus* (Appendix A).

A principle component analysis with chemical and physical factors affecting the river (pH, specific conductivity, water temperature, turbidity, river discharge, and river gage height), precipitation, and salmonellae prevalence revealed that turbidity and salmonellae prevalence were correlated, while precipitation and salmonellae prevalence might be correlated one day prior to sampling (Appendix B). This was confirmed using multiple linear regression with the chemical and physical factors listed above and precipitation as independent variables and salmonellae prevalence as the response variable. Since *Salmonella* cells are ingested and have been observed to reach peak levels in the feces 36 hr after ingestion in *H. plecostomus*, a Pearson's product moment correlation for chemical and physical factors affecting the San Marcos River and salmonellae prevalence was performed the day of, one day prior to, two days prior to, and three days prior to sampling (Table 4). This revealed that turbidity and salmonellae prevalence continue to be correlated even with measurements three days prior to sampling. However, precipitation is not correlated with salmonellae prevalence in *H.*

plecostomus up to three days prior to sampling. This should however be confirmed with more sampling. Several studies have been done attempting to correlate salmonellae to rainfall with mixed results (Gaertner 2009, Gaertner 2011, Sha 2011, McEgan 2013, Thomas 2013), although the majority of these studies involve analyzing surface water samples rather than fish intestinal contents. A seasonal variation in intestinal bacterial flora in tilapia was related to water temperature, with salmonellae only being detected in the winter (Al-Harbi 2004). In Maryland, a correlation was observed between extreme ambient temperatures and precipitation in salmonellosis in humans, with a more pronounced risk in coastal communities (Jiang 2015). Another study found that there was no correlation between salmonellae detection in surface water samples and rainfall, but rather *E. coli* and the presence of other fecal coliforms have a significant impact (McEgan 2013). Our study did not evaluate for the presence of other bacteria as a possible source for competition and should be considered for future studies since competition between *Salmonella* and *E. coli* has been studied at length (Winfield 2003).

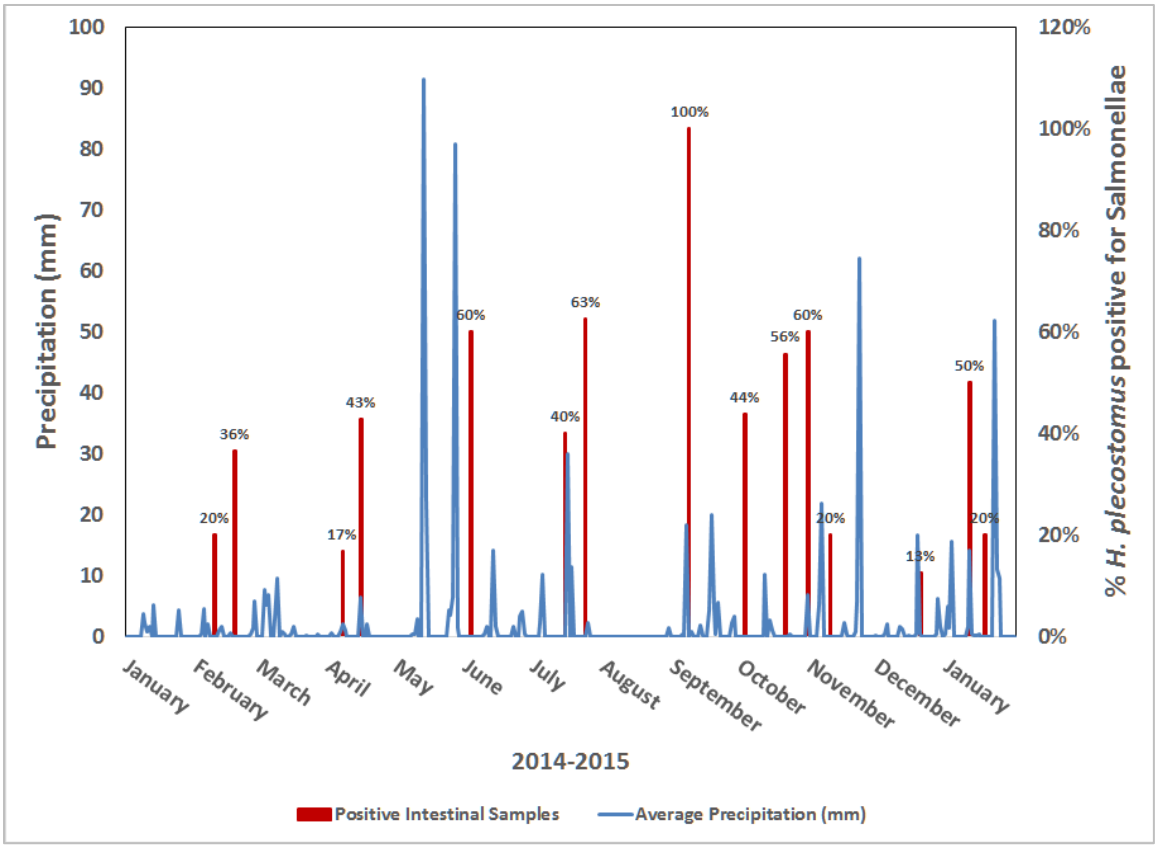

Figure 2: Precipitation data averaged from two weather station (data obtained from http://www.ncdc.noaa.gov/oa/ncdc.html for US1TXHYS074 and USC00417983) located <5km from sampling site are reflected in blue and the percentage of *H. plecostomus* intestinal samples positive for salmonellae after semi-selective enrichment and qPCR detection.

Table 5: Pearson product moment correlations coefficients (p-value) determined for chemical and physical factors affecting the San Marcos River as related to salmonellae prevalence for the day of, one day prior, two days prior, and three days prior to sampling

	Salmonellae Prevalence			
	day of	*1 day prior*	*2 days prior*	*3 days prior*
HDO (mg)	0.215 (0.441)	0.226 (0.419)	0.237 (0.394)	0.205 (0.464)
pH	0.251 (0.367)	0.252 (0.364)	0.253 (0.363)	0.252 (0.364)
Specific Conductivity	0.237 (0.396)	0.241 (0.387)	0.234 (0.400)	0.237 (0.395)
Temperature (°C)	0.318 (0.247)	0.331 (0.228)	0.327 (0.234)	0.326 (0.235)
Turbidity	**0.678** (0.005)	**0.672** (0.006)	**0.680** (0.005)	**0.723** (0.002)
Discharge	-0.206 (0.461)	-0.217 (0.437)	-0.199 (0.477)	-0.179 (0.523)
Gage Height	-0.295 (0.286)	-0.302 (0.275)	-0.295 (0.286)	-0.284 (0.305)
Precipitation	0.123 (0.663)	0.252 (0.3850	-0.232 (0.406)	-0.131 (0.642)

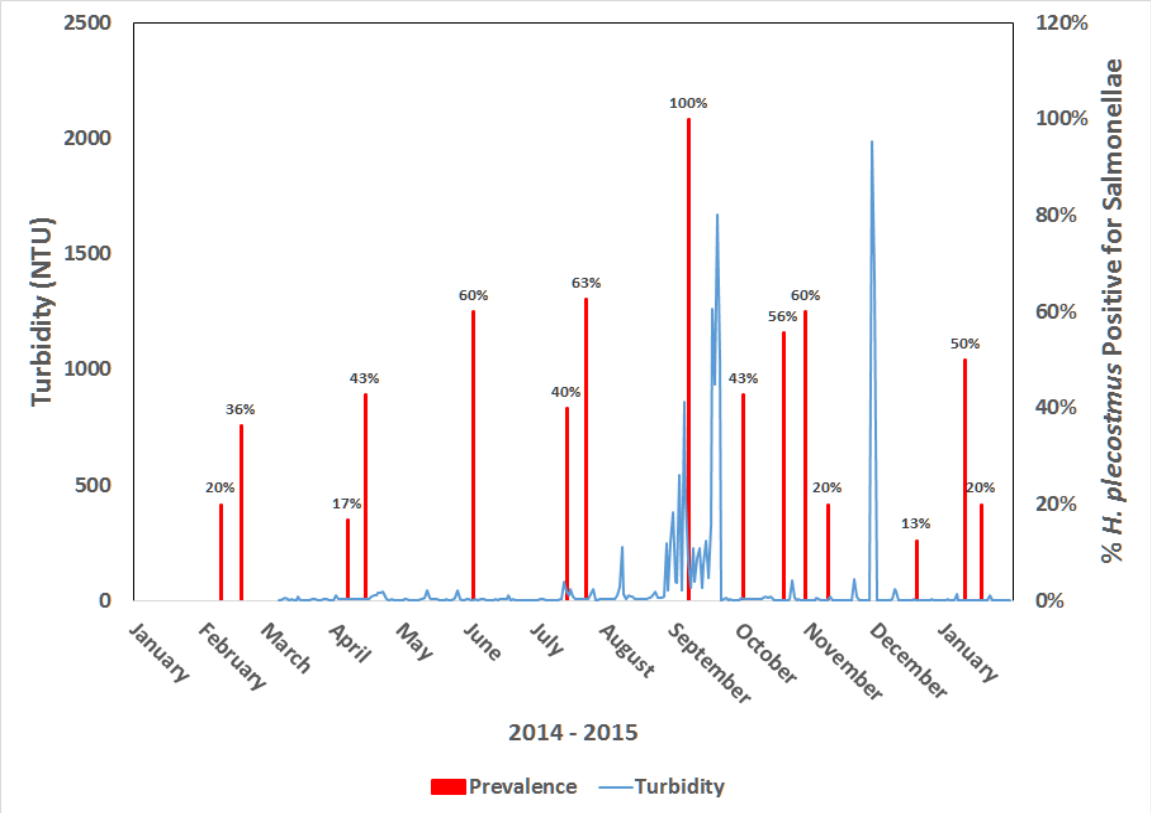

Figure 3: Turbidity measurements taken via 6920 V2-2 Multi-Parameter Water Quality Sonde placed at 29.8800, -97.9333 are reflected in blue and the percentage of *H. plecostomus* intestinal samples positive for salmonellae after semi-selective enrichment and qPCR detection.

Characterization of isolates

Rep-PCR was utilized for characterization of salmonellae isolates using the BoxA1R primer. This primer is complimentary to short, repetitive Box sequences found throughout the bacterial genome and is used to generate DNA fingerprints that allow for discrimination between serotypes and strains (Albufera 2009). All samples in which the *inv*A gene was detected via *q*PCR after semi-selective enrichment were plated onto bismuth sulfite agar to obtain individual isolates of *Salmonella*. Isolates were screened for the presence of the *inv*A gene via *q*PCR, in order to confirm that the isolate was *Salmonella*. A minimum of ten isolates were obtained for all salmonellae positive

samples except Fish#6 sampled on April 15, 2014, Fish#4 sampled on July 24, 2016, Fish#1 and Fish#4 sampled on October 3, 2014, Fish#7 sampled on October 21, 2014, and Fish#4 sampled on January 11, 2015. For all samples listed above, at least one isolate was obtained for characterization, except for Fish#4 sampled on July 24, 2014. A total of 728 salmonellae isolates were obtained for visual analysis via Rep-PCR. Methods of enrichment and background microflora simultaneously enriched with these procedures can impact the diversity dynamics of *Salmonella* serotypes isolated (Gorski 2012). It is possible that some salmonellae serotypes may have been inhibited or unable to be isolated due to competition from other serotypes or even other bacterial genus.

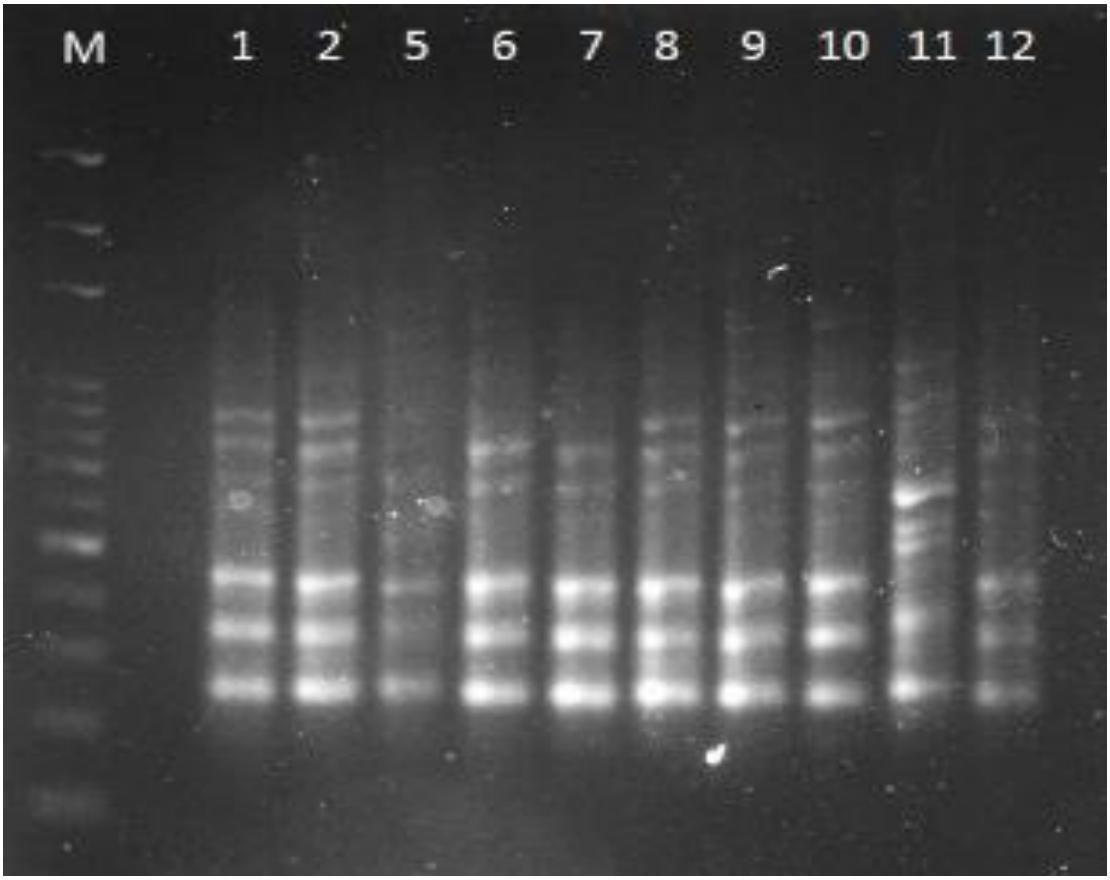

Figure 4: Rep-PCR products for salmonellae positive isolates from Fish#3 collected September 9, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

Multiple banding patterns were observed in numerous *H. plecostomus* samples, indicating multiple infections with different *Salmonella* strains, as seen in Figure 4. Isolate 11 has a seemingly different banding pattern than the other nine isolates characterized. In order to compare salmonellae from different samples, representatives of each pattern were re-characterized using Rep-PCR and analyzed with the Bioanalyzer that provides much more accurate pattern assignments (Figure 5). A total of 180 representative isolate rep-PCR samples were analyzed using the Bioanalyzer. The representative isolates for Fish#3 from February 9, 2014 evaluated via the Bioanalyzer confirmed that Isolate 11 and Isolate 12 had different banding patterns. The Bioanalyzer is a computer chip based technology that analyzes nucleic acid samples using migration plots and displays data as a virtual gel (Panaro 2000). Multiple banding patterns were not observed for surface water salmonellae isolates obtained, however, the salmonellae positive sediment sample displayed two distinct banding patterns.

Rep-PCR as a technique for molecular characterization is one of the earliest DNA subtyping tools used for salmonellae (Wittiau 2011). This technique can be utilized for outbreak source tracking to determine genetic "relatedness" and can provide more differentiation information than serotyping alone (Harbottle 2006). Data bases have been created for serotype prediction using this method coupled with pulse-field gel electrophoresis (PFGE), however, this technique is subjective as it relies on visual analysis (Wittiau 2011). Coupling rep-PCR with the Agilent Bioanalyzer can reduce this subjectivity as the analyzing software allows for overlay of electropherogram profiles of rep-PCR amplicons (Panaro 2000, Wise 2009). However, a single serotype can provide multiple banding patterns, especially if isolated from different sources (Harbottle 2006).

In this way, rep-PCR can be very useful for short-term prevalence studies, whereas serotyping could beneficial for a broader scale comparison (Maiden 1998, Urwin 2003).

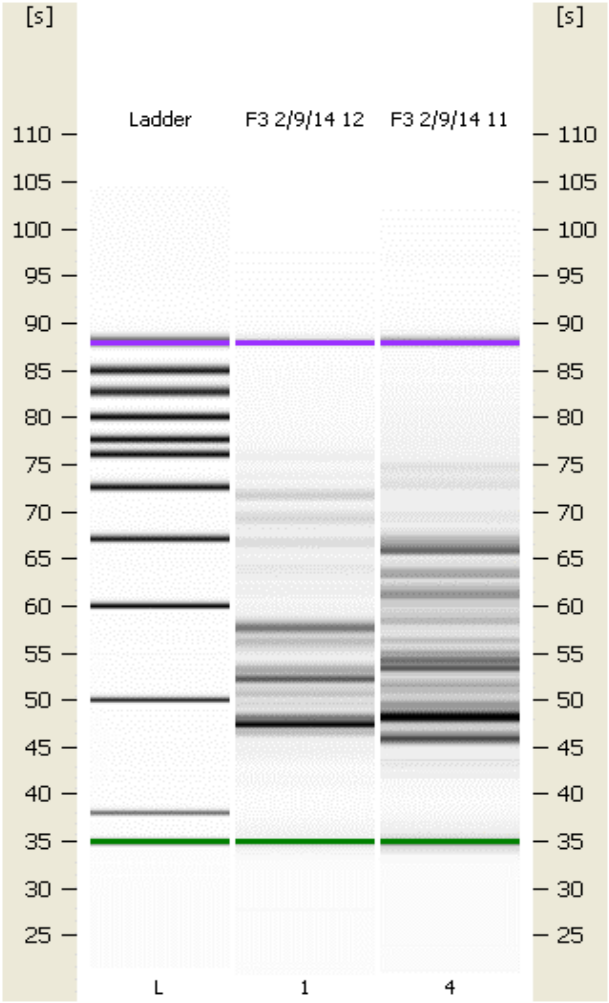

Figure 5: Rep-PCR characterization of Fish#3 sampled on February 9, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

Serotype identification

Multilocus Sequence Typing (MLST) was utilized to identify the serotypes of unique salmonellae isolates isolated from *H. plecostomus* intestinal contents and environmental samples. From the 180 representative isolates analyzed using rep-PCR

and the Bioanalyzer, 112 isolates were determined to be unique and underwent serotype identification via MLST. Eight unique isolates have been observed to occur over multiple sampling dates, with U21 occurring the most frequent, at five sampling dates. Four other isolates, U2, U20, U25, and U32, each occurred in 3 sampling dates (Figure 6). *H. plecostomus* sampled on September 8, 2014 yielded 42 unique isolates, which is the most yielded by sample date (Figure 7). Although, this may be due to high number of salmonellae positive catfish analyzed.

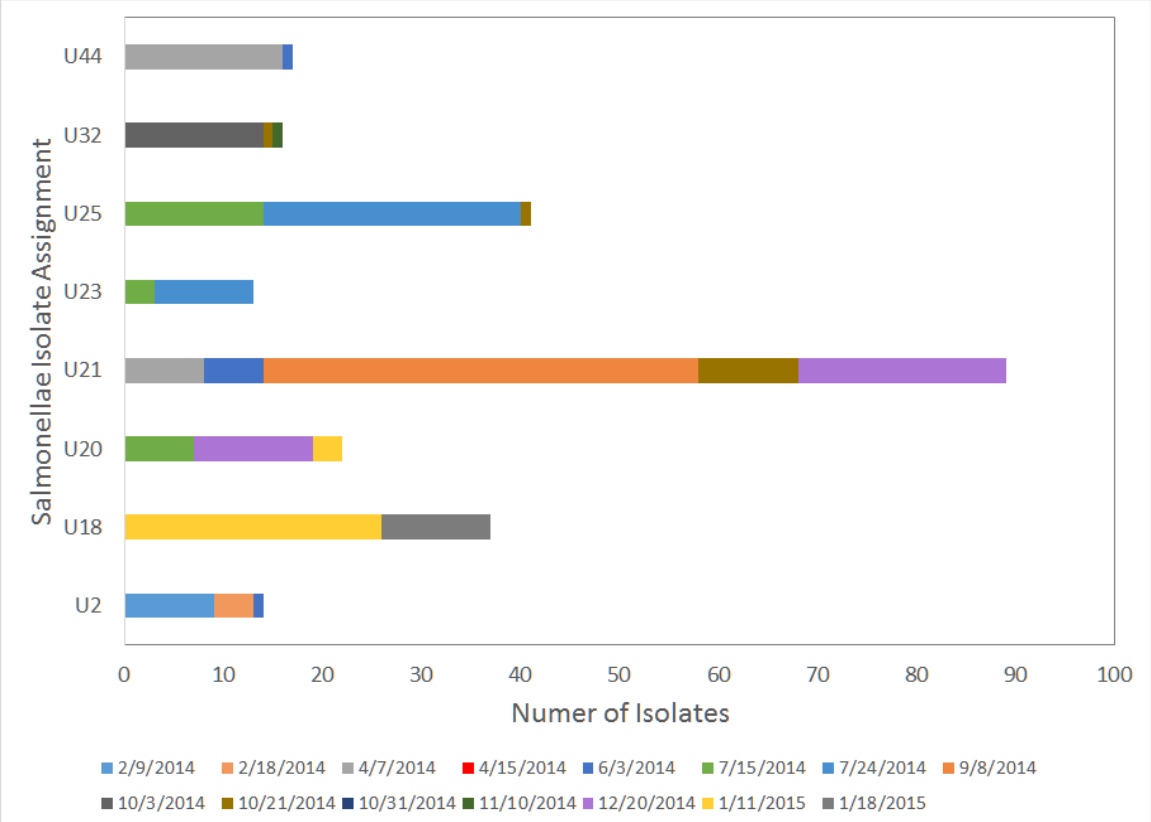

Figure 6: *Salmonella enterica* unique isolates and the frequency of isolation from *H. plecostomus* intestinal samples and environmental samples.

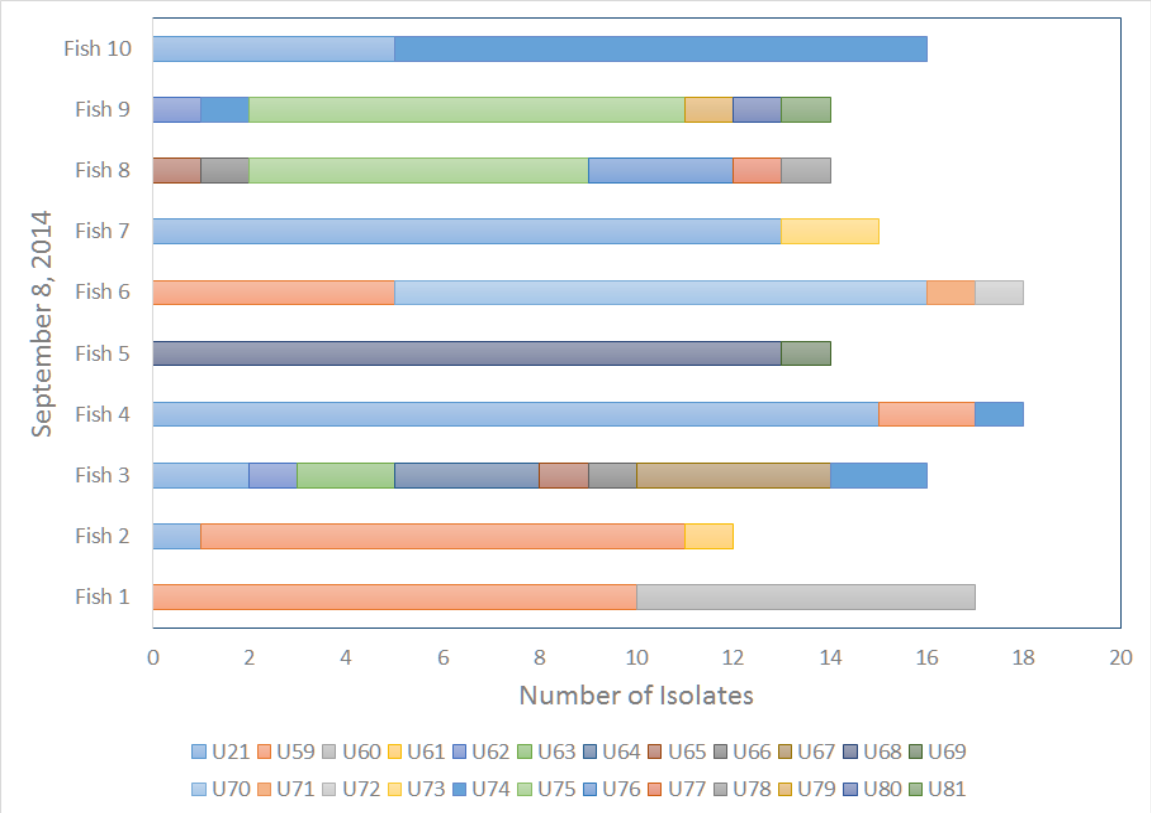

Figure 7: *Salmonella enterica* unique isolates and the frequency of isolation from *H. plecostomus* intestinal samples sampled on September 8, 2014.

To date, 46% of these samples have been completed and have been identified. The remaining samples are in varying degrees of completion and will be finished prior to publication of this study. 14 different serotypes have been identified from *H. plecostomus* (Figure 8). One surface water sample was identified as *S.* Newport, while the other two remaining environmental samples are in the process of completion. Of the number of isolates identified thus far, *S.* Newport accounts for the most number of isolates, followed by *S.* Give (Figure 8). *S.* Newport has also been detected in seven sampling dates thus far and has been the most frequently occurring serotype thus far (Figure 9). Two important clinical serotypes, *S.* Paratyphi B and *S.* Typhimurium have been identified from *H. plecostomus* intestinal samples, (Figure 8, 9). The only sampling

date that has had all isolates collected identified is July 15, 2014 (Figure 10). When comparing the number of Rep-PCR unique isolates to the number of serotype identified for this sampling date, Fish#1 and Fish#2 both had differing quantities (Figure 10, 11). Fish#1 was observed to have five unique isolates while only four different *Salmonella* serotypes were identified. The same is observed for Fish#2, where three unique isolates were observed, but only two different serotypes were identified. This may be due to the more distinguishable genetic differences Rep-PCR can discern over serotype identification (Harbottle 2006). For example, from Fish#1 sampled on July 15, 2014, unique isolates U28 and U29 were observed to be the same *Salmonella* serotype, *S.* Muenchen. Since these unique isolates produced two different banding patterns via rep-PCR, it is possible that two different strains of *S.* Muenchen may be infecting the same *H. plecostomus*. Some *H. plecostomus* are infected by multiple salmonellae serotypes, with *S.* Newport occurring in 75% of salmonellae positive catfish sampled on July 15, 2014 (Figure 10).

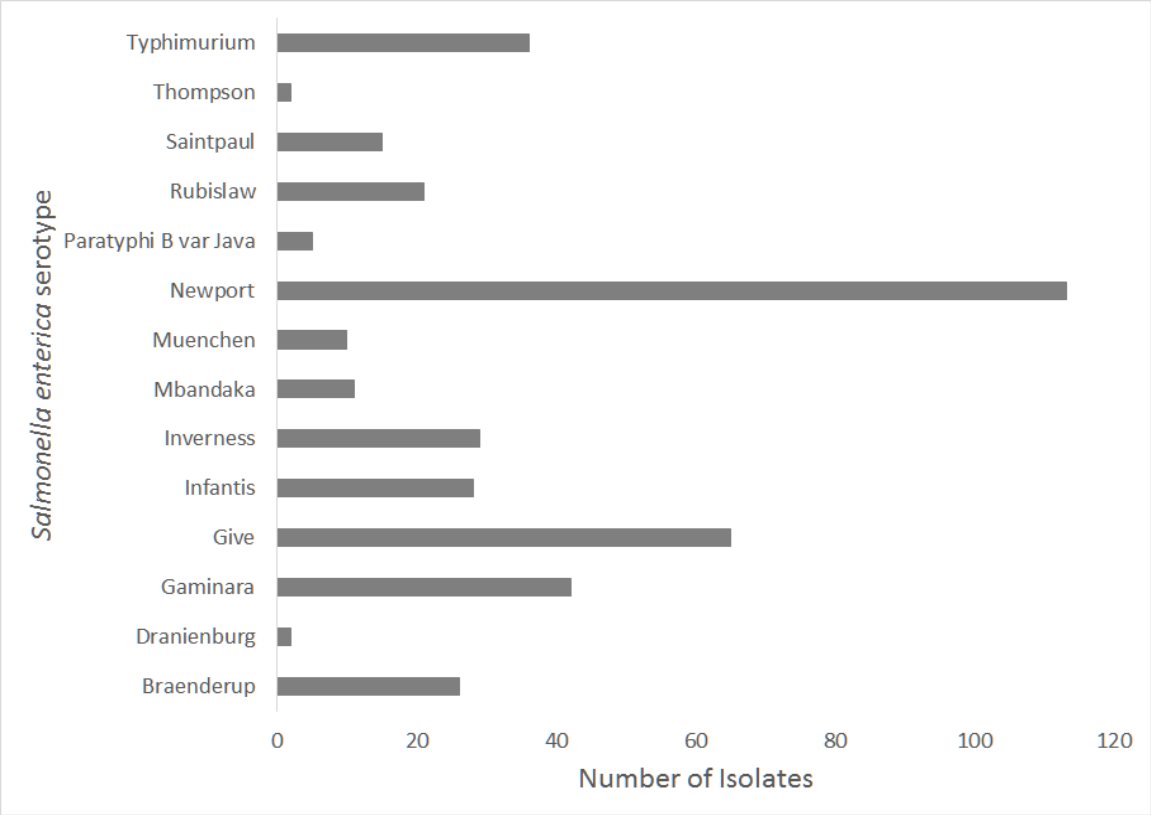

Figure 8: *Salmonella enterica* serotypes identified thus far and the frequency of isolation from *H. plecostomus* intestinal samples and environmental samples.

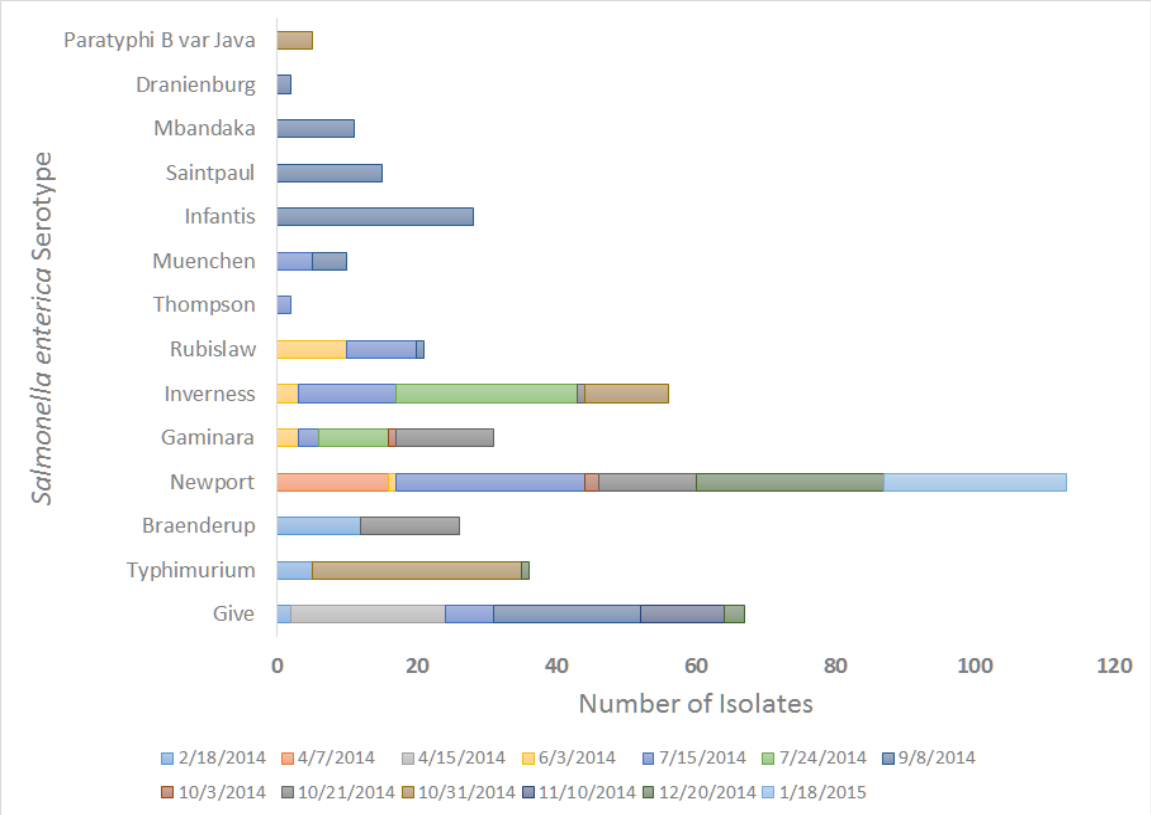

Figure 9: *Salmonella enterica* serotypes identified thus far and the frequency of isolation from *H. plecostomus* intestinal samples and environmental samples as it relates to sample dates.

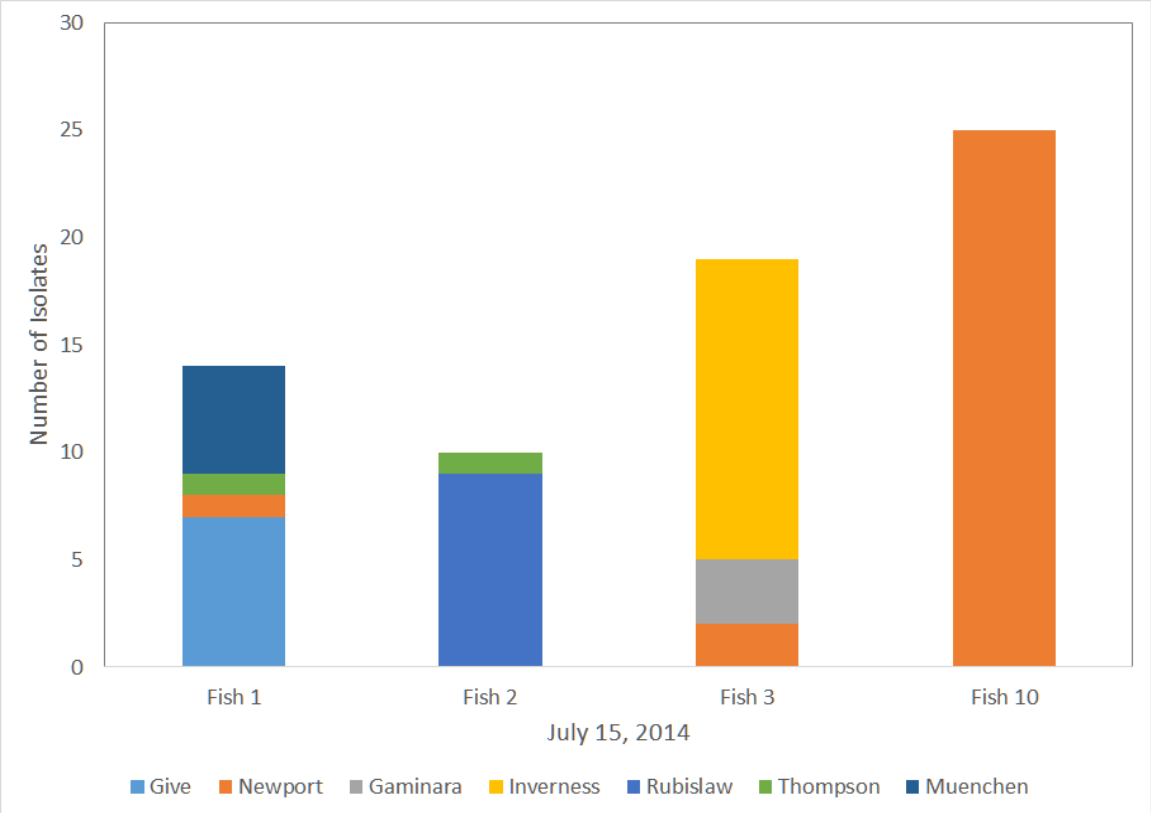

Figure 10: *Salmonella enterica* serotypes identified and the frequency of isolation from *H. plecostomus* collected on July 15, 2014.

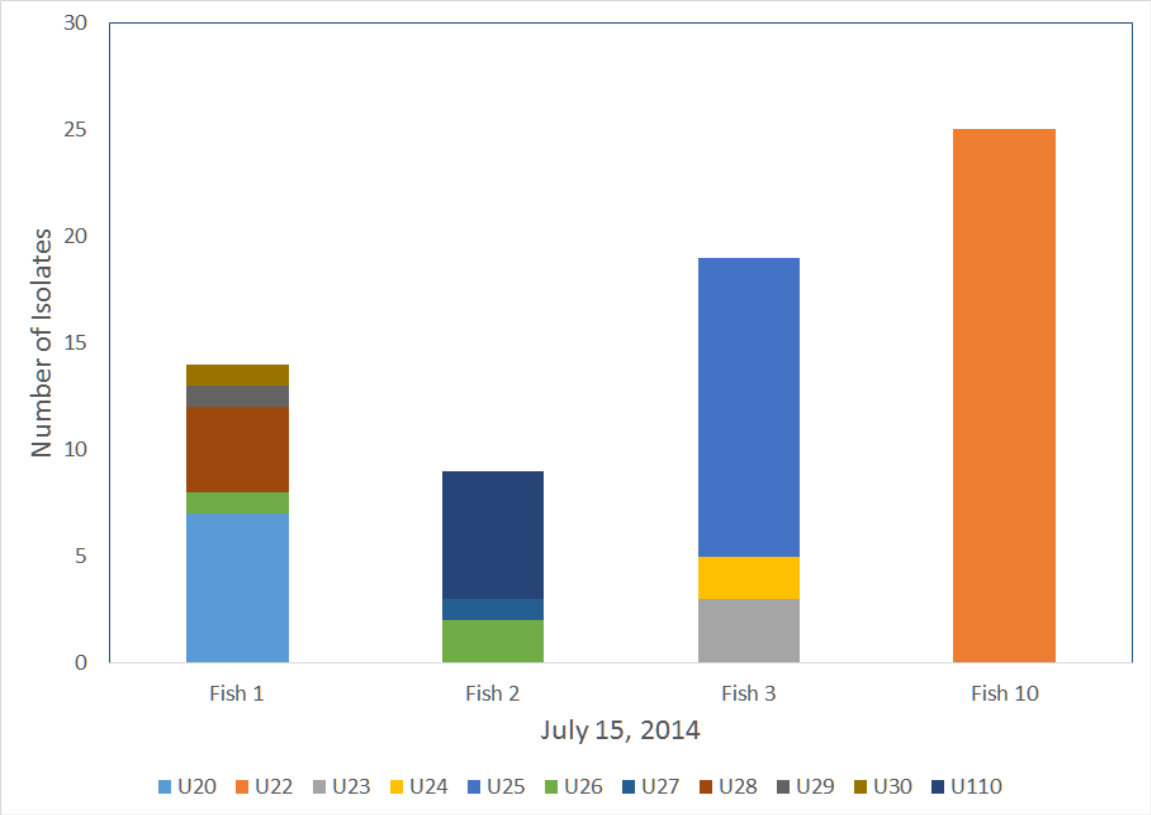

Figure 11: *Salmonella enterica* unique isolates identified and the frequency of isolation from *H. plecostomus* collected on July 15, 2014.

Identification of salmonellae isolates to the serotype level is crucial to evaluate whether any clinically import strains are present and evaluate these results on a broader scale (Urwin 2003). Previous studies have characterized *Salmonella* isolates obtained from fish samples with varying degrees of diversity: two different serotypes were identified in fish in Iran (Rahimi 2011), 34 serotypes from fish sampled in Ouagadougou, Burkina Faso (Traore 2015), and 3 serotypes in fish sampled in Indonesia (Kusumaningrum 2012). All these studies identified *S.* Typhimurium as one of the serotypes carried by these fish samples. In Iran, *S.* Paratyphi B was also identified as a serotype carried by the fish sampled (Rahimi 2011). Between 2000 and 2003, Quebec saw an increase in *S.* Paratyphi B var Java infections that was linked to tropical fish

aquariums (Gaulin 2005), while between 2010 and 2011, Spain saw outbreaks of this virulent strain due to human exposure to turtles (Hernandez 2012).

This study found that *H. plecostomus* are carriers of the pathogen, *Salmonella enterica*, and can carry environmental serotypes, as well as, more virulent serotypes. Fish can play an important role as indicator organisms (Novotny 2004), as well as vectors for spreading these pathogens (Sha 2013a). This study also found that these salmonellae strains do not appear to be introduced into the San Marcos River, but are rather consistently present. These findings can impact human recreational activities related to this aquatic system, allow dissemination to wildlife, and potentially impact fish farms that use this river water economically.

APPENDIX SECTION

Appendix A - Figures of chemical and physical factors affecting the San Marcos River as they compare to salmonellae prevalence in *H. plecostomus*

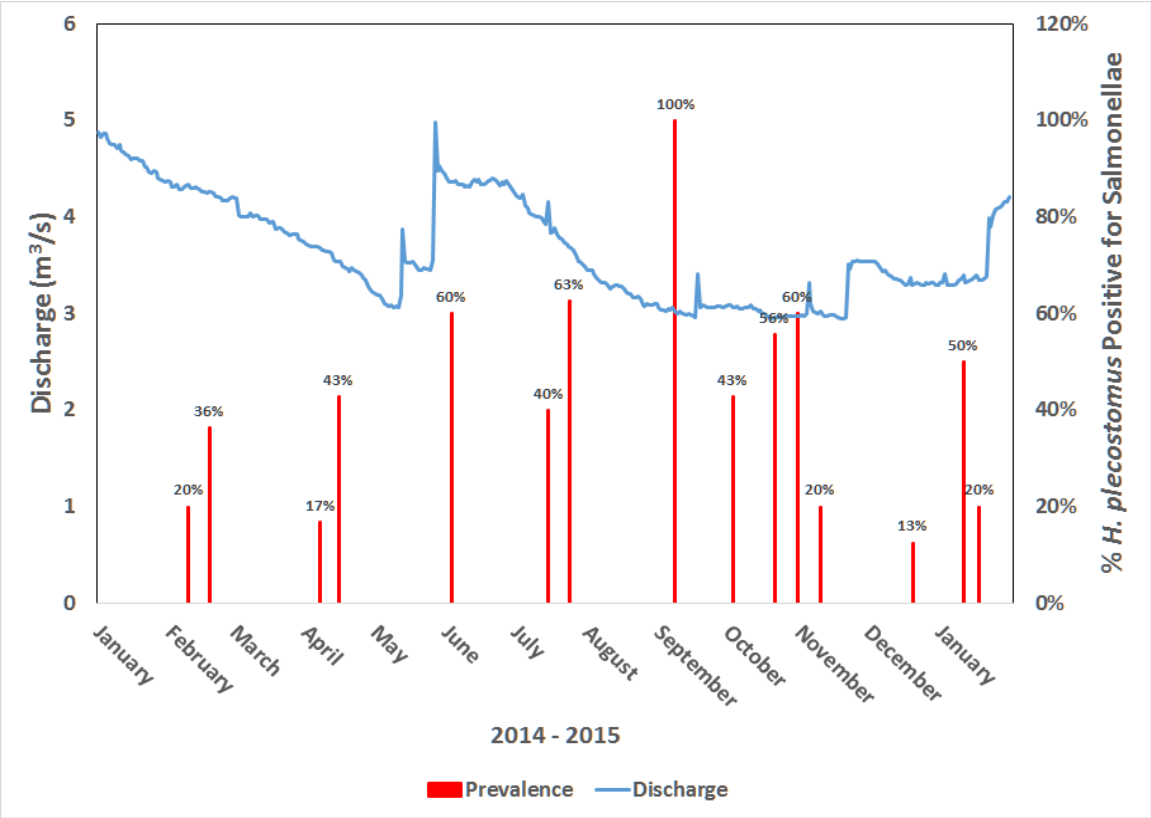

Figure A-1: River discharge taken from USGS are reflected in blue and the percentage of *H. plecostomus* intestinal samples positive for salmonellae after semi-selective enrichment and *q*PCR detection.

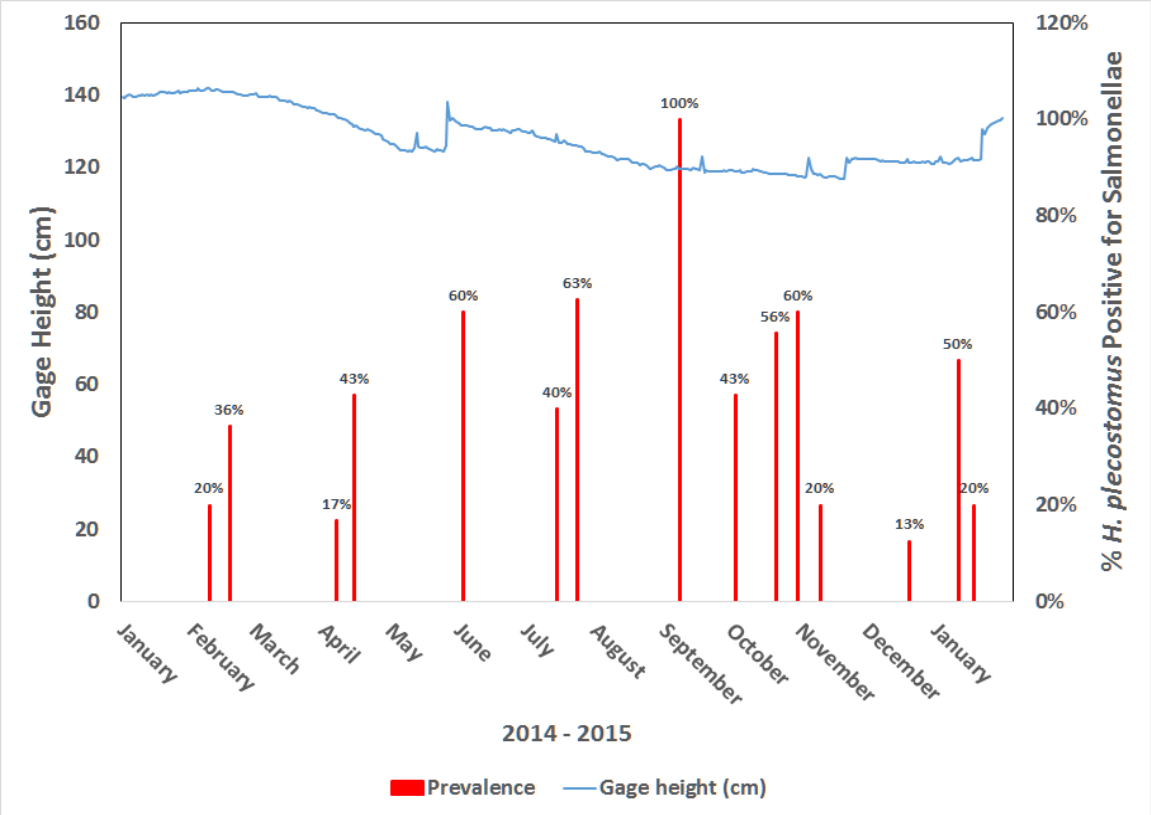

Figure A-2: River gage height taken from USGS are reflected in blue and the percentage of *H. plecostomus* intestinal samples positive for salmonellae after semi-selective enrichment and *q*PCR detection.

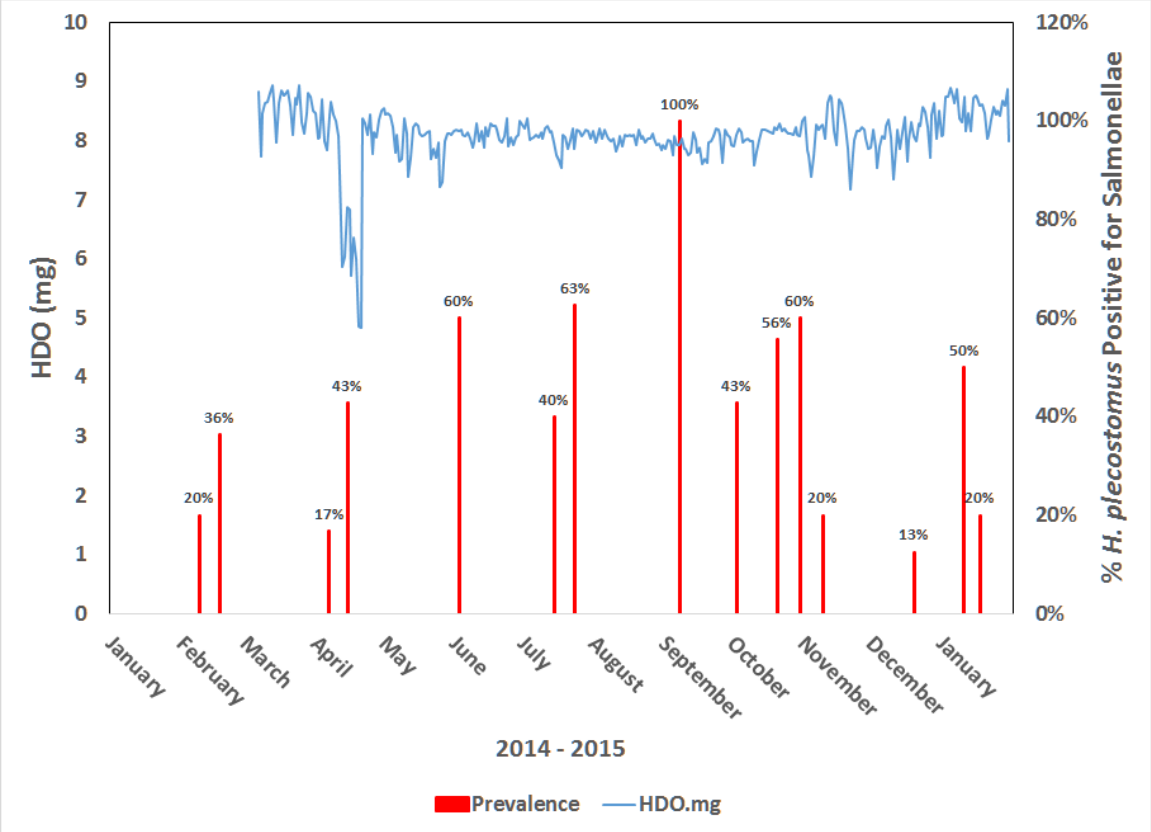

Figure A-3: HDO measurements taken via 6920 V2-2 Multi-Parameter Water Quality Sonde placed at 29.8800, -97.9333 are reflected in blue and the percentage of *H. plecostomus* intestinal samples positive for salmonellae after semi-selective enrichment and *q*PCR detection.

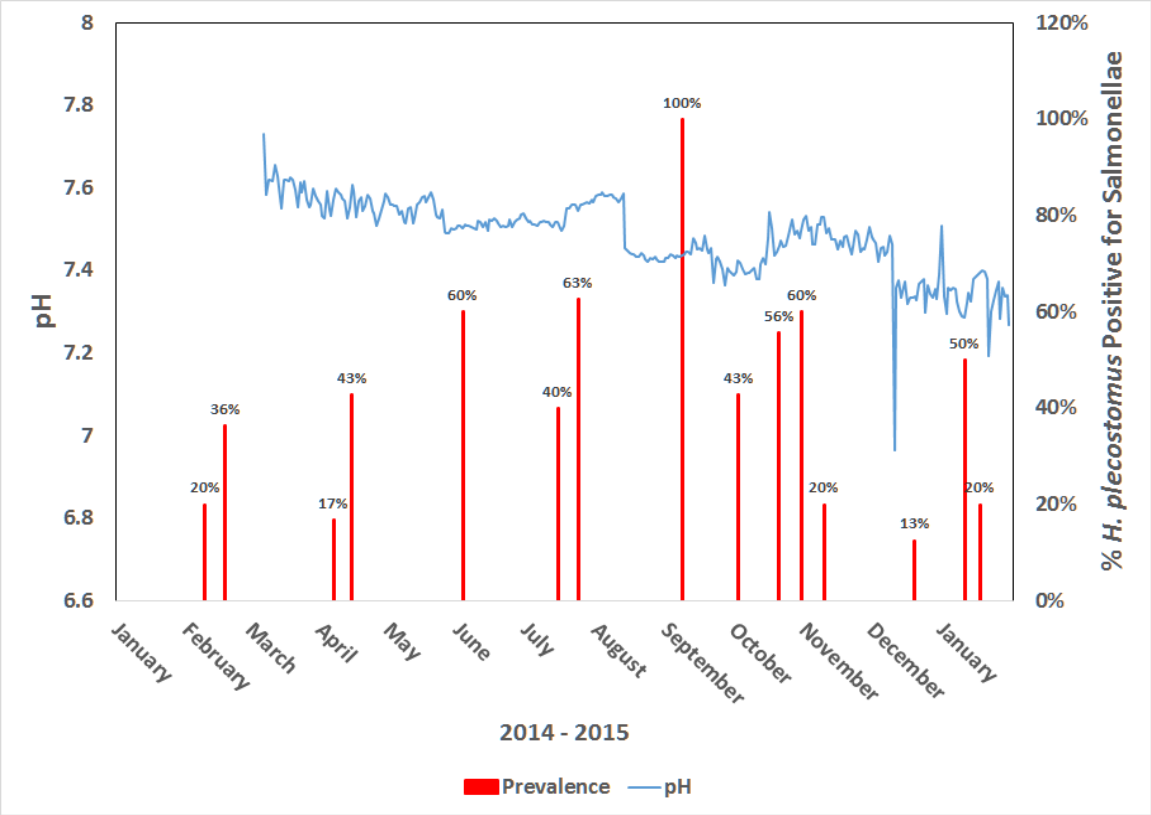

Figure A-4: pH measurements taken via 6920 V2-2 Multi-Parameter Water Quality Sonde placed at 29.8800, -97.9333 are reflected in blue and the percentage of *H. plecostomus* intestinal samples positive for salmonellae after semi-selective enrichment and *q*PCR detection.

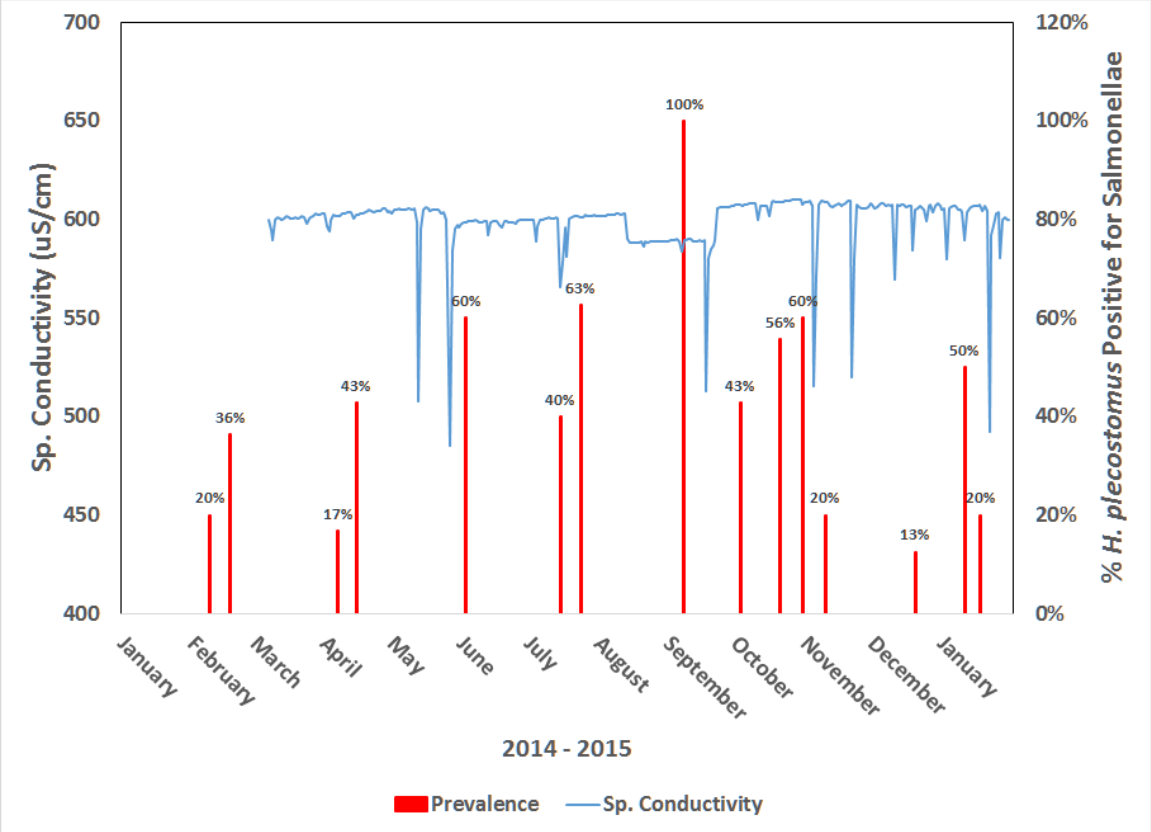

Figure A-5: Specific conductivity measurements taken via 6920 V2-2 Multi-Parameter Water Quality Sonde placed at 29.8800, -97.9333 are reflected in blue and the percentage of *H. plecostomus* intestinal samples positive for salmonellae after semi-selective enrichment and *q*PCR detection.

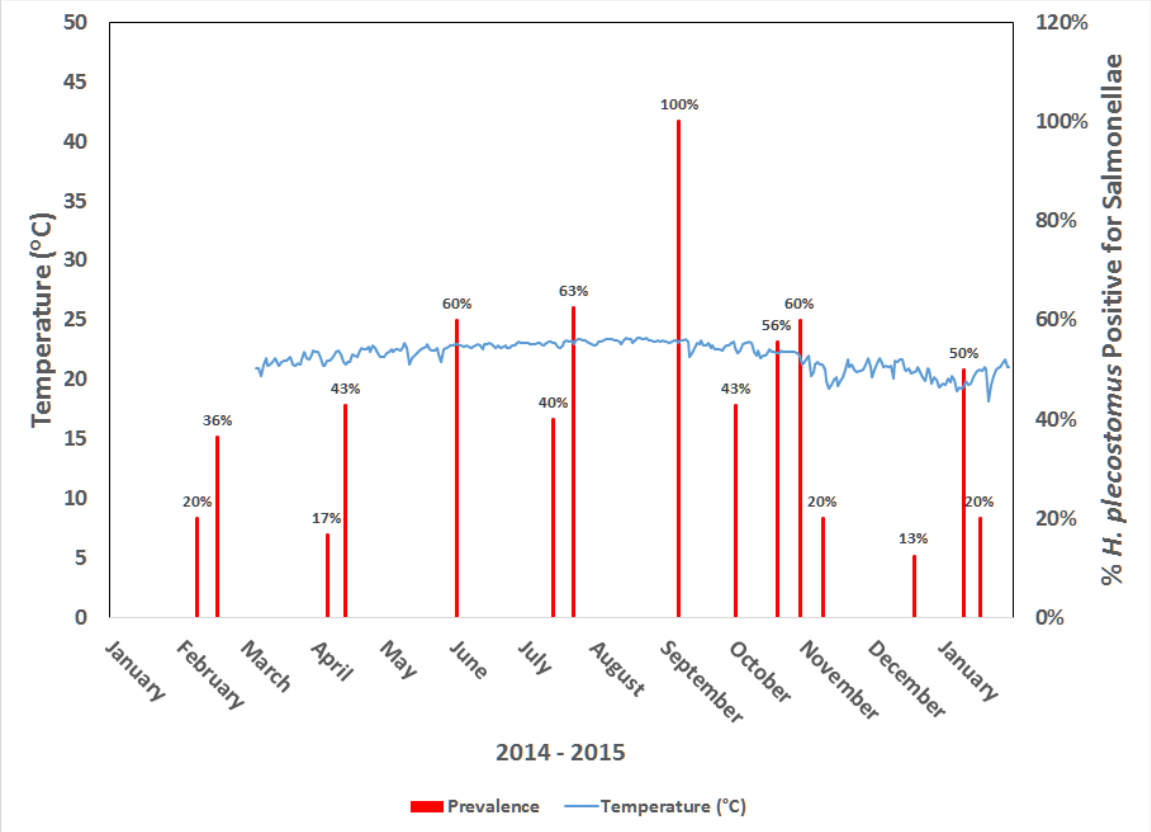

Figure A-6: Water temperature measurements taken via 6920 V2-2 Multi-Parameter Water Quality Sonde placed at 29.8800, -97.9333 are reflected in blue and the percentage of *H. plecostomus* intestinal samples positive for salmonellae after semi-selective enrichment and *q*PCR detection.

Appendix B – Figures and tables of statistical analysis

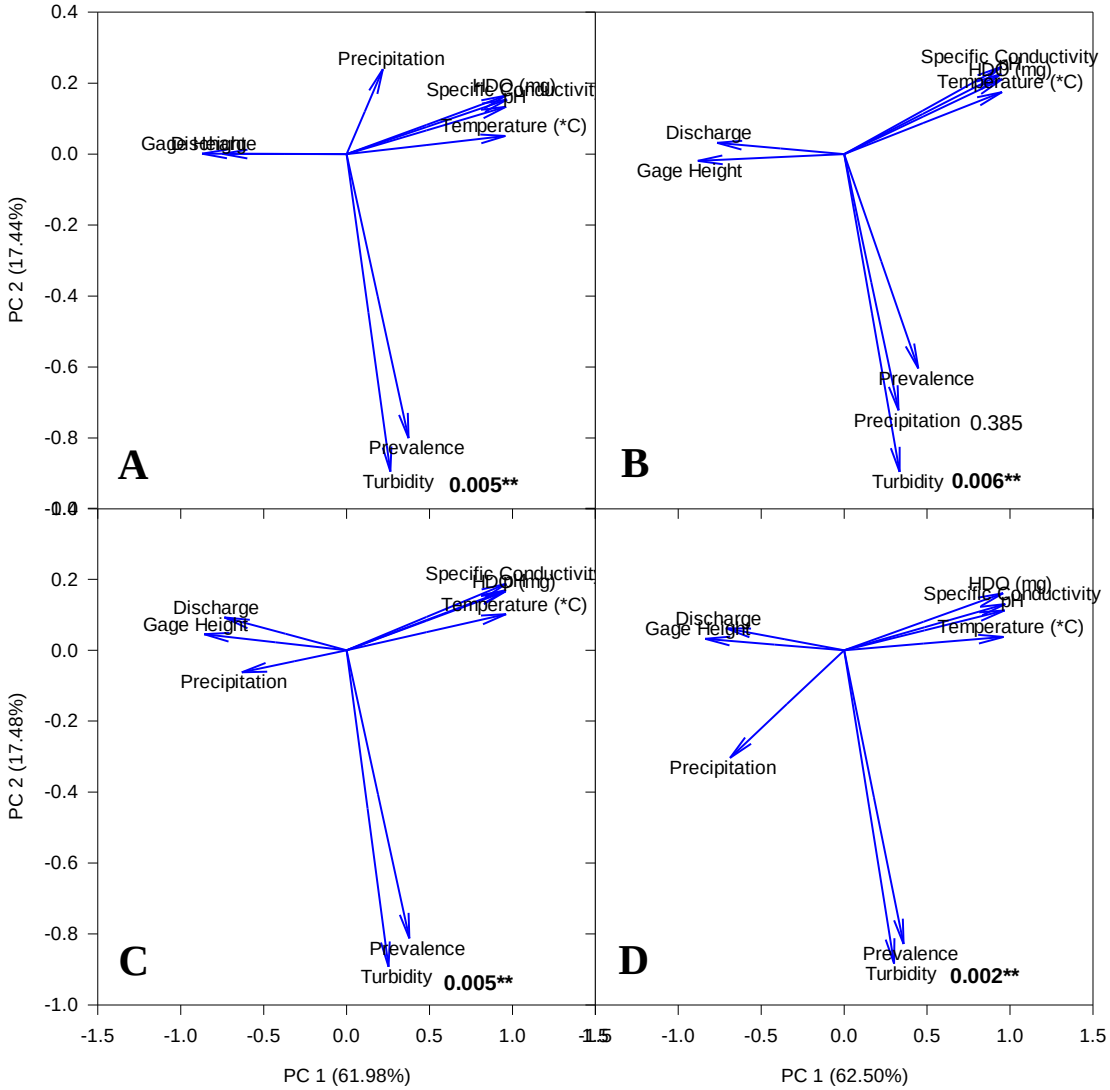

Figure B-1: Principle Component Analysis (PCA) of physical and chemical facotrs affecting the San Marcos River in correlation with salmonellae prevalence on the sampling day (A), one day prior to sampling (B), two days prior to sampling (C), and three days prior to sampling(D). P-vales of of correlated vactors were calculated using Pearson's product moment correlation.

Appendix C – Rep-PCR with ethidium bromide gel electrophoresis images

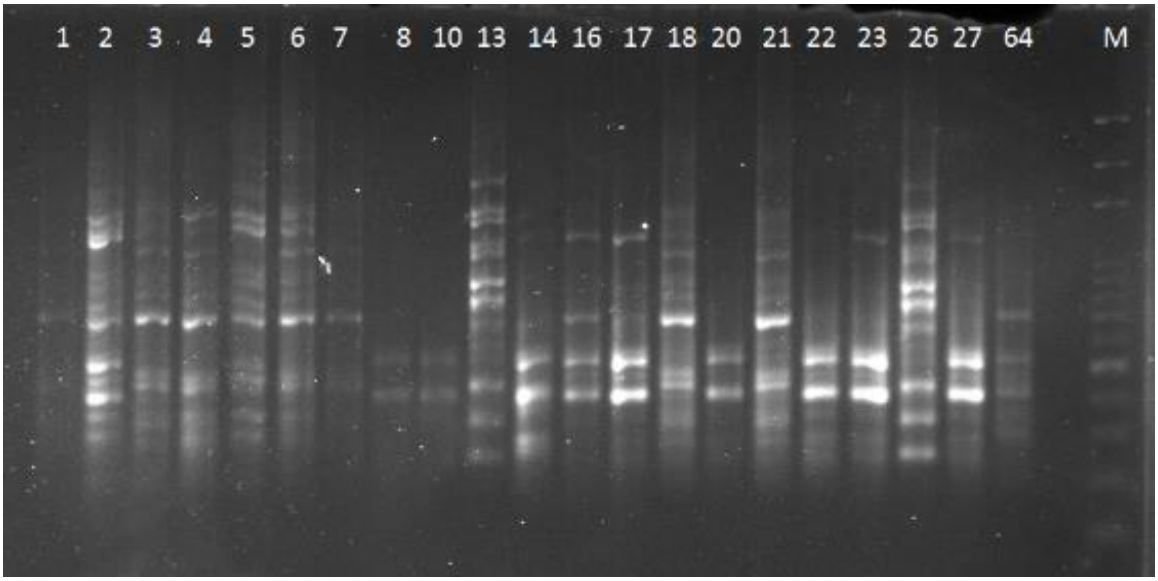

Figure C-1: Rep-PCR products for salmonellae positive isolates from Fish #3 collected September 18, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

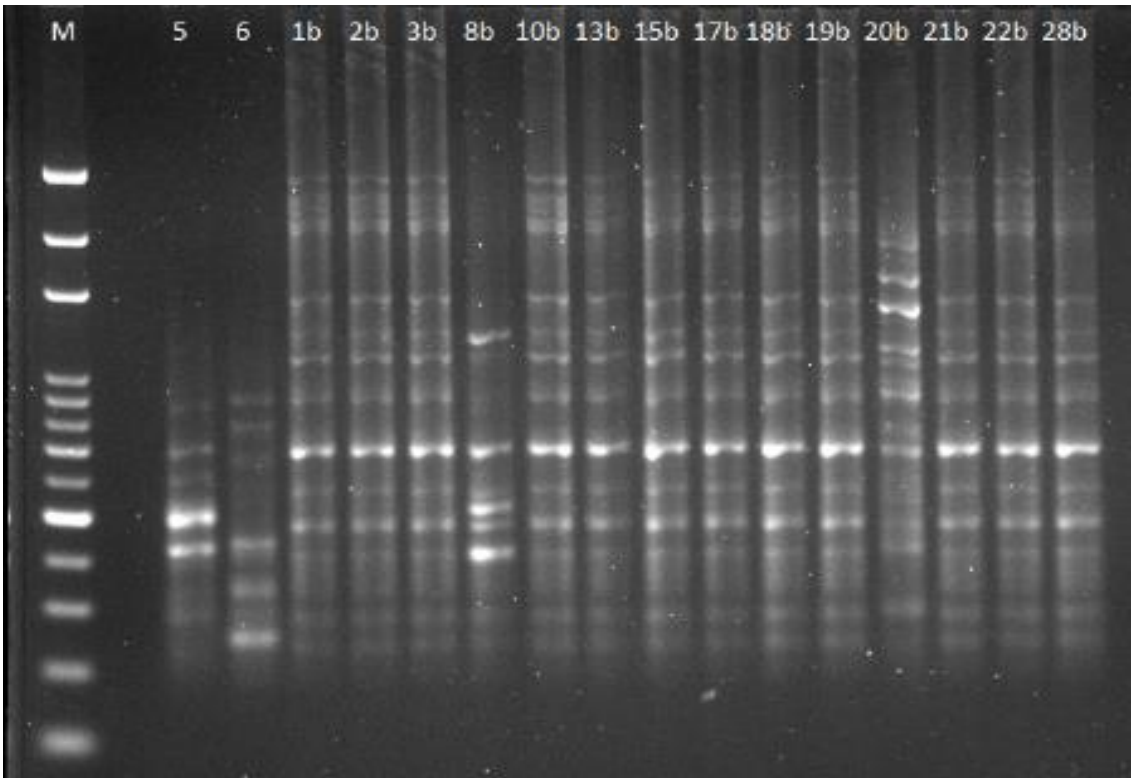

Figure C-2: Rep-PCR products for salmonellae positive isolates from Fish #4 collected September 18, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

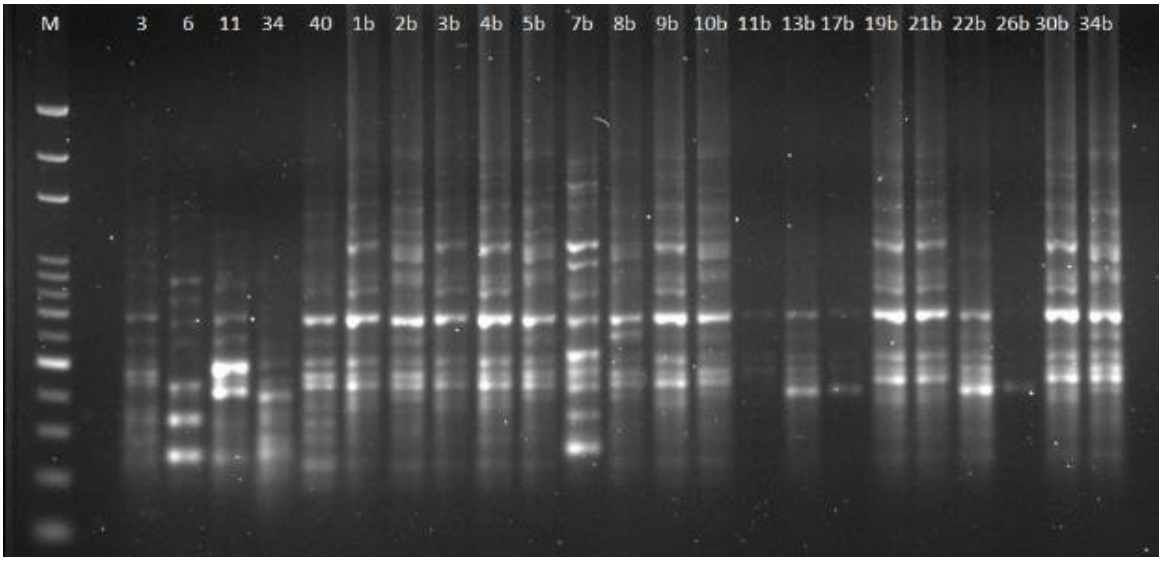

Figure C-3: Rep-PCR products for salmonellae positive isolates from Fish #8 collected September 18, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

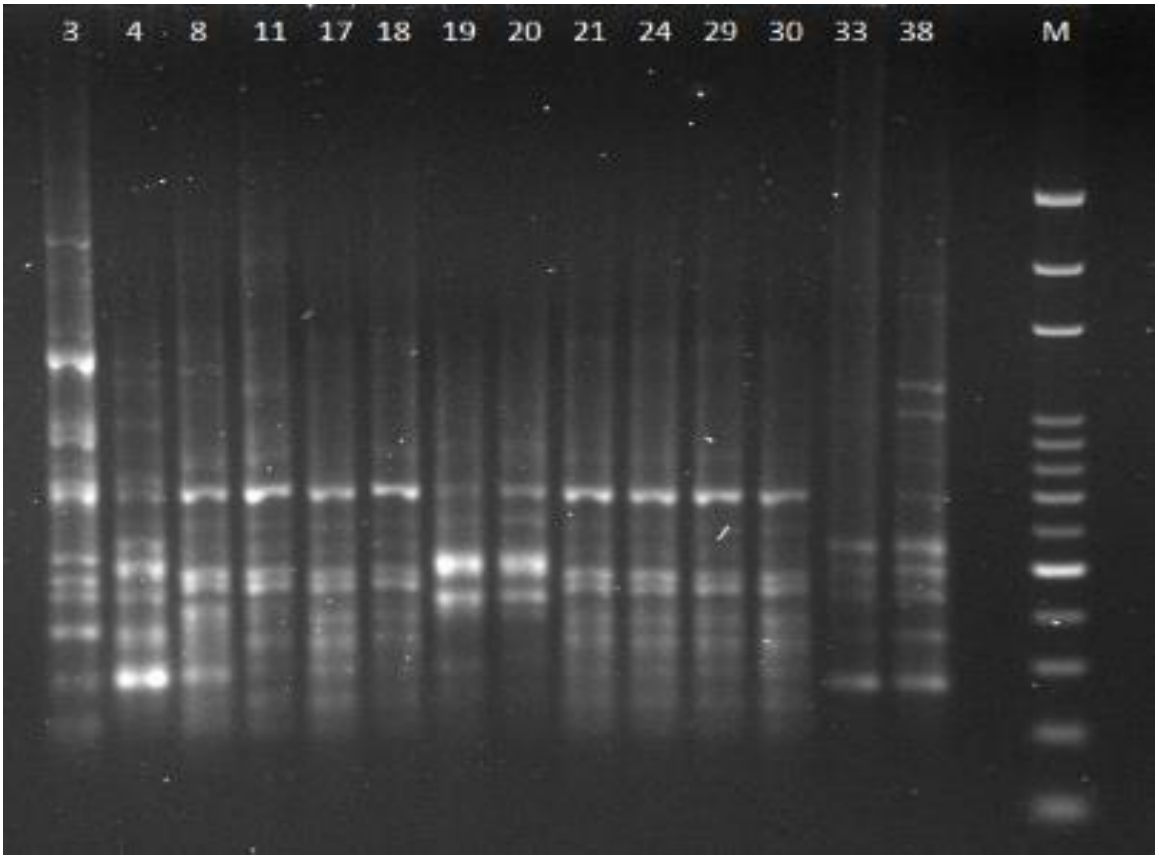

Figure C-4: Rep-PCR products for salmonellae positive isolates from Fish #10 collected September 18, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

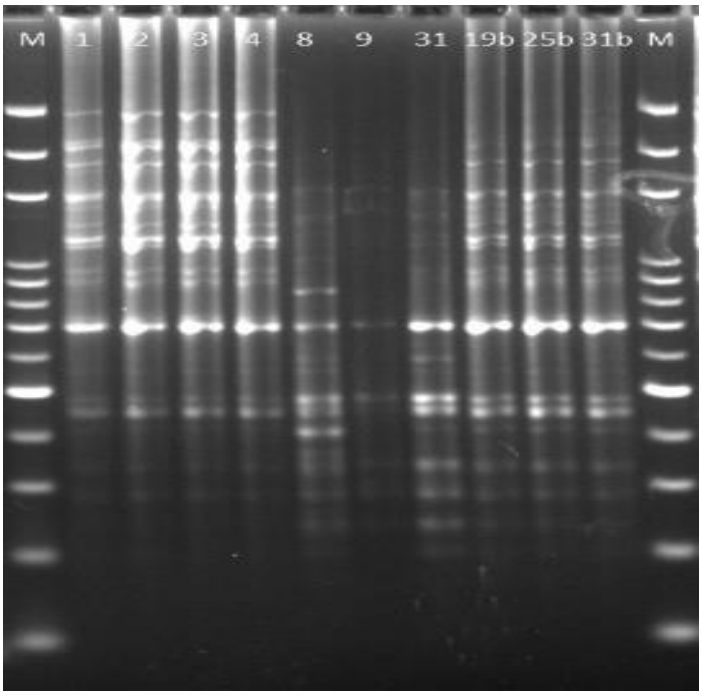

Figure C-5: Rep-PCR products for salmonellae positive isolates from Fish #4 collected April 7, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

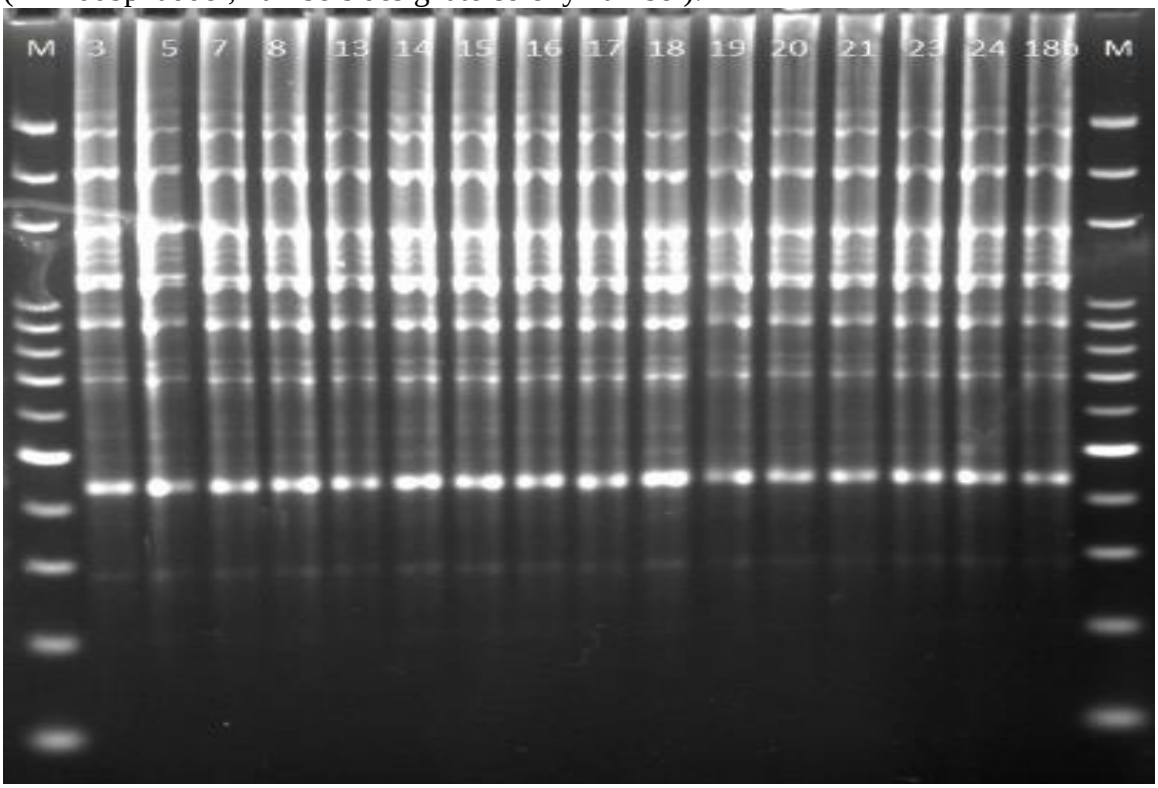

Figure C-6: Rep-PCR products for salmonellae positive isolates from surface water collected April 7, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number)

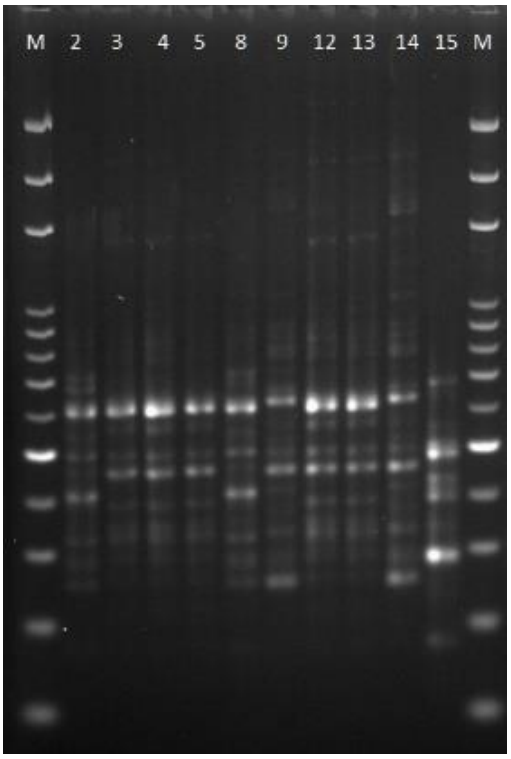

Figure C-7: Rep-PCR products for salmonellae positive isolates from Fish #2 collected April 15, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

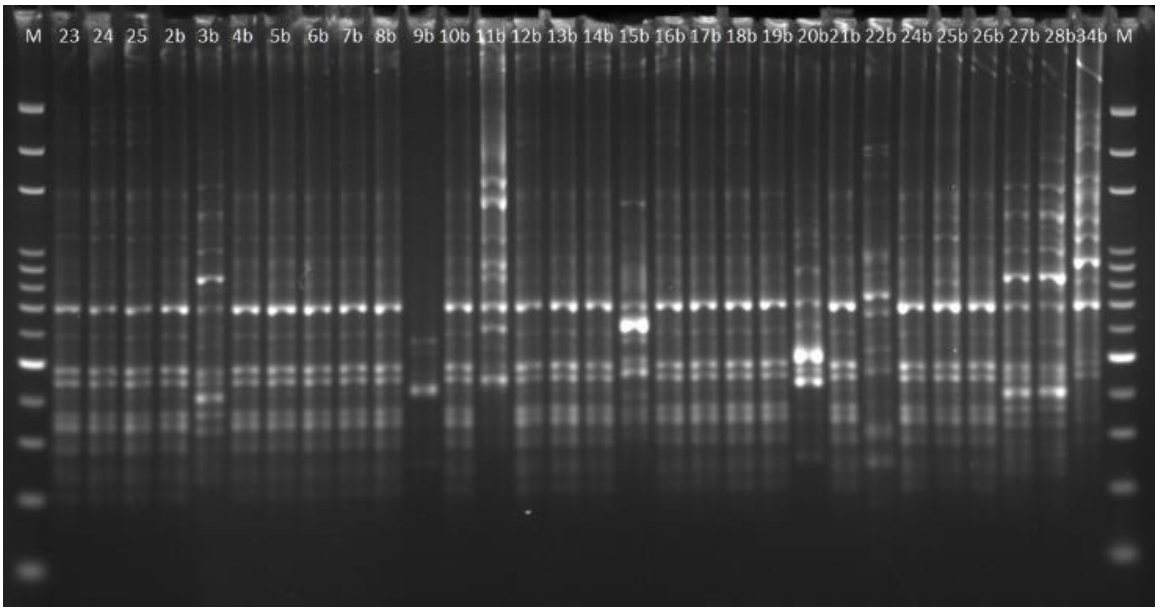

Figure C-8: Rep-PCR products for salmonellae positive isolates from Fish #7 collected April 15, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

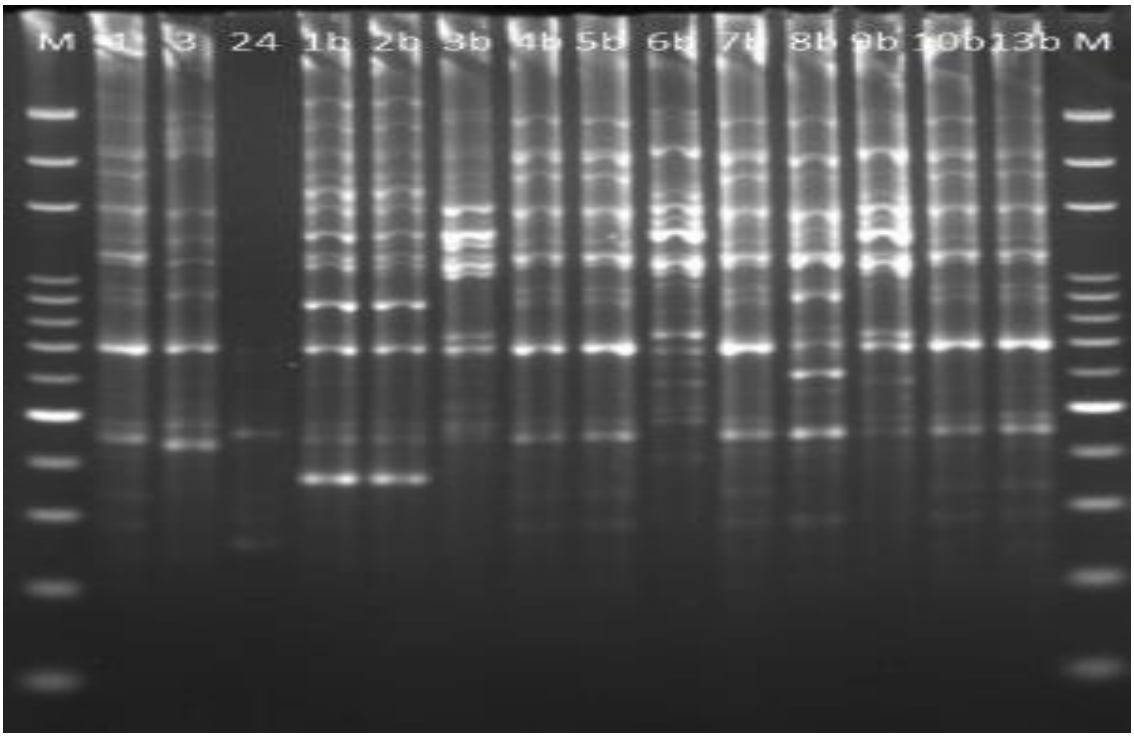

Figure C-9: Rep-PCR products for salmonellae positive isolates from Fish #1 collected June 3, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

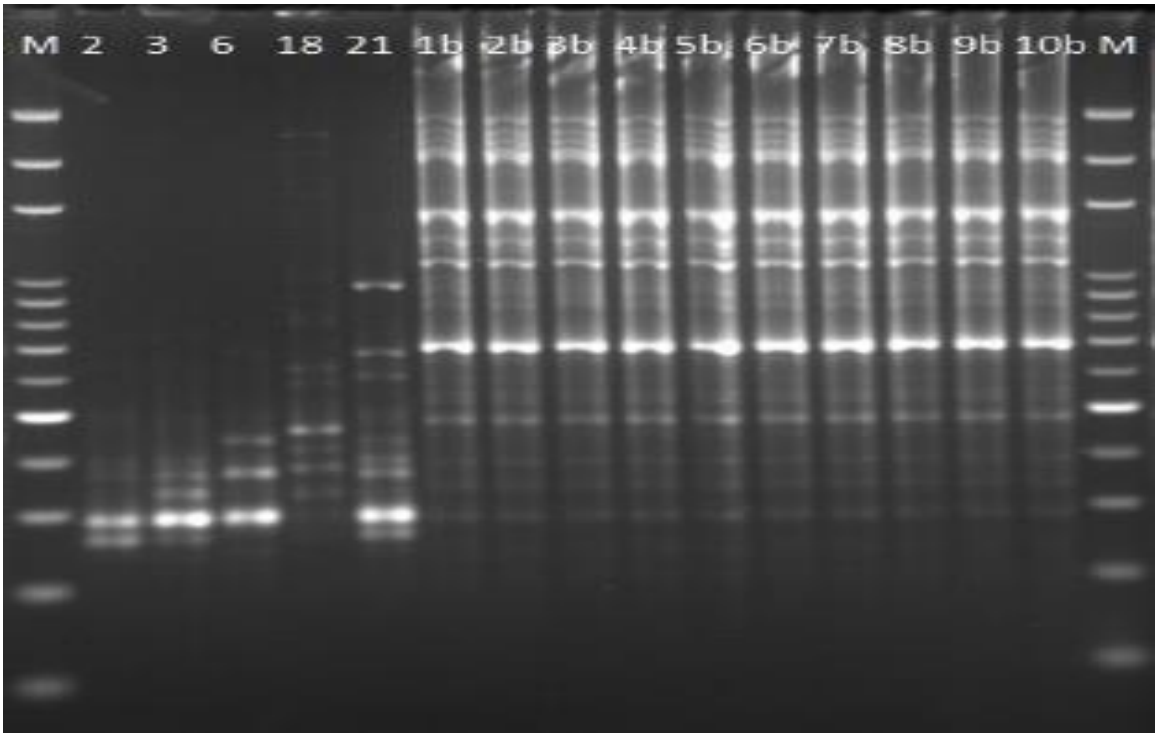

Figure C-10: Rep-PCR products for salmonellae positive isolates from Fish #3 collected June 3, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

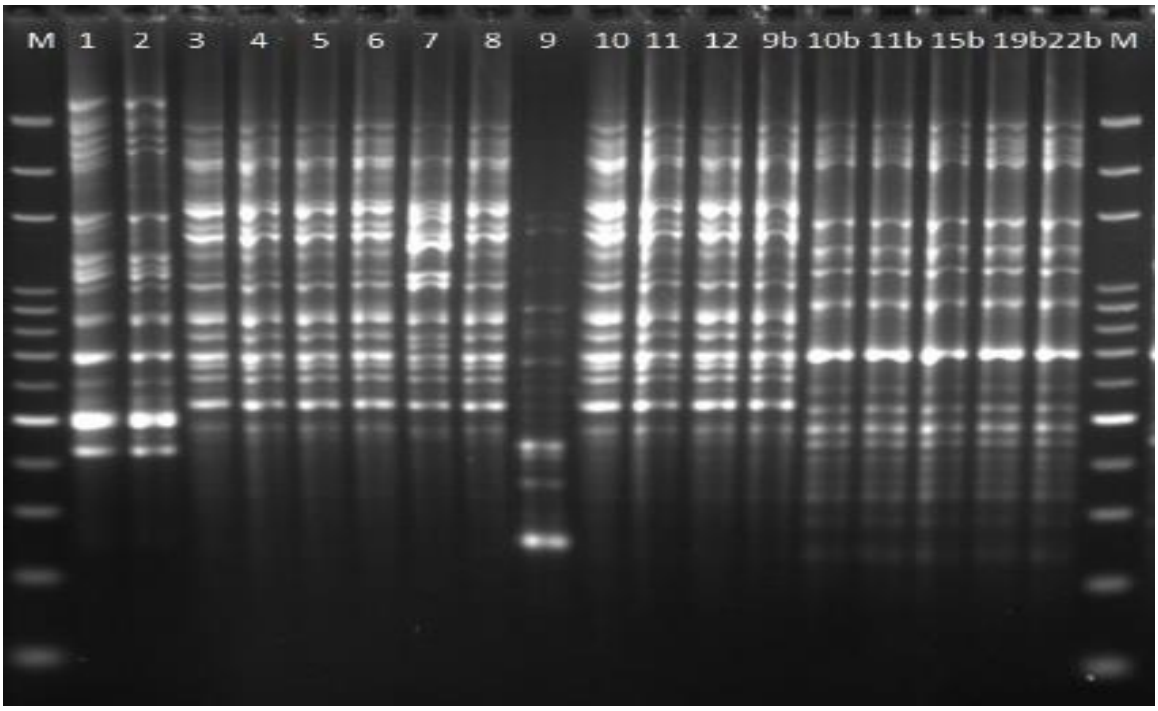

Figure C-11: Rep-PCR products for salmonellae positive isolates from Fish #5 collected June 3, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

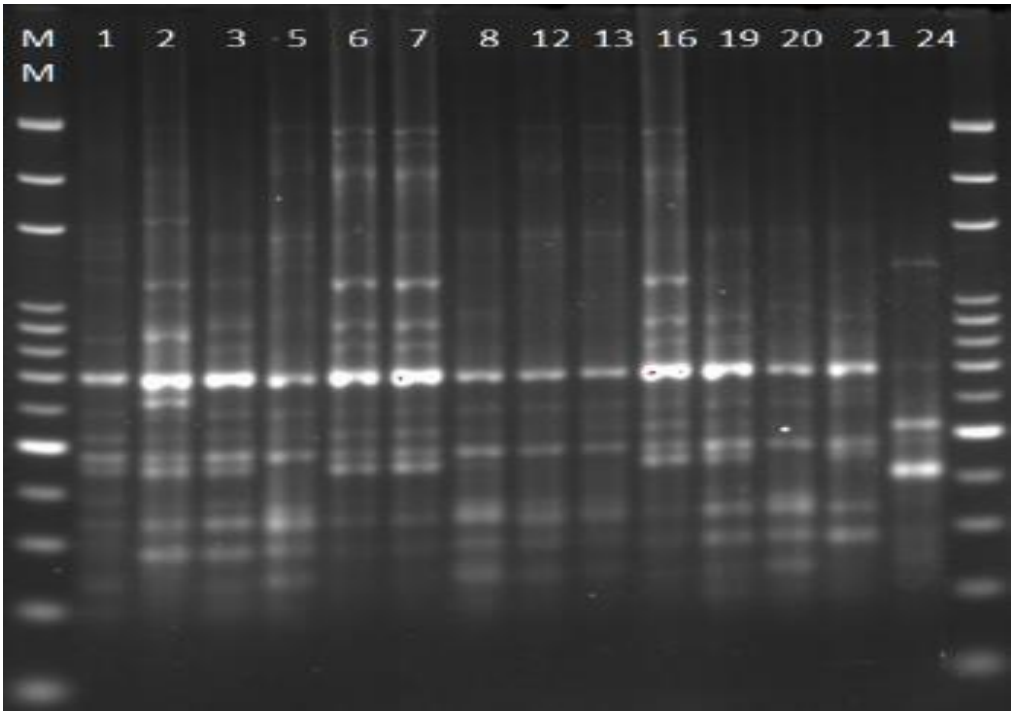

Figure C-12: Rep-PCR products for salmonellae positive isolates from Fish#1 collected July 15, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

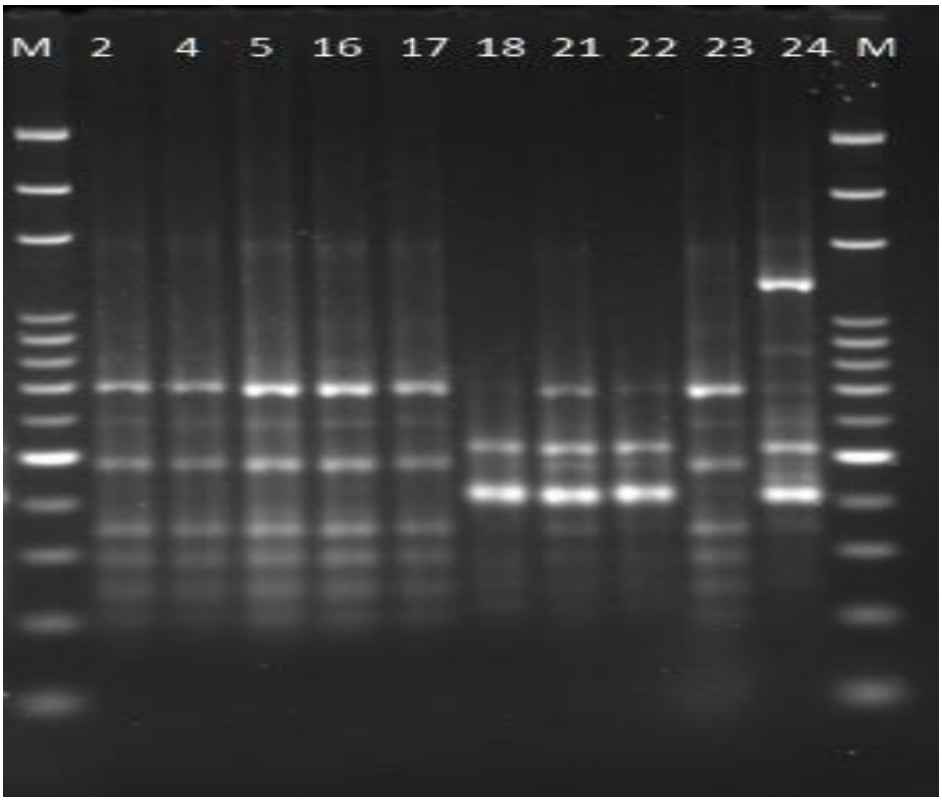

Figure C-13: Rep-PCR products for salmonellae positive isolates from Fish #2 collected July 15, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

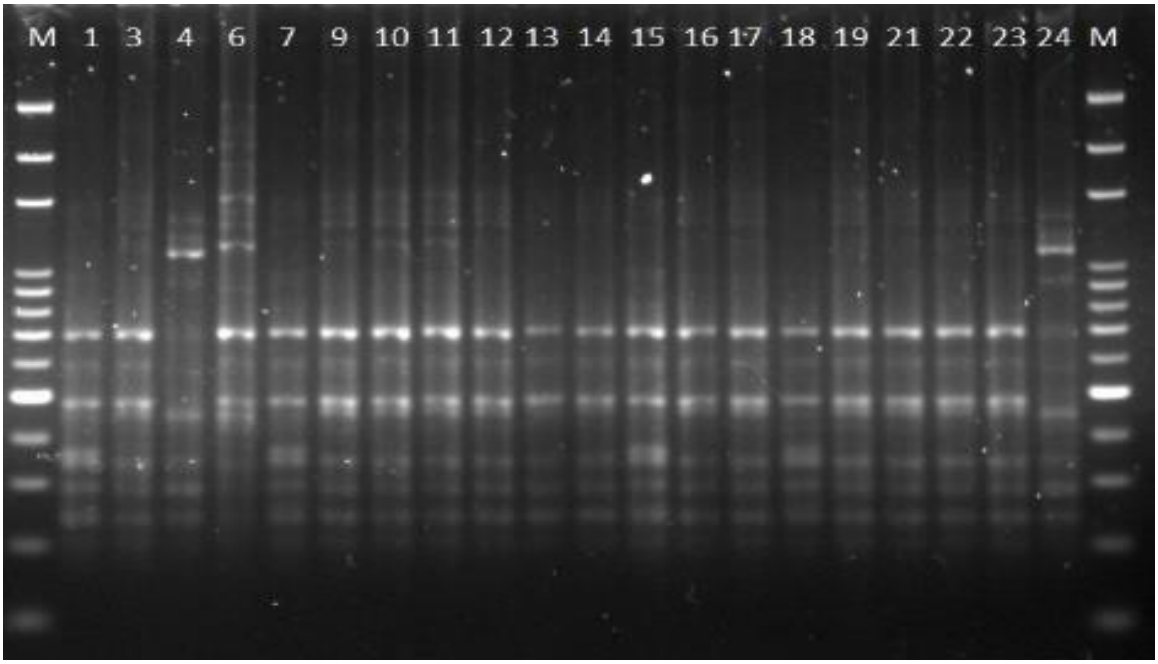

Figure C-14: Rep-PCR products for salmonellae positive isolates from Fish #3 collected July 15, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

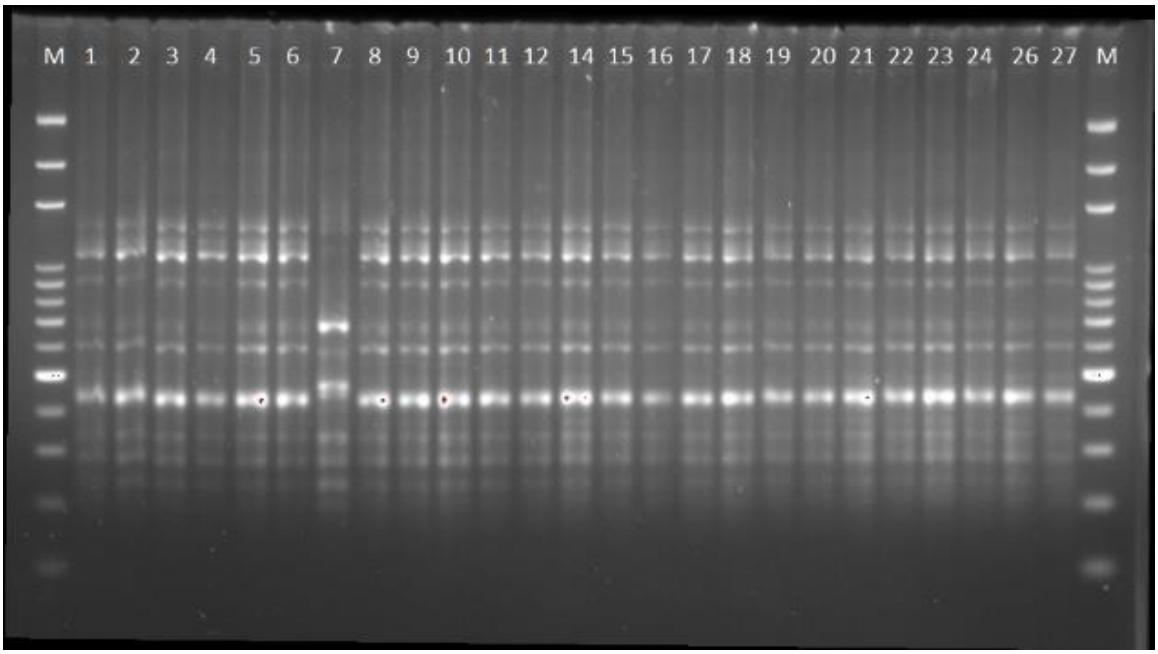

Figure C-15: Rep-PCR products for salmonellae positive isolates from Fish #10 collected July 15, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

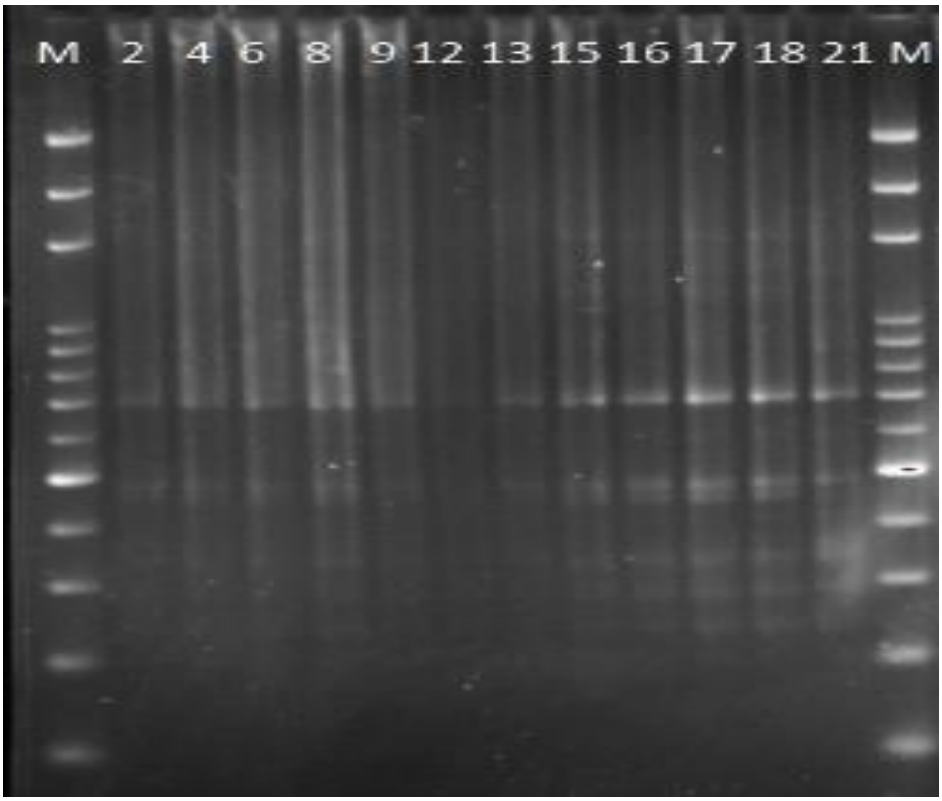

Figure C-16: Rep-PCR products for salmonellae positive isolates from Fish #2 collected July 24, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

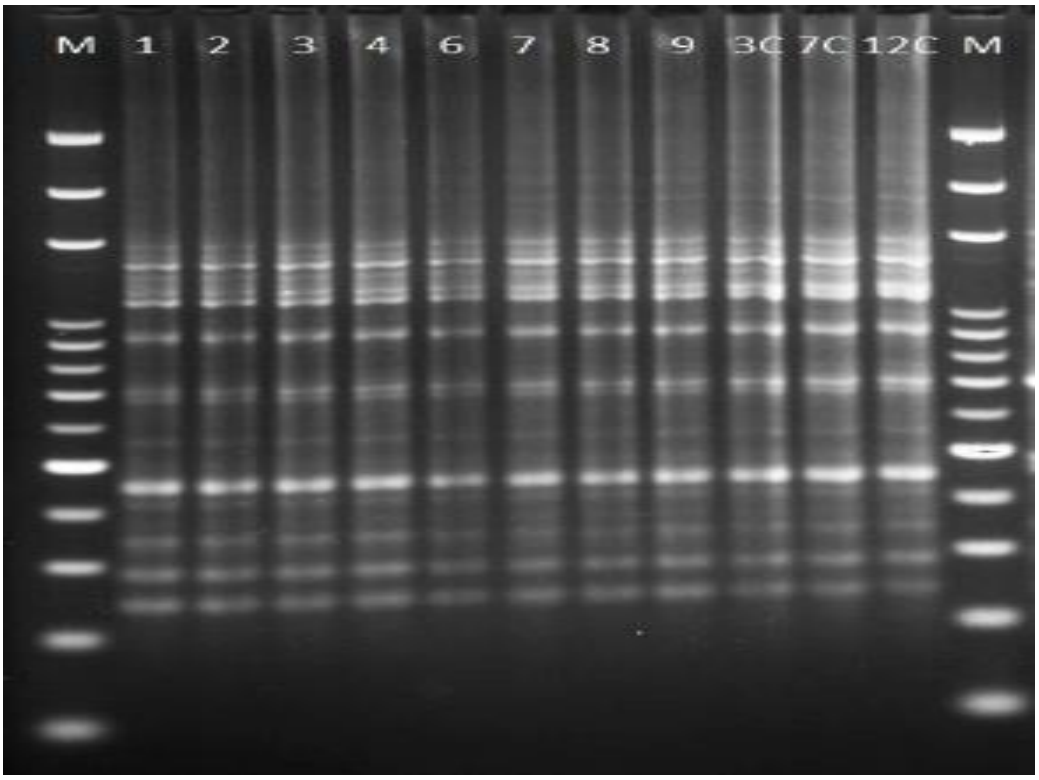

Figure C-17: Rep-PCR products for salmonellae positive isolates from Fish #3 collected July 24, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

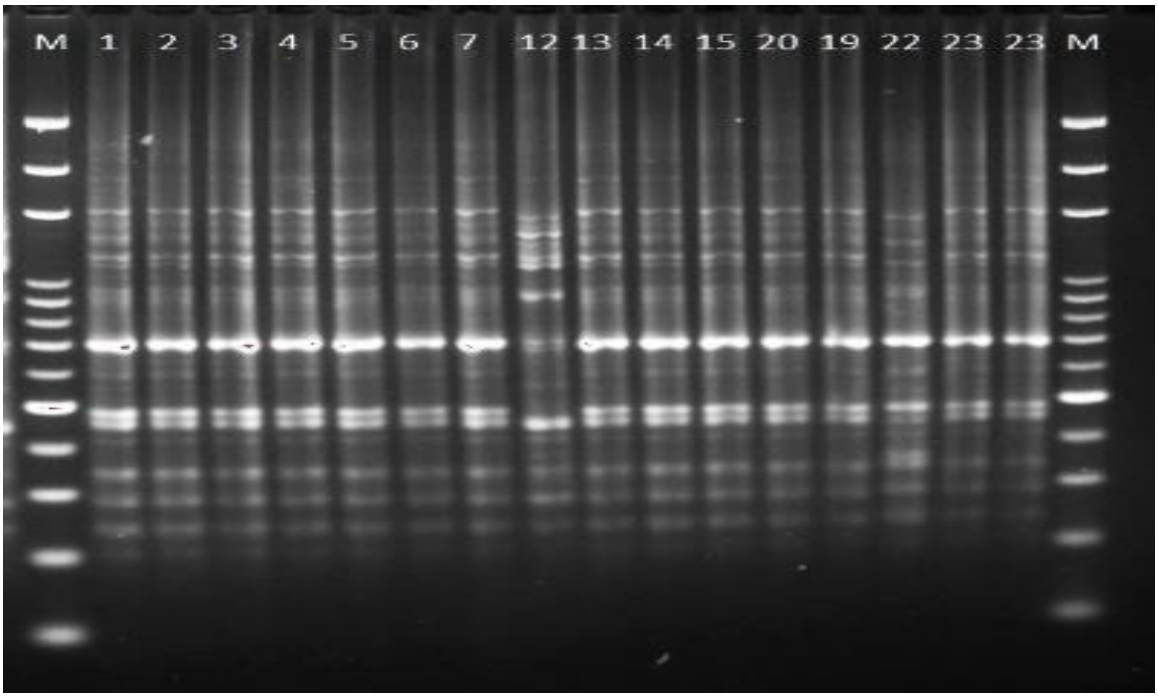

Figure C-18: Rep-PCR products for salmonellae positive isolates from Fish #6 collected July 24, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

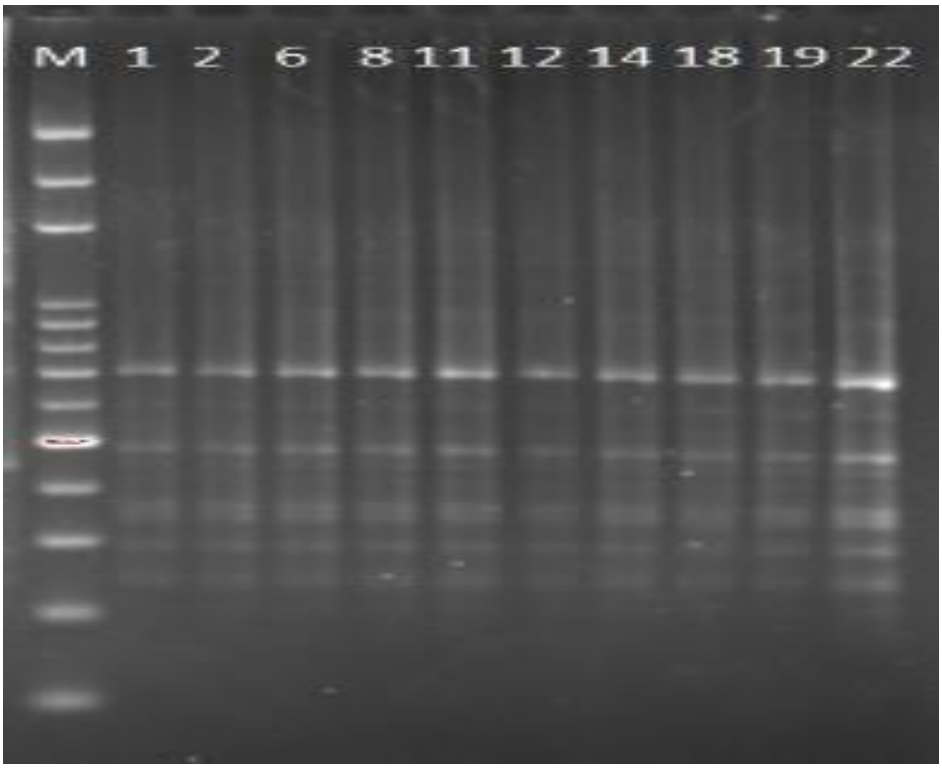

Figure C-19: Rep-PCR products for salmonellae positive isolates from Fish #7 collected July 24, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

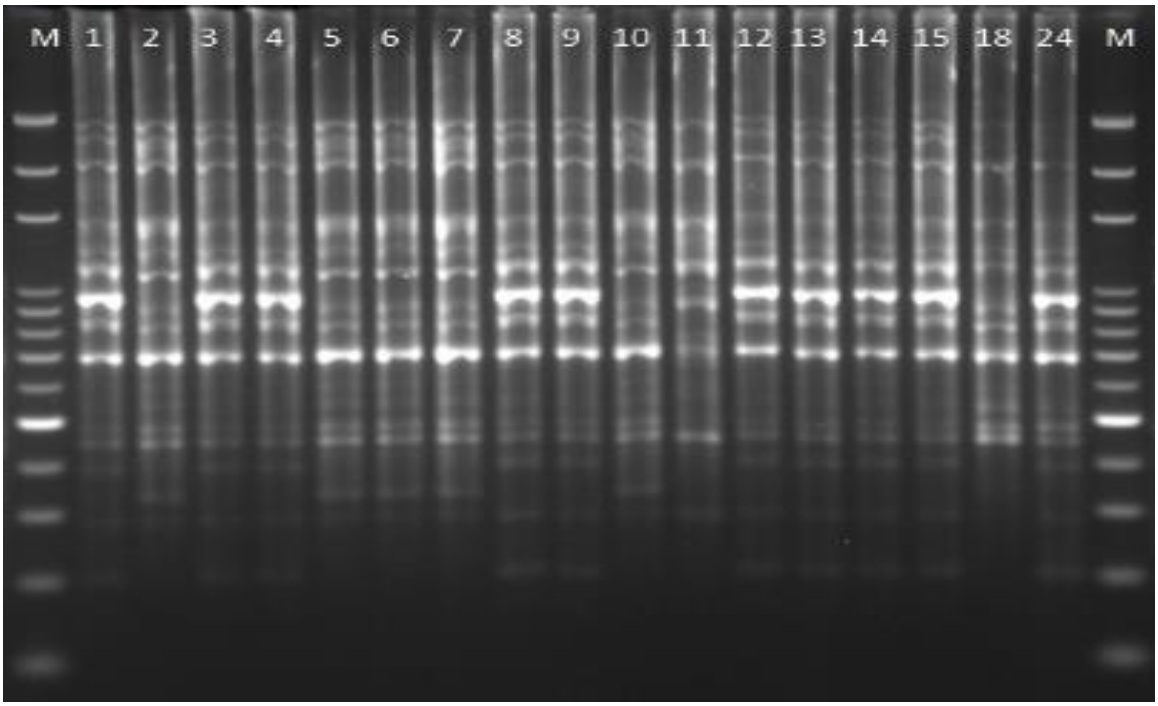

Figure C-20: Rep-PCR products for salmonellae positive isolates from Fish #1 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

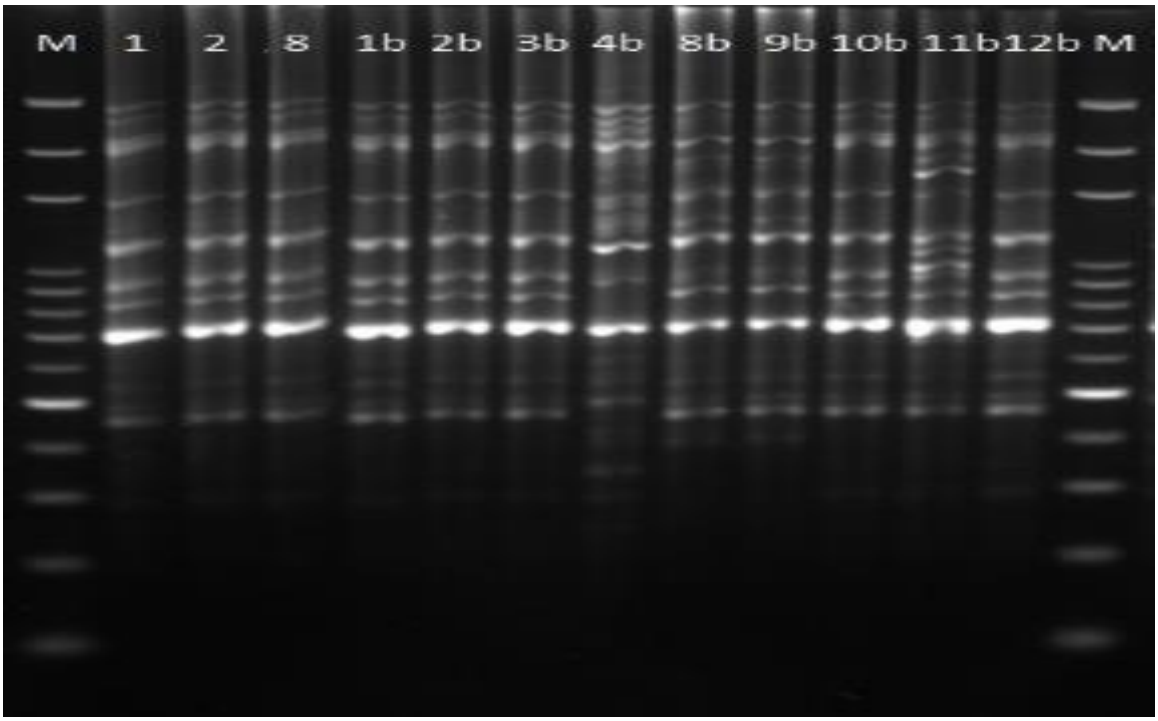

Figure C-21: Rep-PCR products for salmonellae positive isolates from Fish #2 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

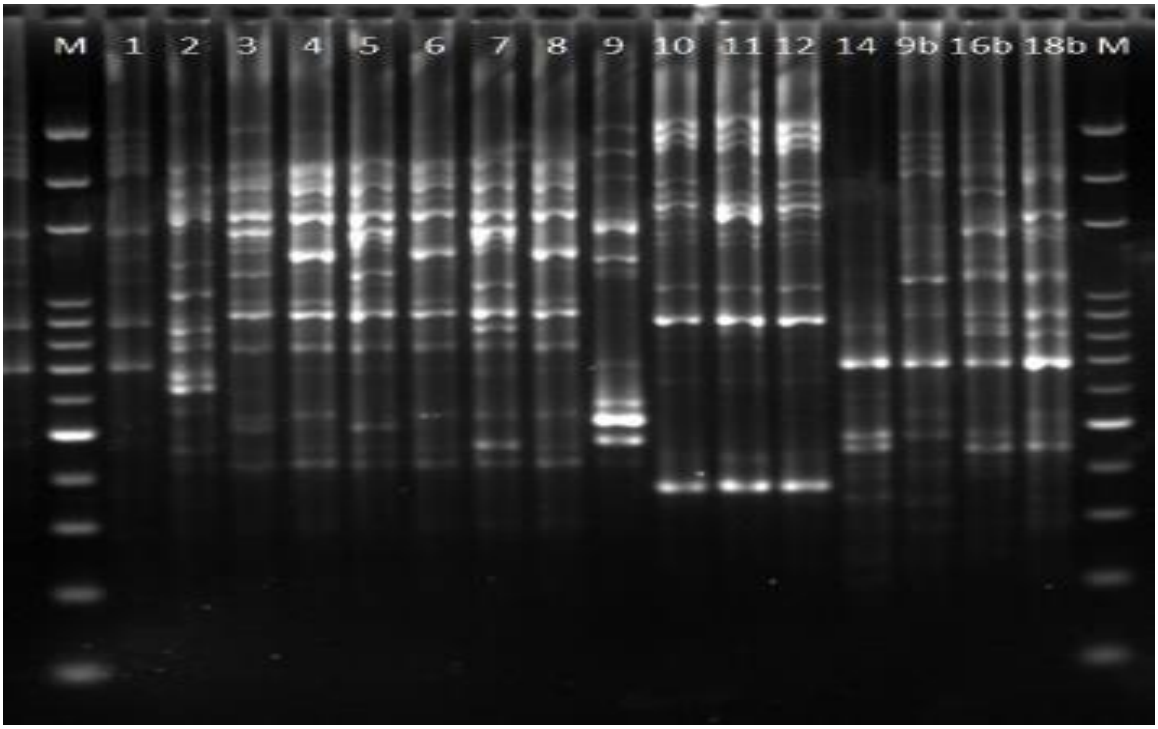

Figure C-22: Rep-PCR products for salmonellae positive isolates from Fish #3 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

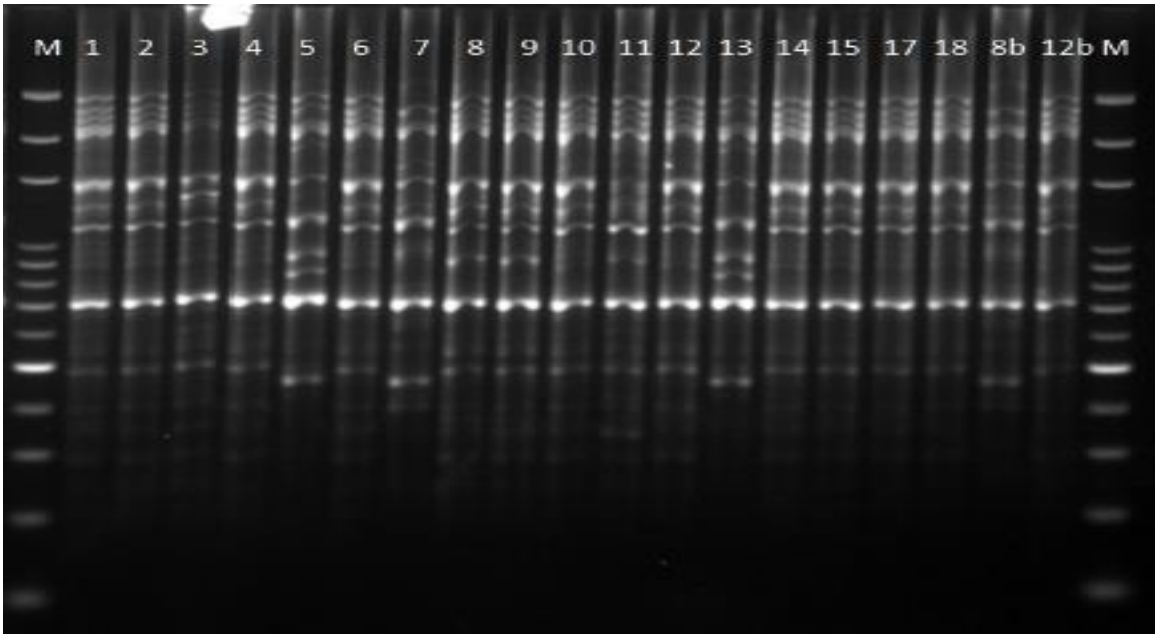

Figure C-23: Rep-PCR products for salmonellae positive isolates from Fish #4 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

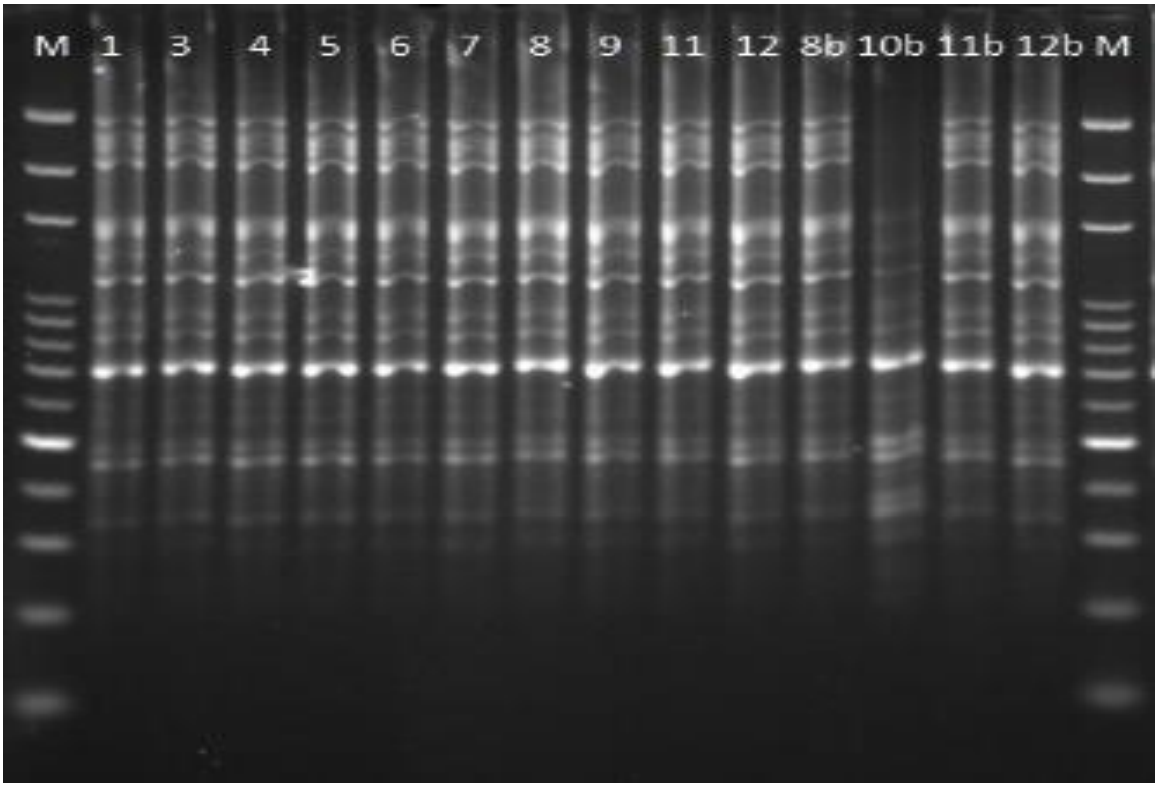

Figure C-24: Rep-PCR products for salmonellae positive isolates from Fish #5 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

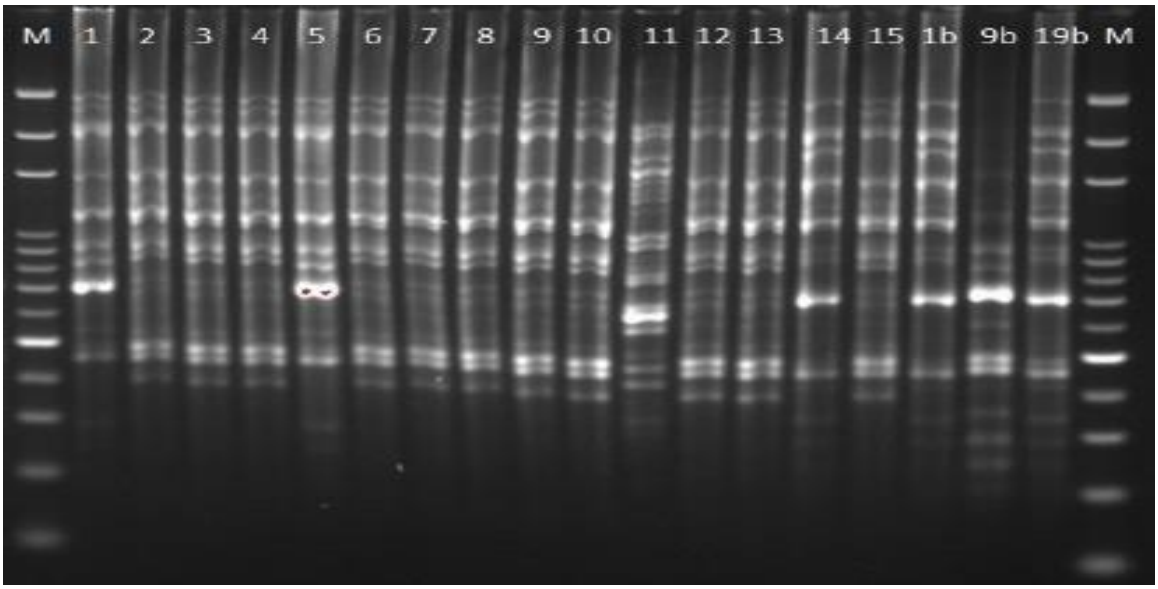

Figure C-25: Rep-PCR products for salmonellae positive isolates from Fish #6 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

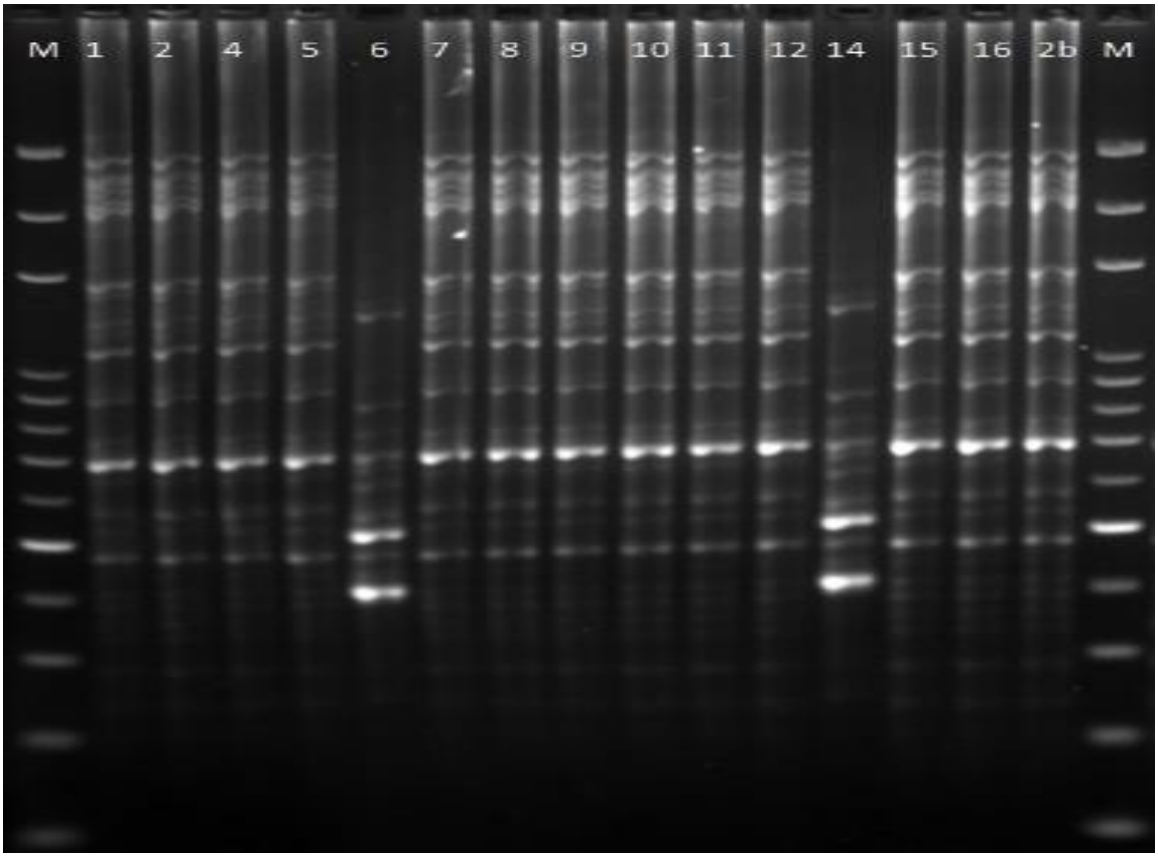

Figure C-26: Rep-PCR products for salmonellae positive isolates from Fish #7 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

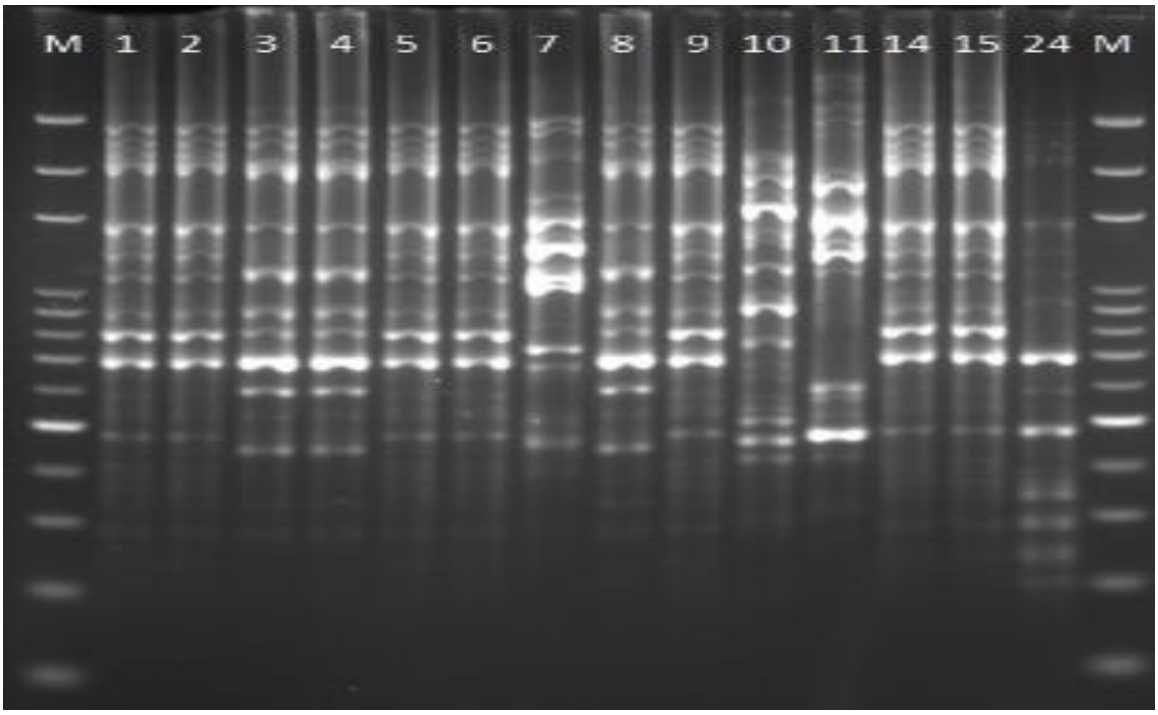

Figure C-27: Rep-PCR products for salmonellae positive isolates from Fish #8 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

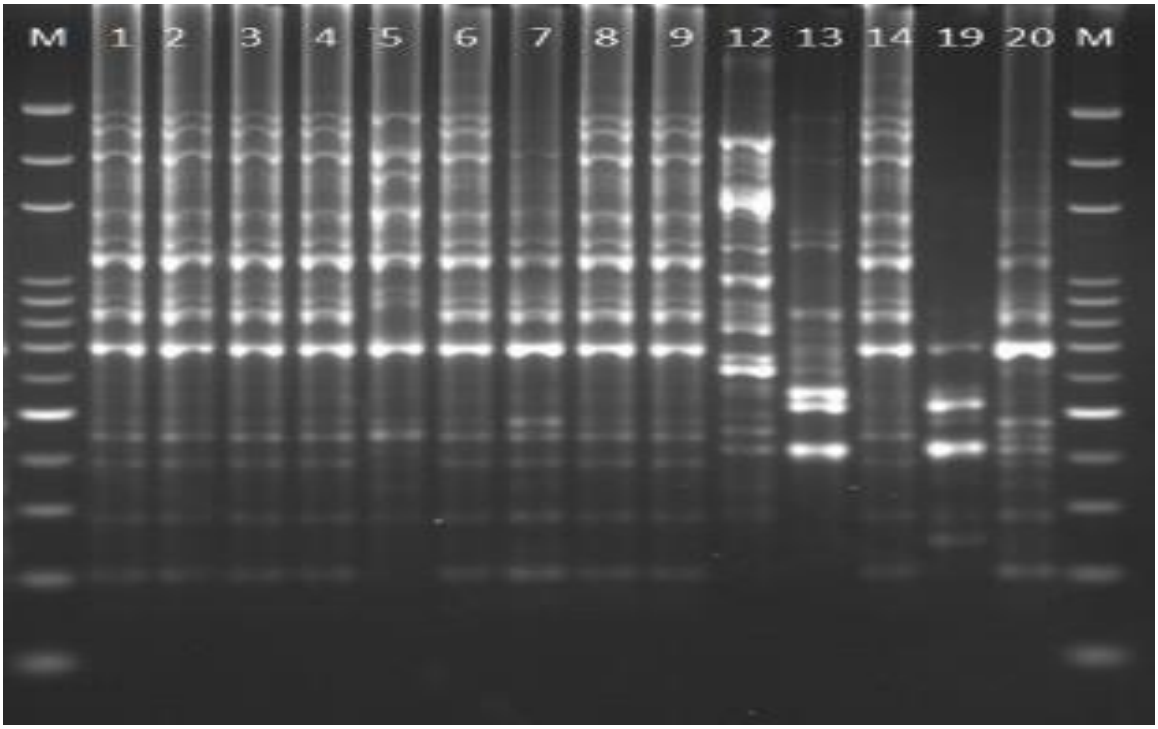

Figure C-28: Rep-PCR products for salmonellae positive isolates from Fish #9 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

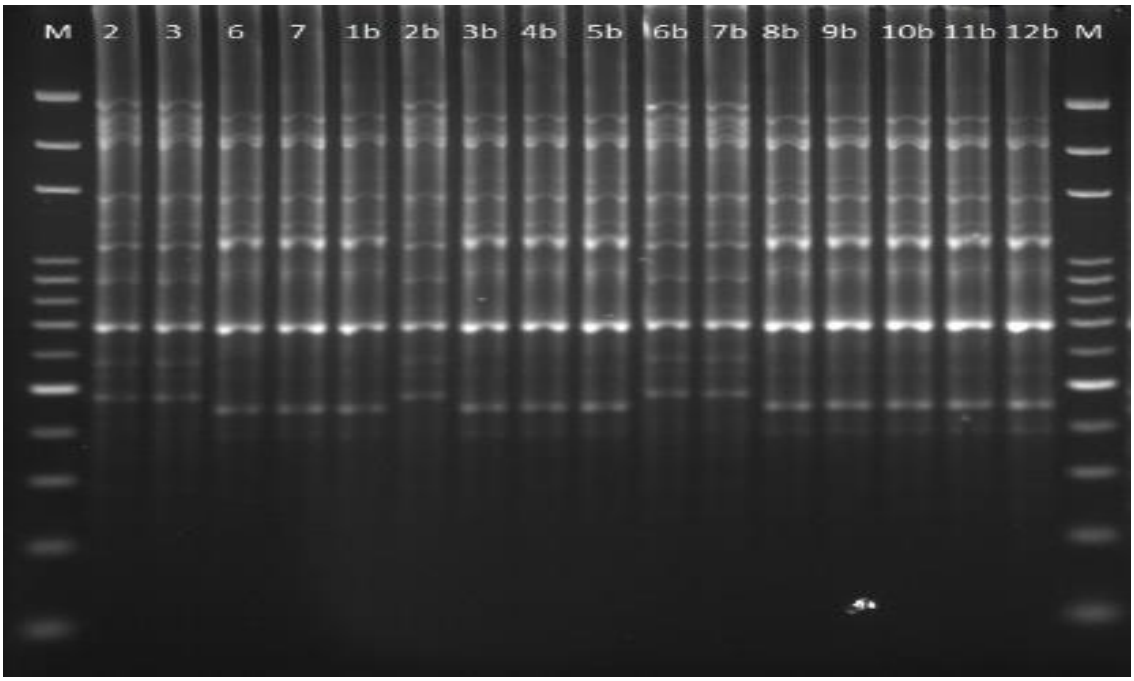

Figure C-29: Rep-PCR products for salmonellae positive isolates from Fish #10 collected September 8, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

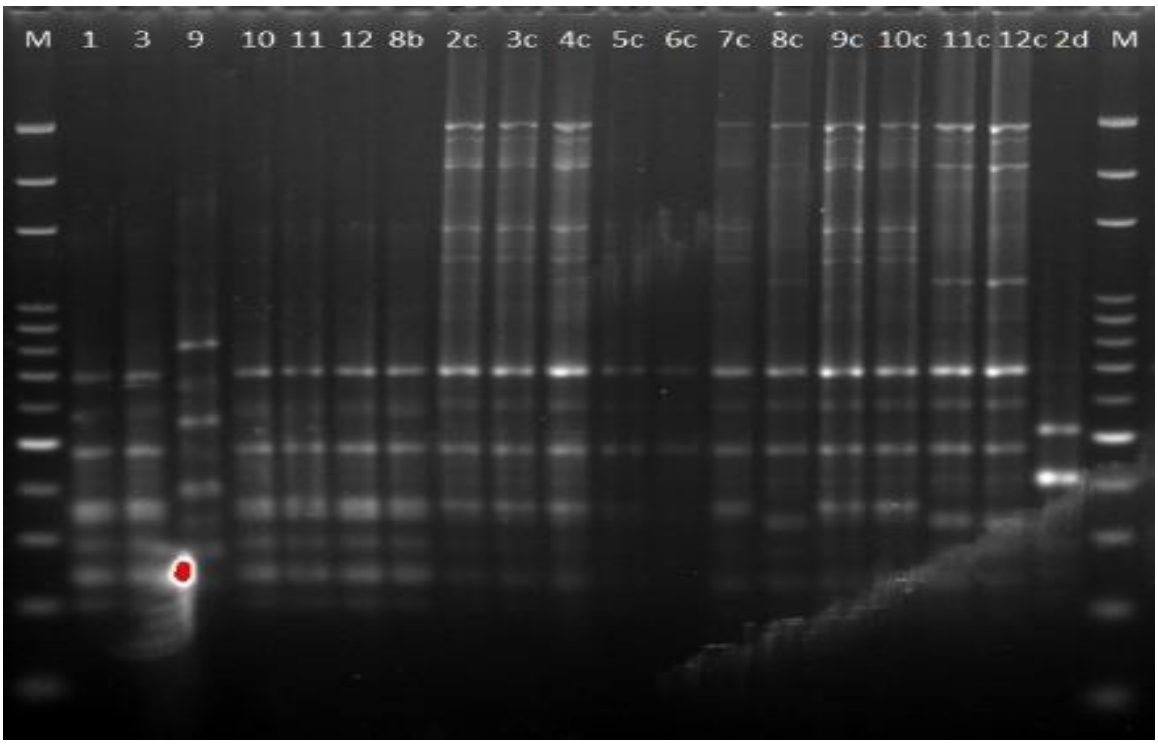

Figure C-30: Rep-PCR products for salmonellae positive isolates from Fish #6 collected October 3, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

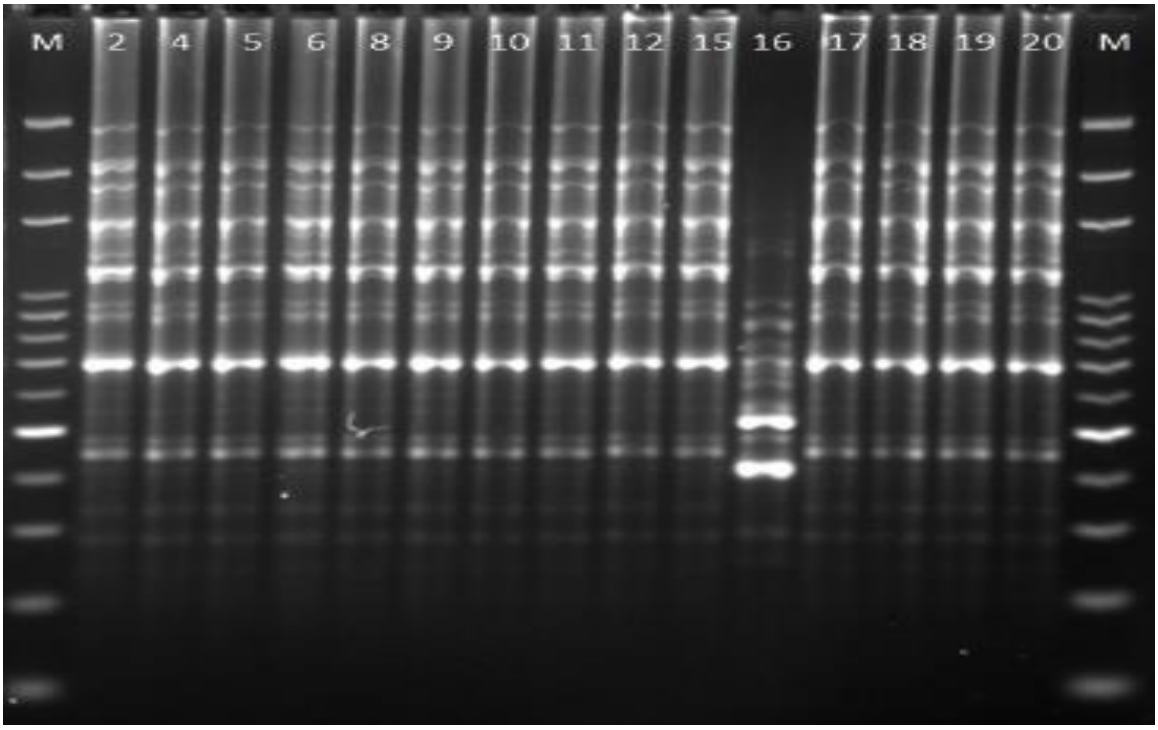

Figure C-31: Rep-PCR products for salmonellae positive isolates from sediment collected October 3, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

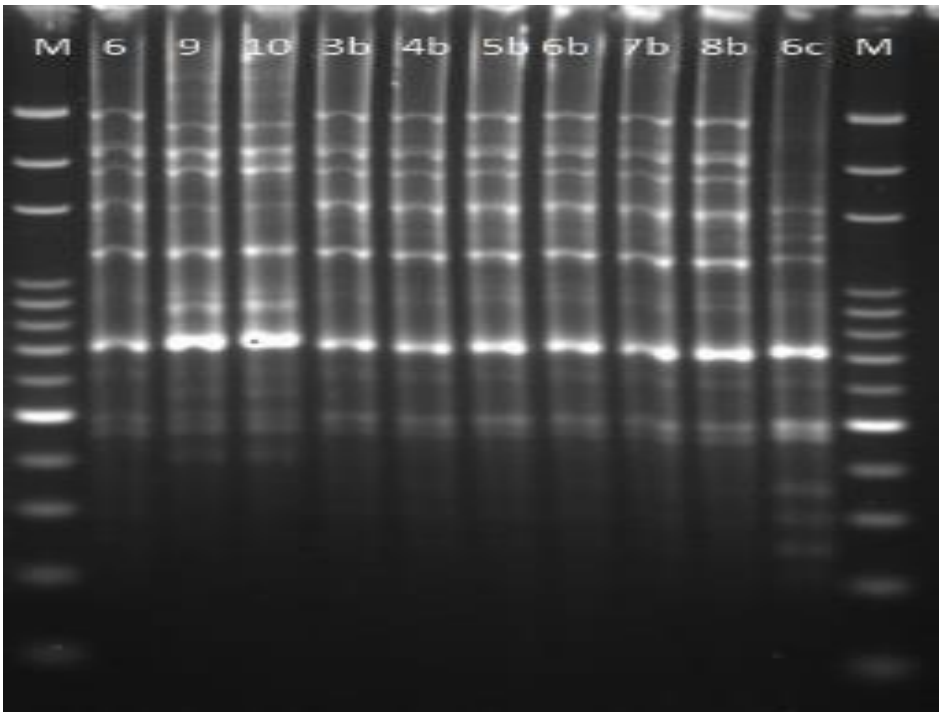

Figure C-32: Rep-PCR products for salmonellae positive isolates from Fish #1 collected October 21, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

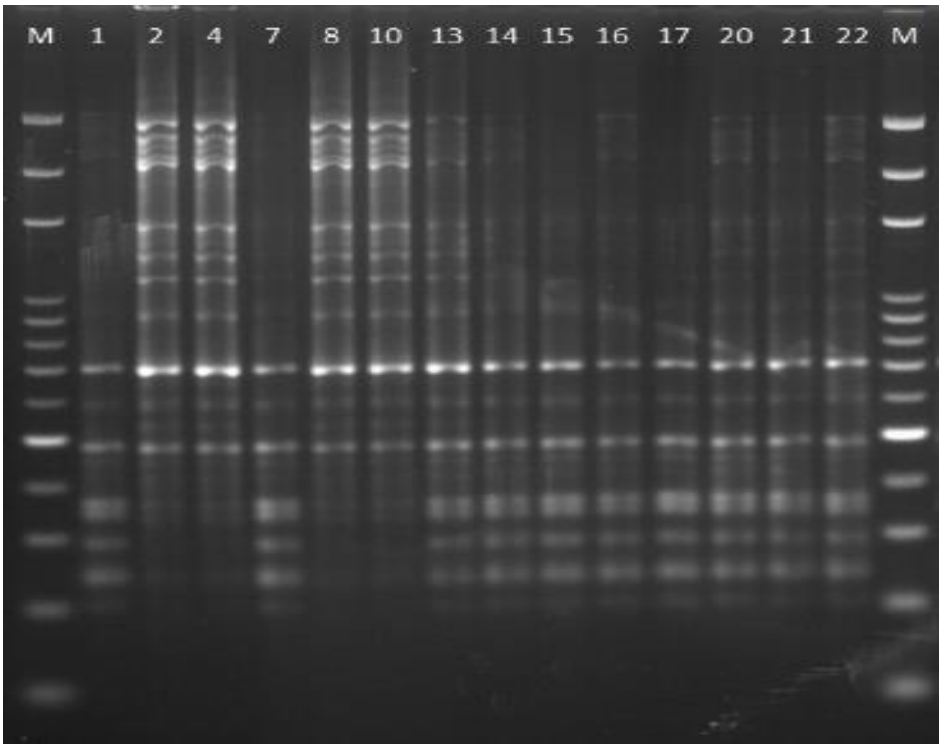

Figure C-33: Rep-PCR products for salmonellae positive isolates from Fish #3 collected October 21, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

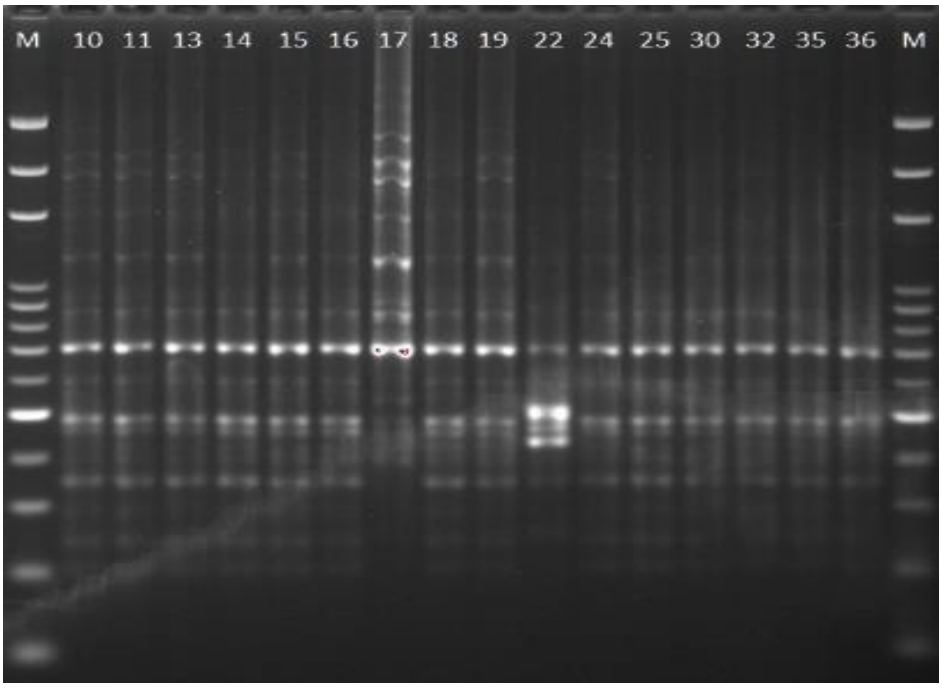

Figure C-34: Rep-PCR products for salmonellae positive isolates from Fish #5 collected October 21, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

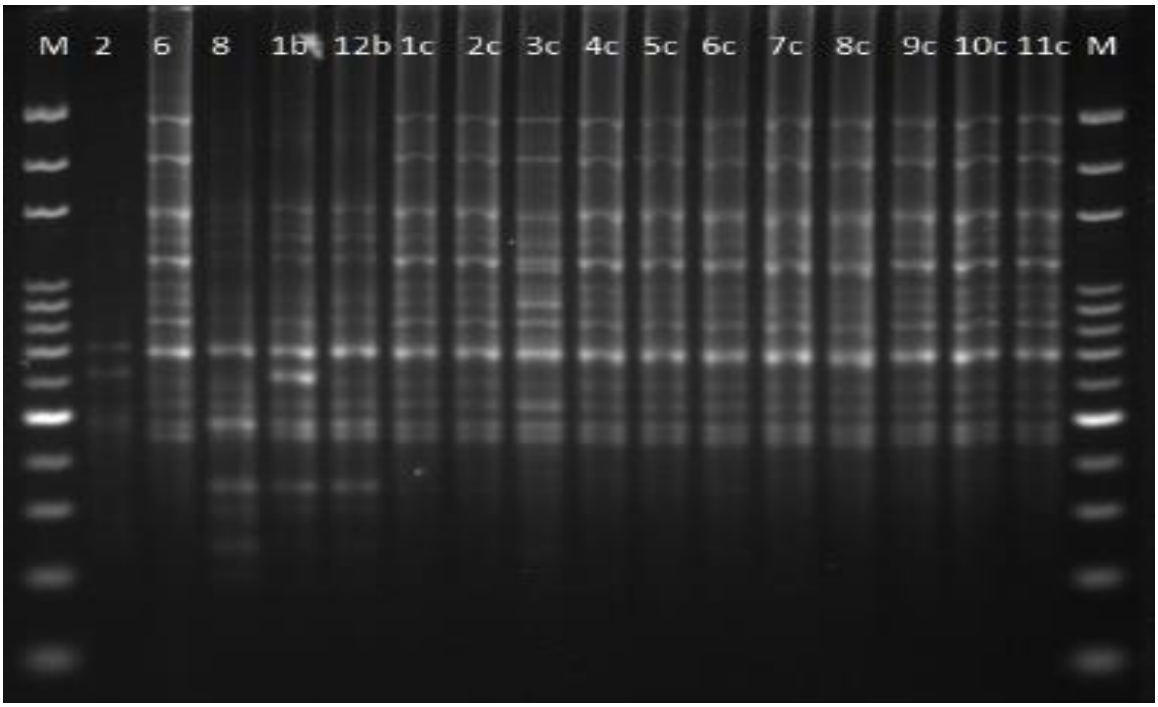

Figure C-35: Rep-PCR products for salmonellae positive isolates from Fish #6 collected October 21, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

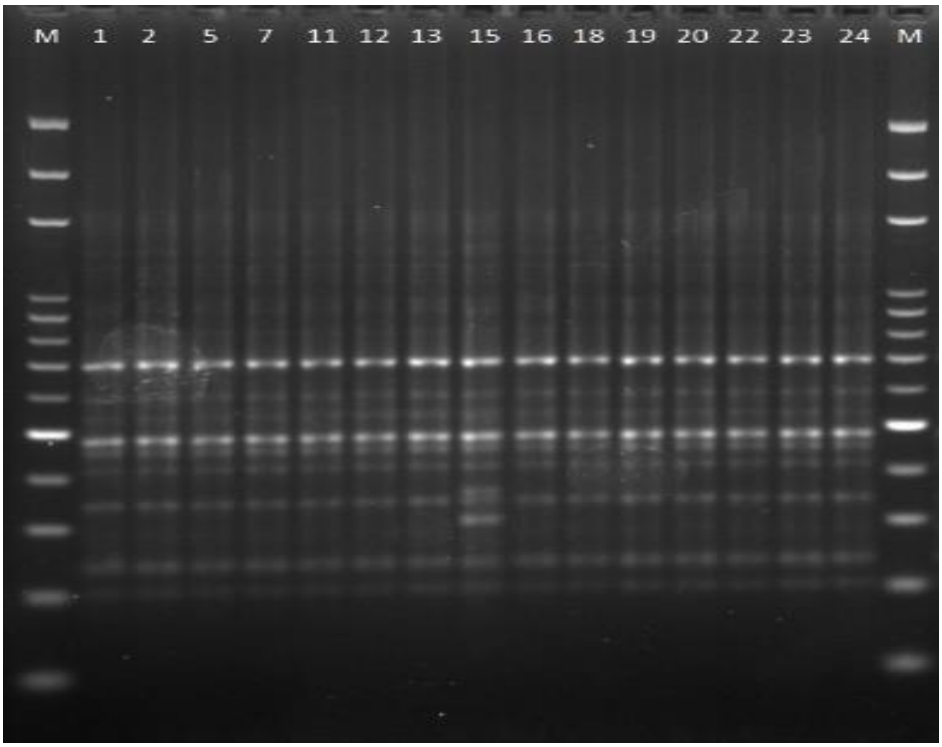

Figure C-36: Rep-PCR products for salmonellae positive isolates from Fish #1 collected October 31, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

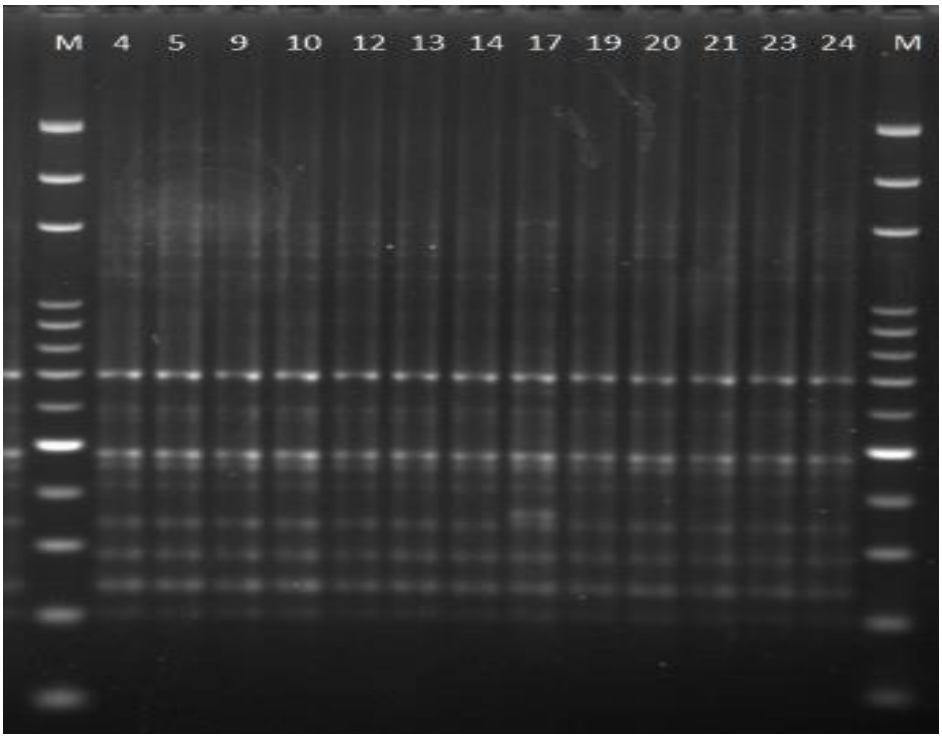

Figure C-37: Rep-PCR products for salmonellae positive isolates from Fish #3 collected October 31, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

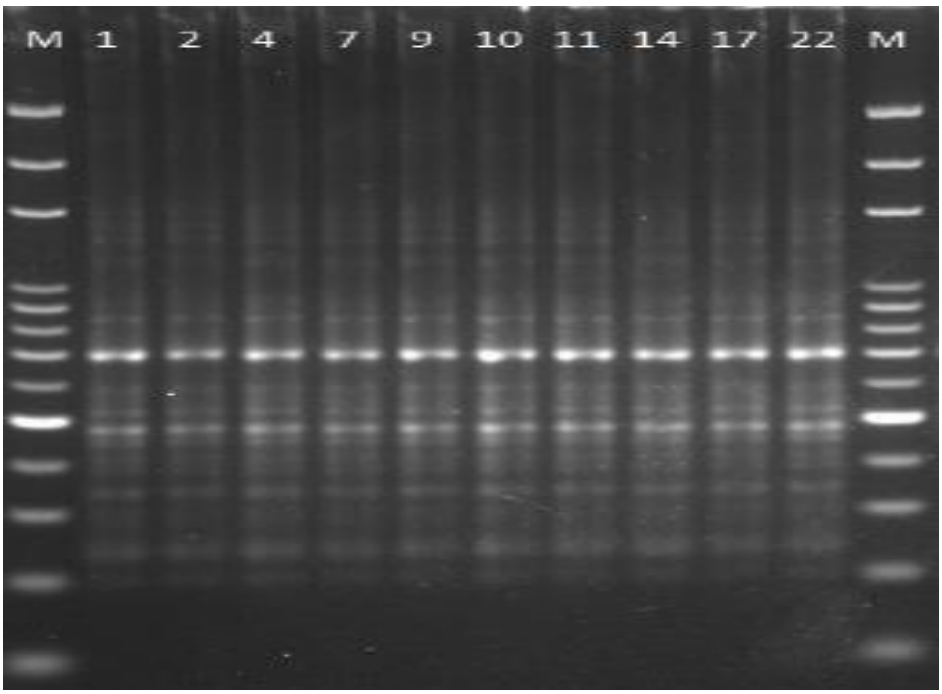

Figure C-38: Rep-PCR products for salmonellae positive isolates from Fish #5 collected October 31, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

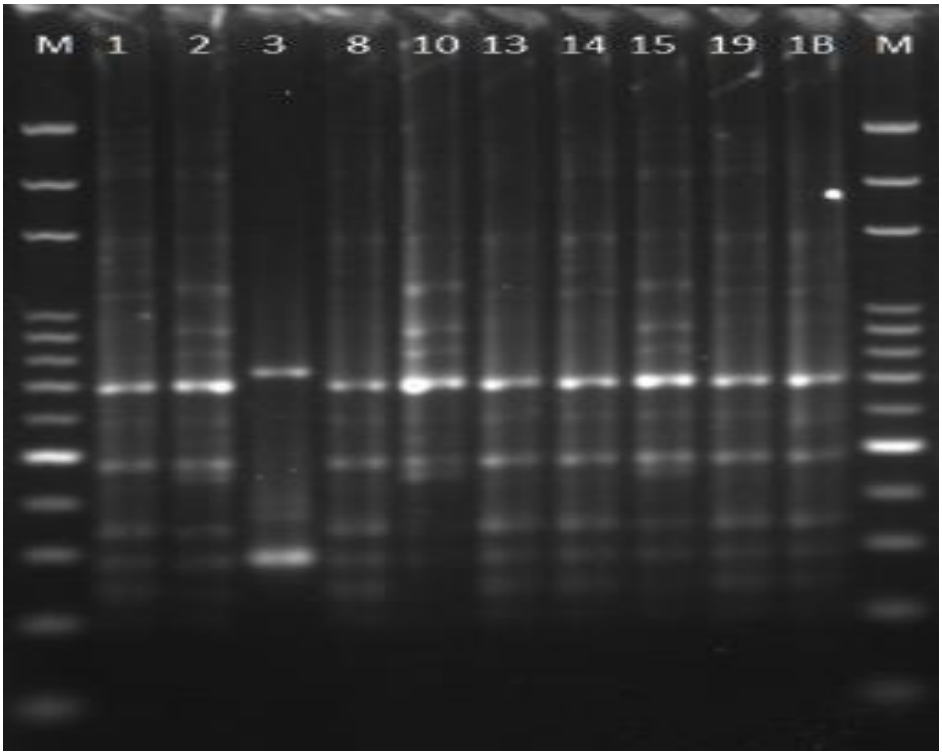

Figure C-39: Rep-PCR products for salmonellae positive isolates from Fish #7 collected October 31, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

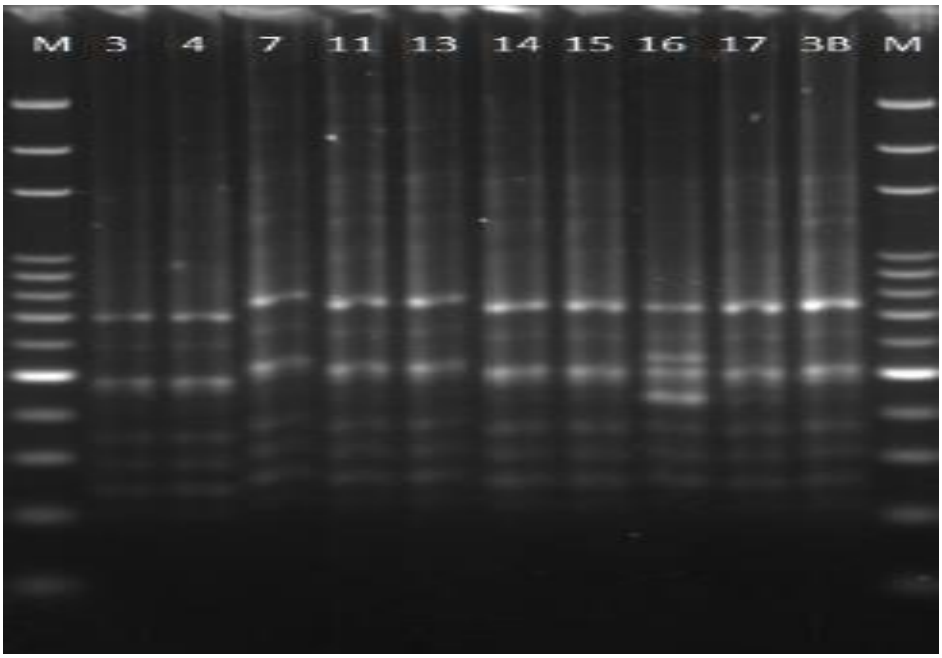

Figure C-40: Rep-PCR products for salmonellae positive isolates from Fish #8 collected October 31, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

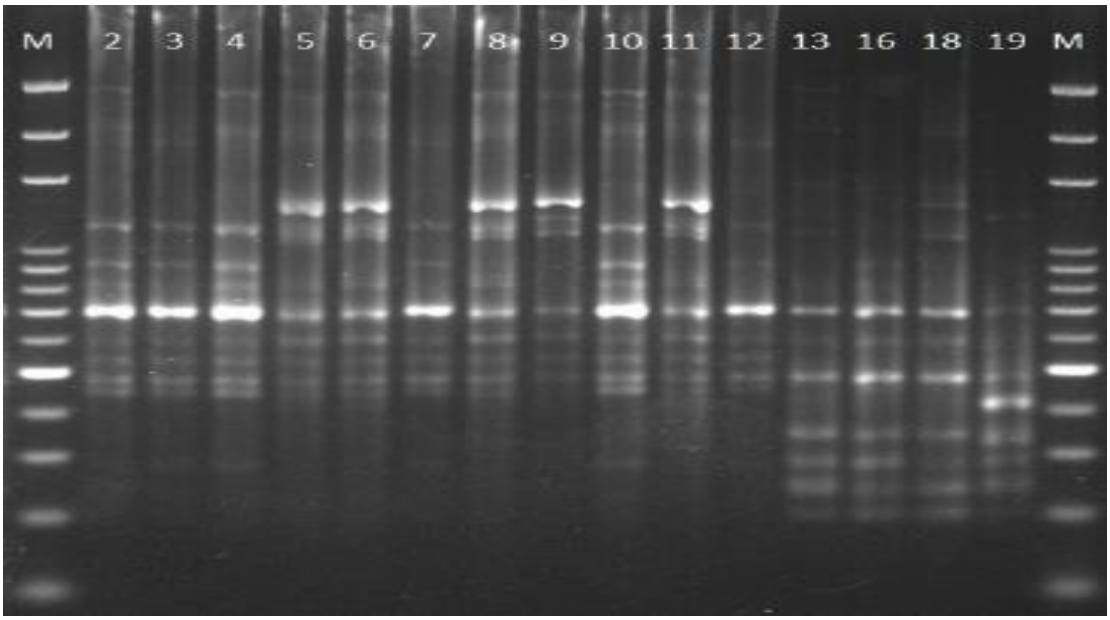

Figure C-41: Rep-PCR products for salmonellae positive isolates from Fish #10 collected October 31, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

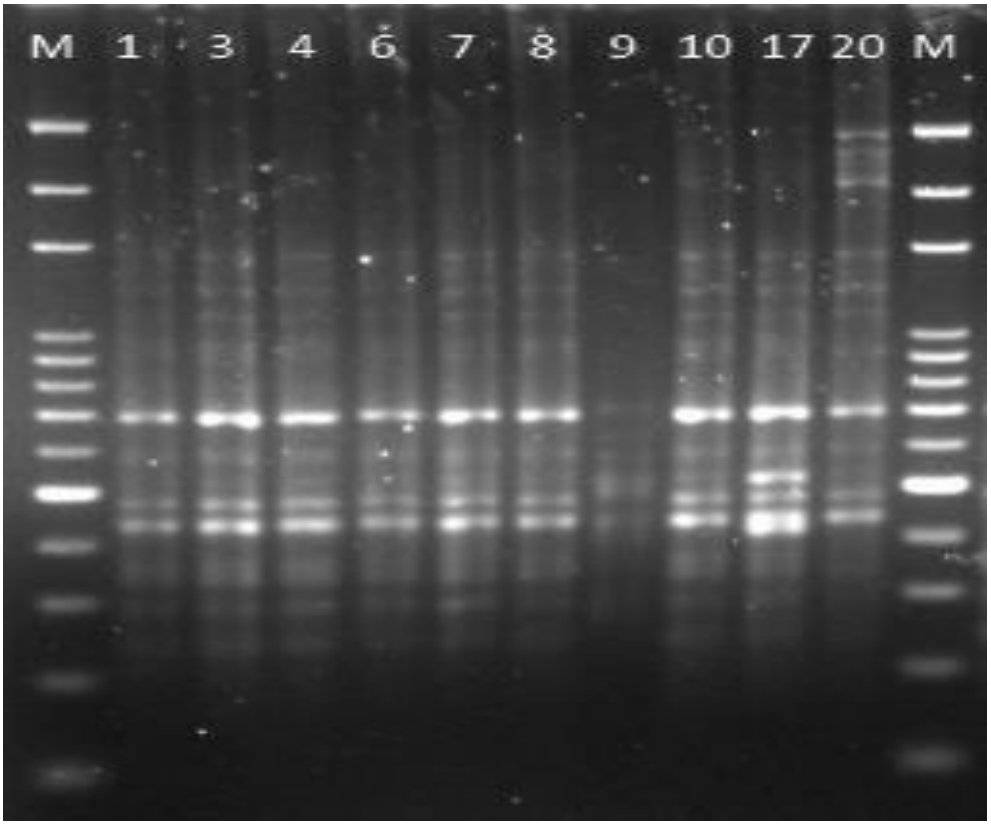

Figure C-42: Rep-PCR products for salmonellae positive isolates from Fish #2 collected November 10, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

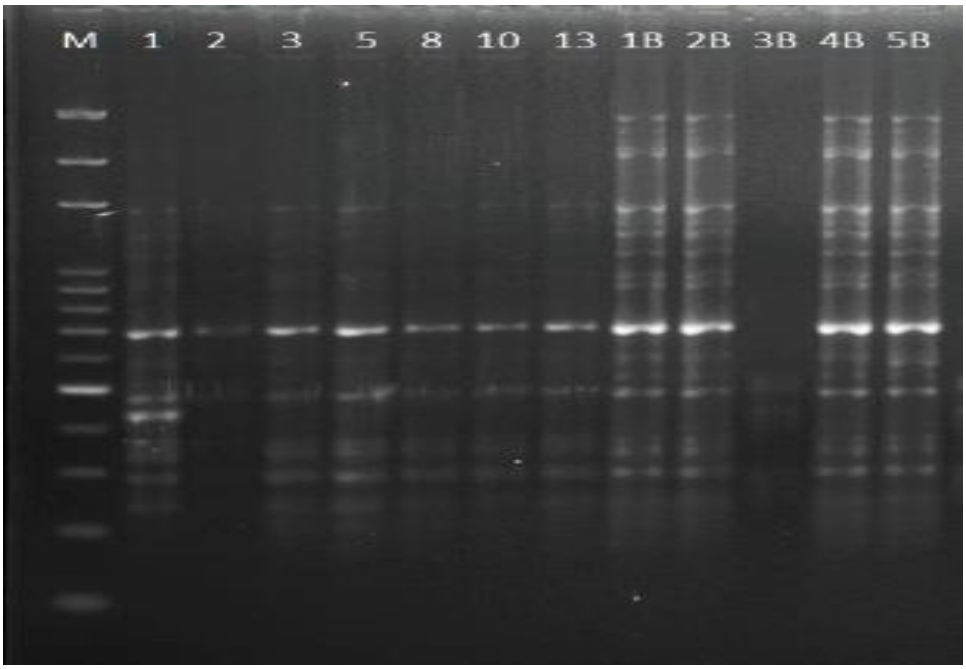

Figure C-43: Rep-PCR products for salmonellae positive isolates from Fish #1 collected December 20, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

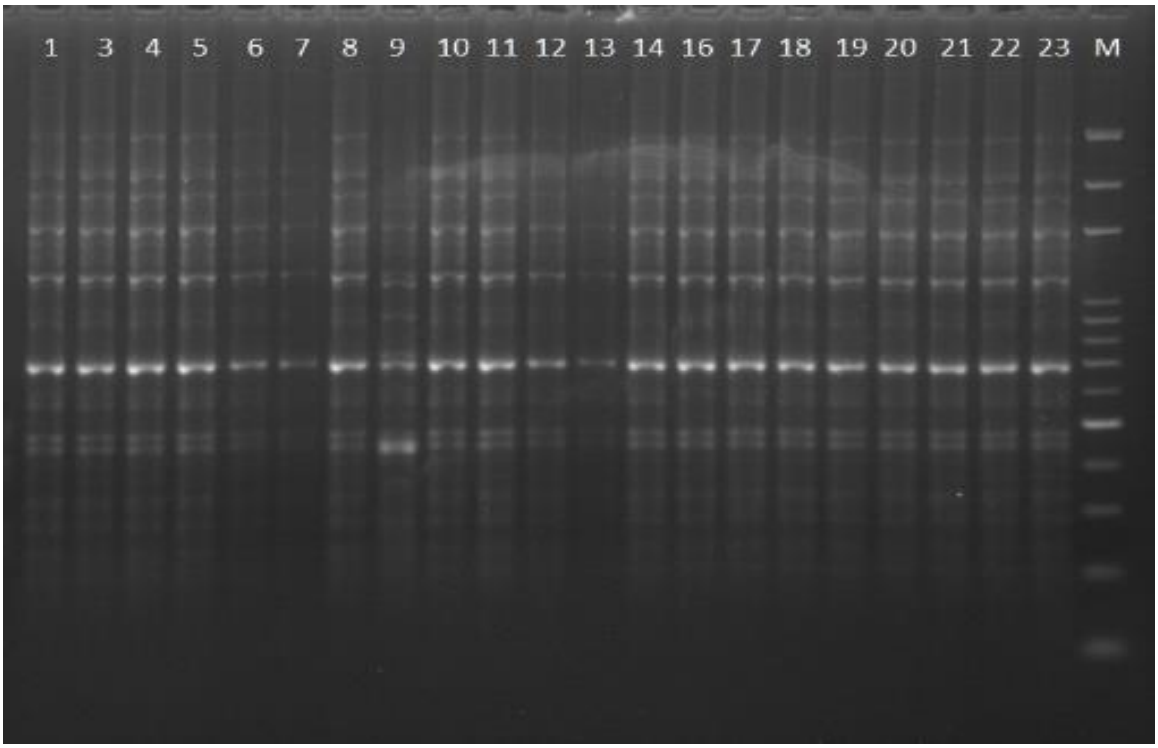

Figure C-44: Rep-PCR products for salmonellae positive isolates from surface water collected December 20, 2014 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

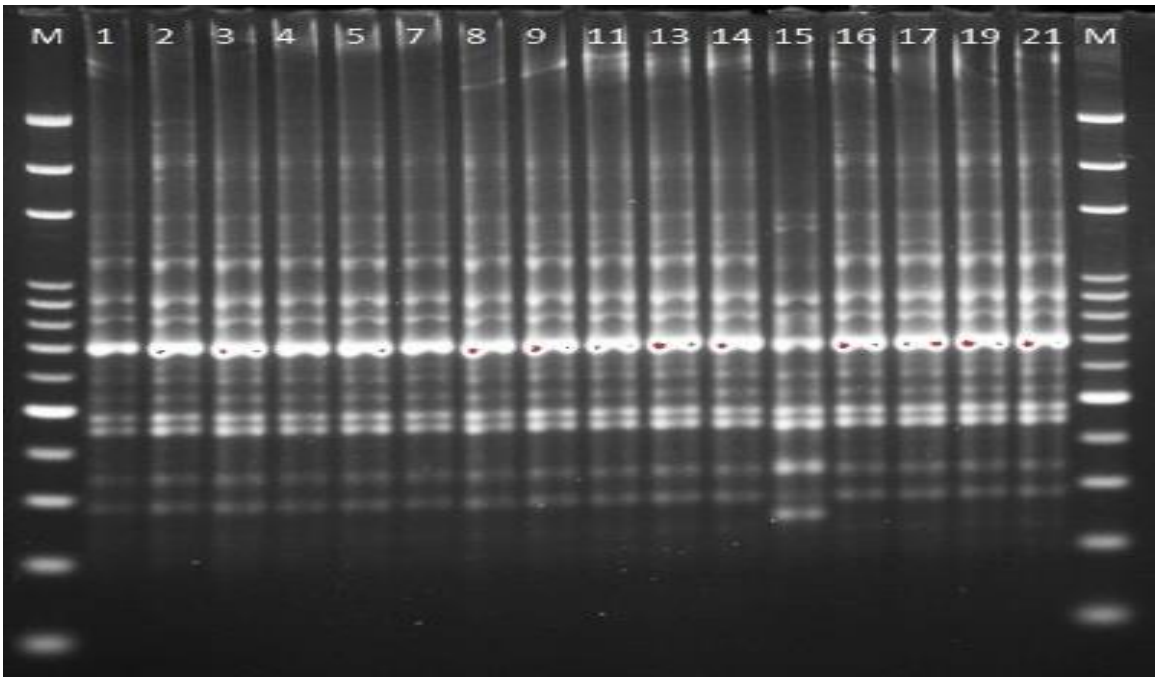

Figure C-45: Rep-PCR products for salmonellae positive isolates from Fish #2 collected January 11, 2015 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

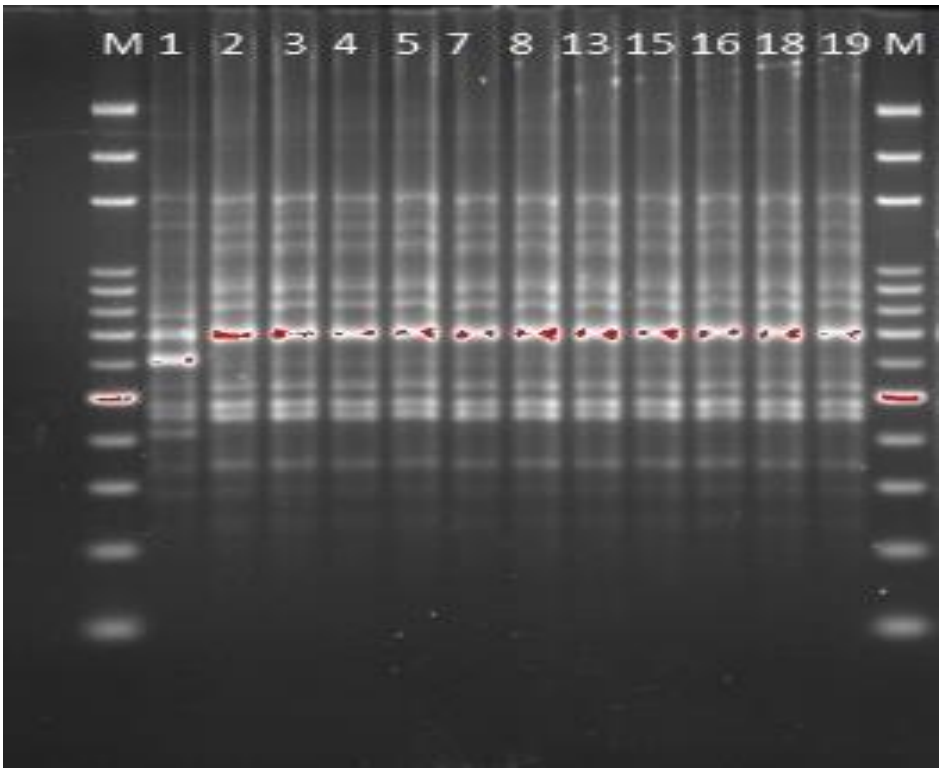

Figure C-46: Rep-PCR products for salmonellae positive isolates from Fish #5 collected January 11, 2015 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

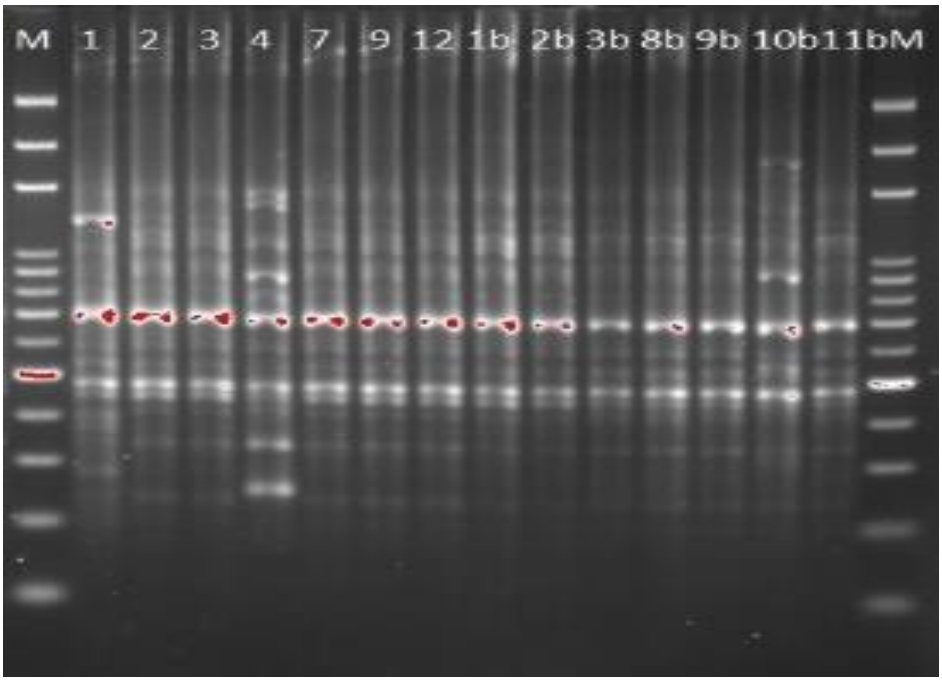

Figure C-47: Rep-PCR products for salmonellae positive isolates from Fish #7 collected January 11, 2015 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

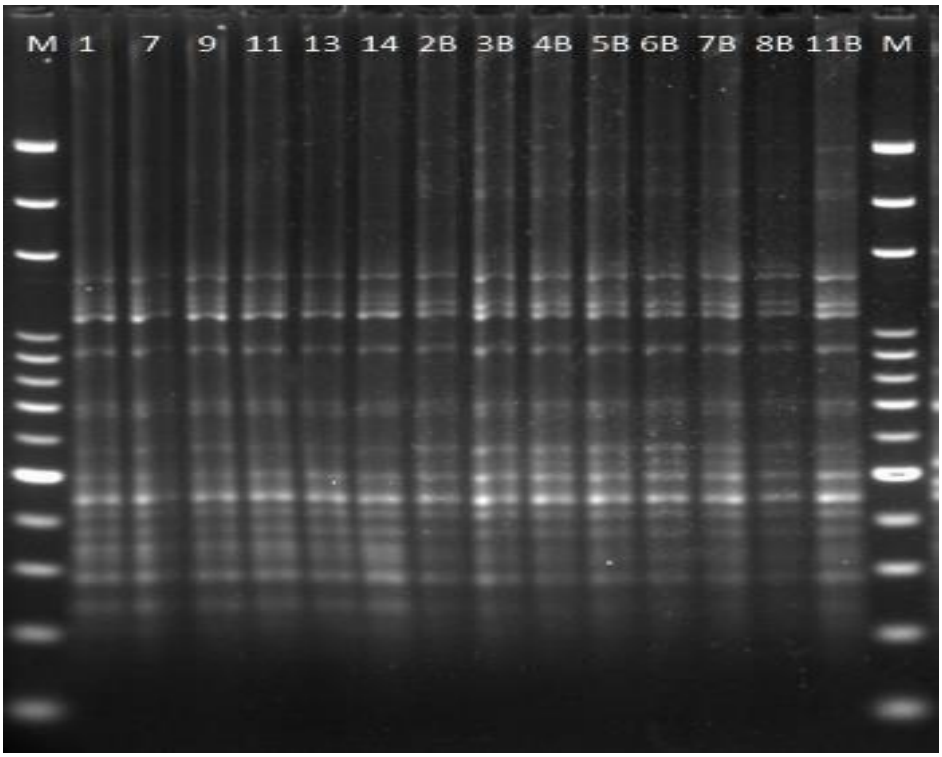

Figure C-48: Rep-PCR products for salmonellae positive isolates from Fish #3 collected January 18, 2015 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

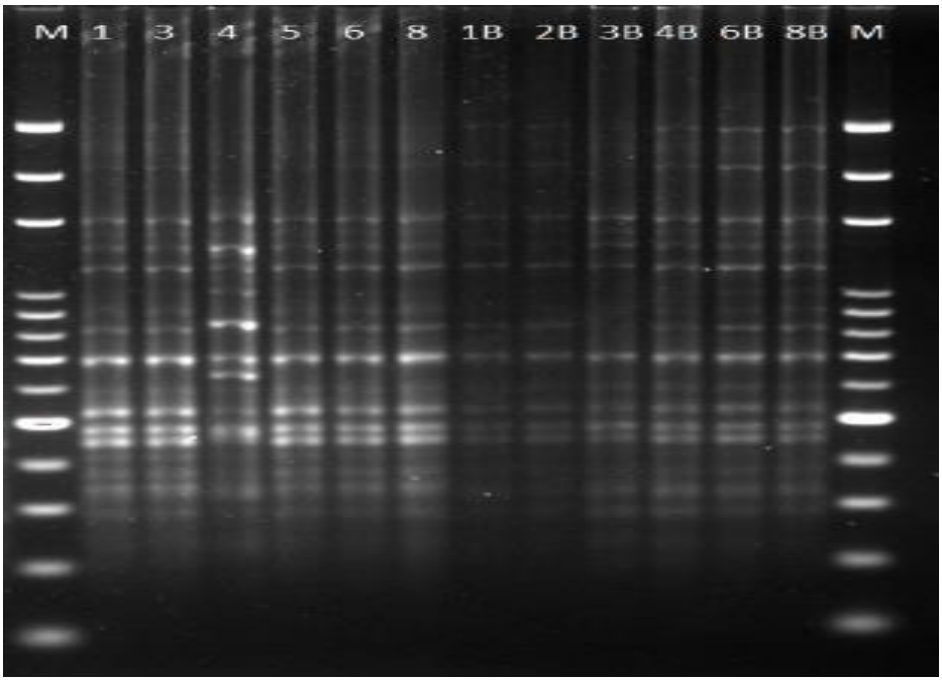

Figure C-49: Rep-PCR products for salmonellae positive isolates from Fish #4 collected January 18, 2015 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

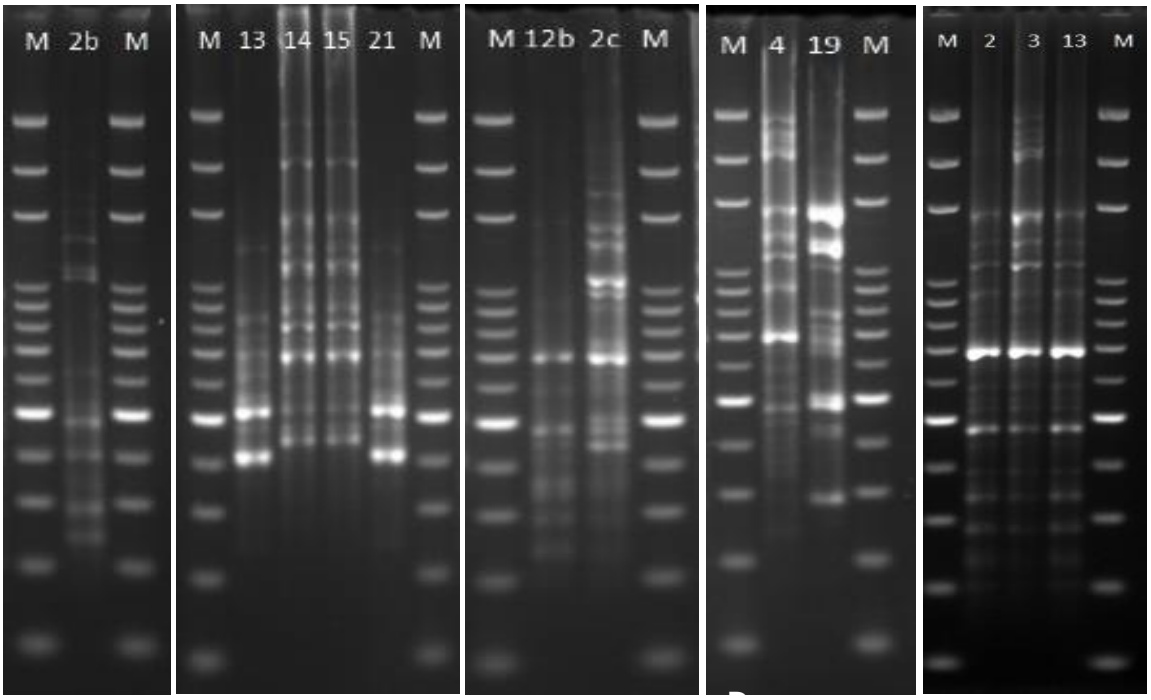

Figure C-50: Rep-PCR products for salmonellae positive isolates from A) Fish#6 collected on April 15, 2014, B)Fish#1 and C)Fish#4 collected on October 3, 2014, D)Fish#7 collected on October 21, 2014, and E)Fish #4 collected January 11, 2015 run on 2% agarose gel with ethidium bromide. (M=100bp ladder, numbers designate colony number).

Appendix D – Rep-PCR analyzed with Agilent Bioanalyzer

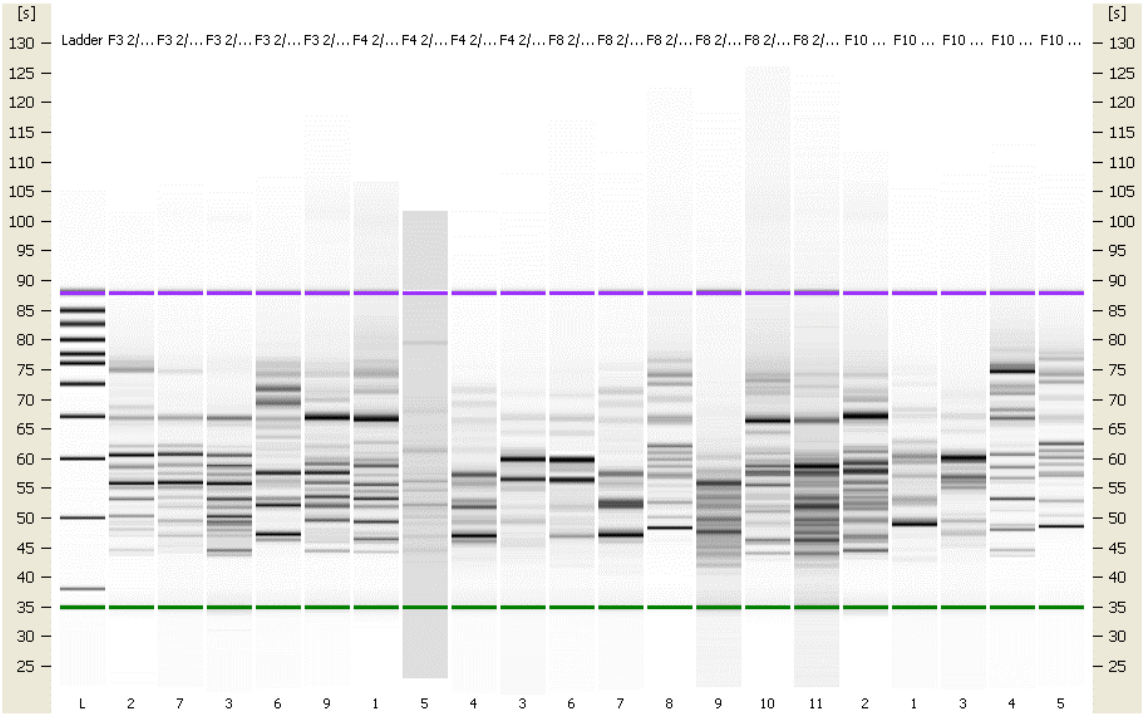

Figure D-1: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on February 18, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

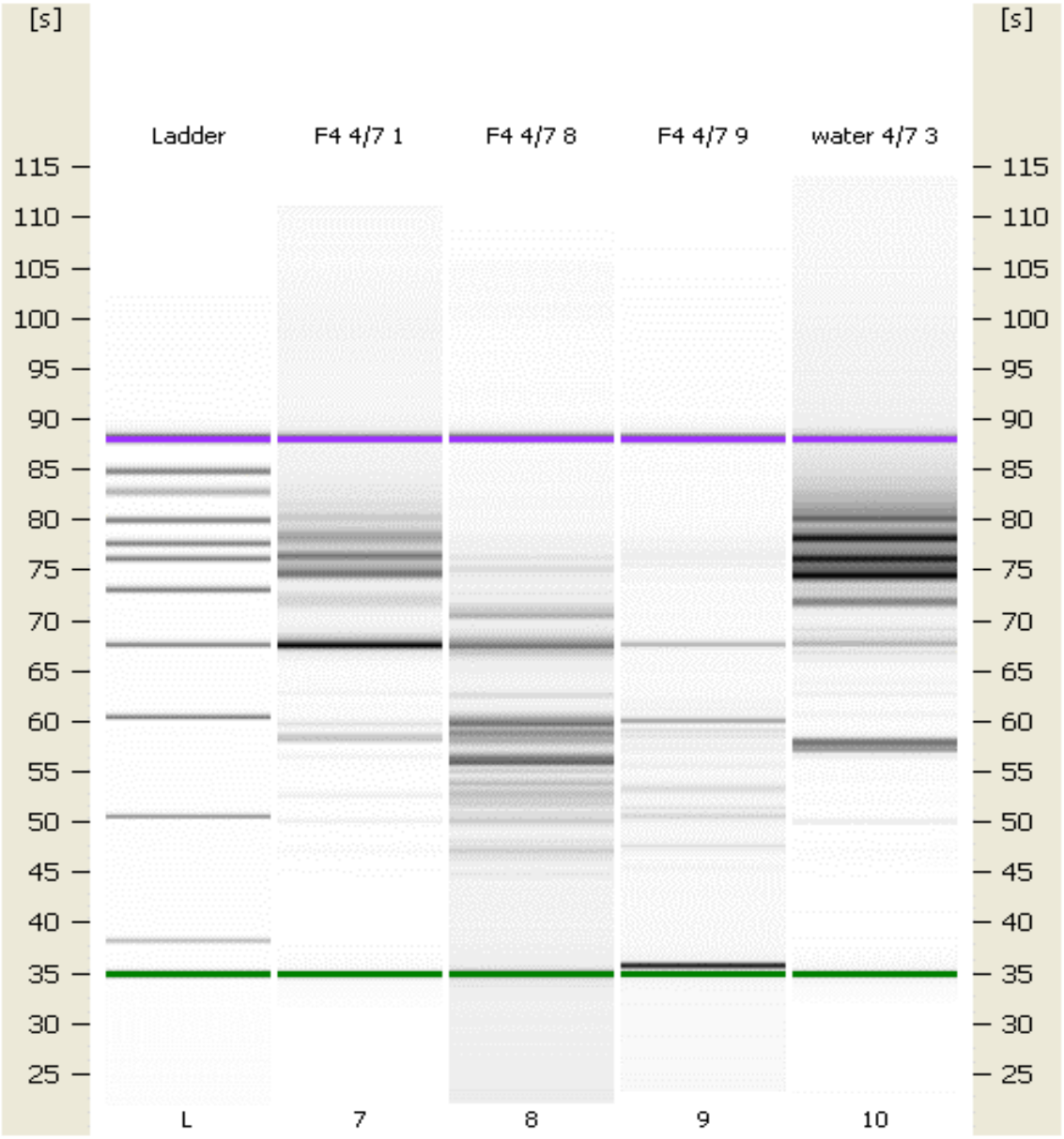

Figure D-2: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* and surface water sampled on April 7, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

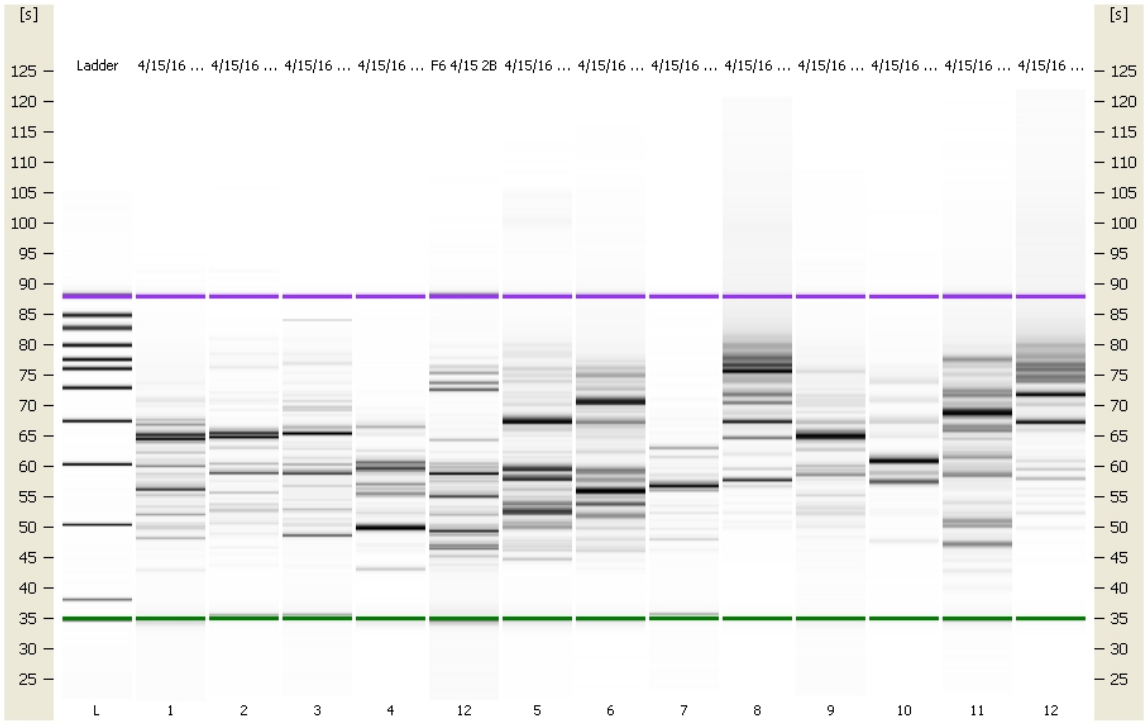

Figure D-3: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on April 15, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

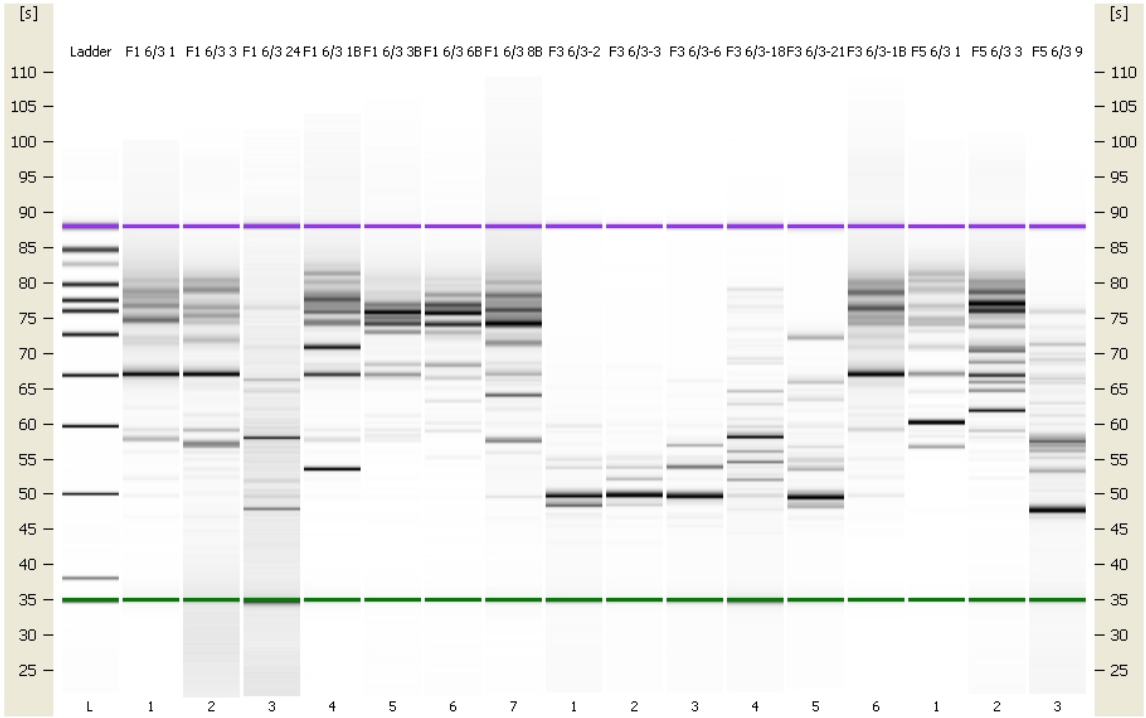

Figure D-4: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on June 3, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

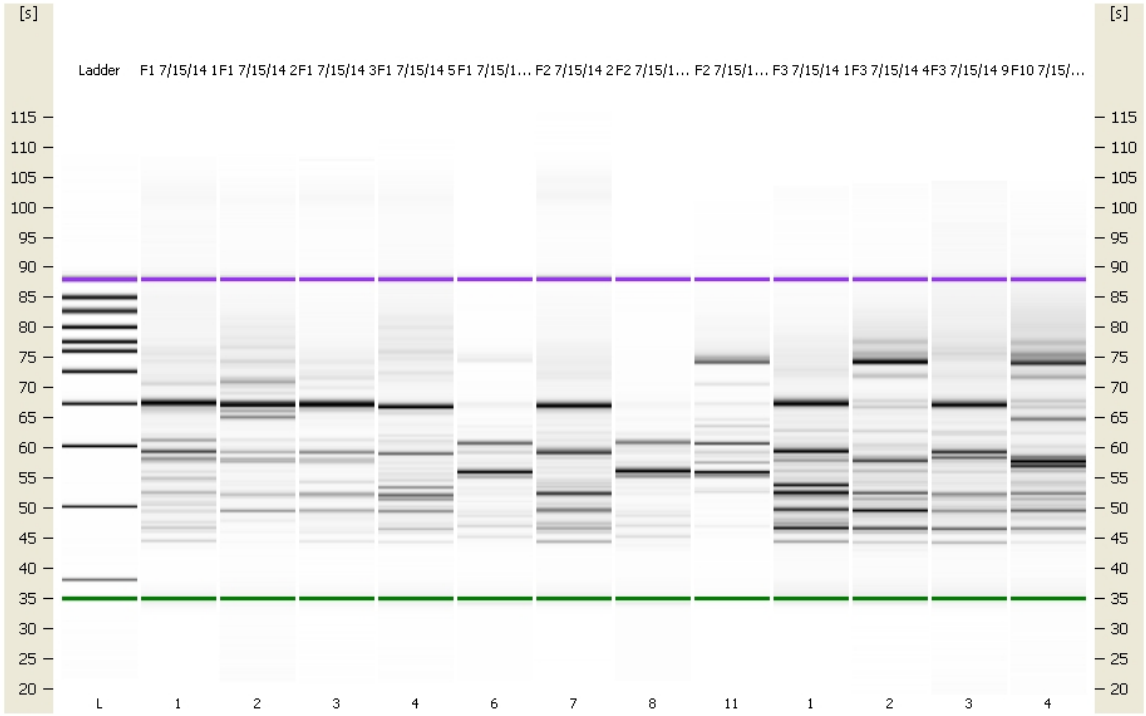

Figure D-5: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on July 15, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

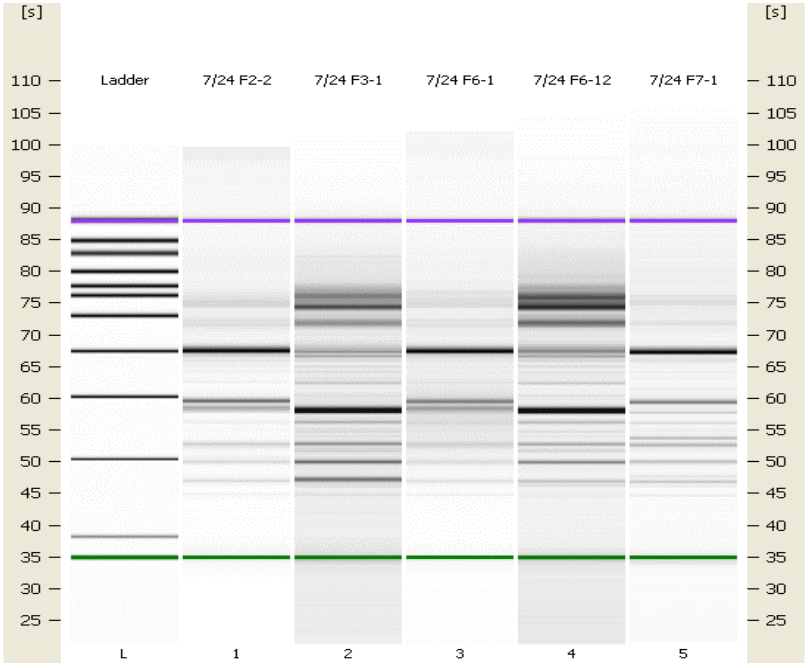

Figure D-6: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on July 24, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

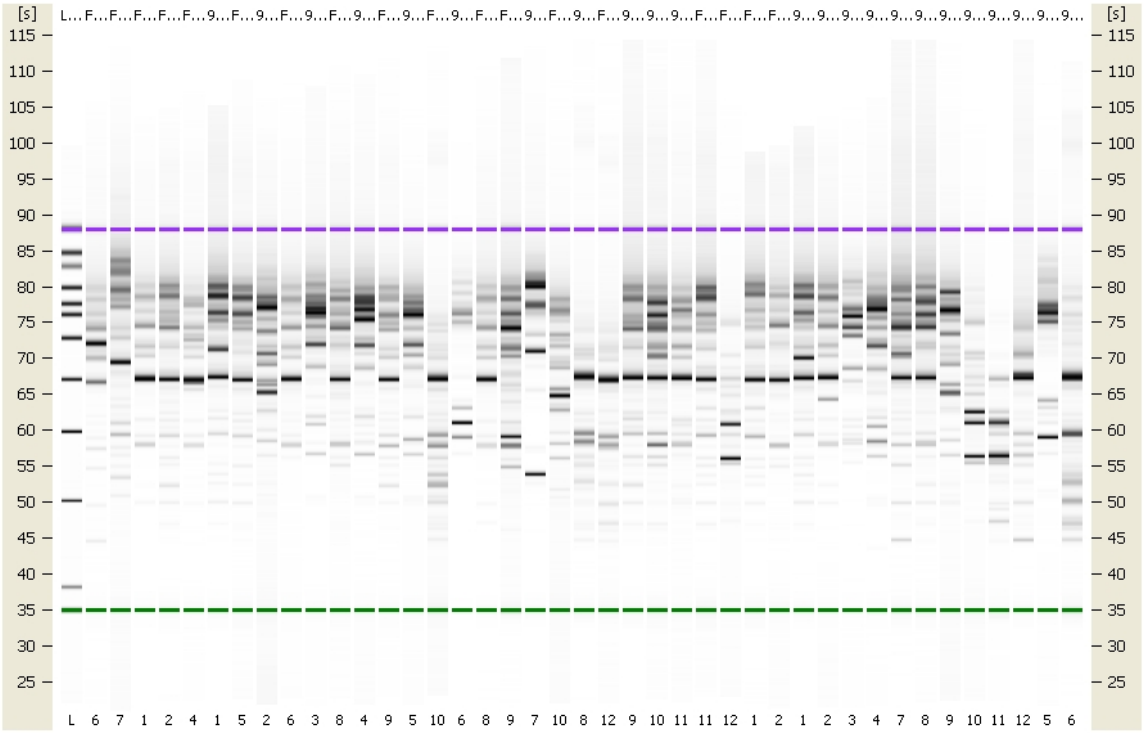

Figure D-7: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on September 8, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

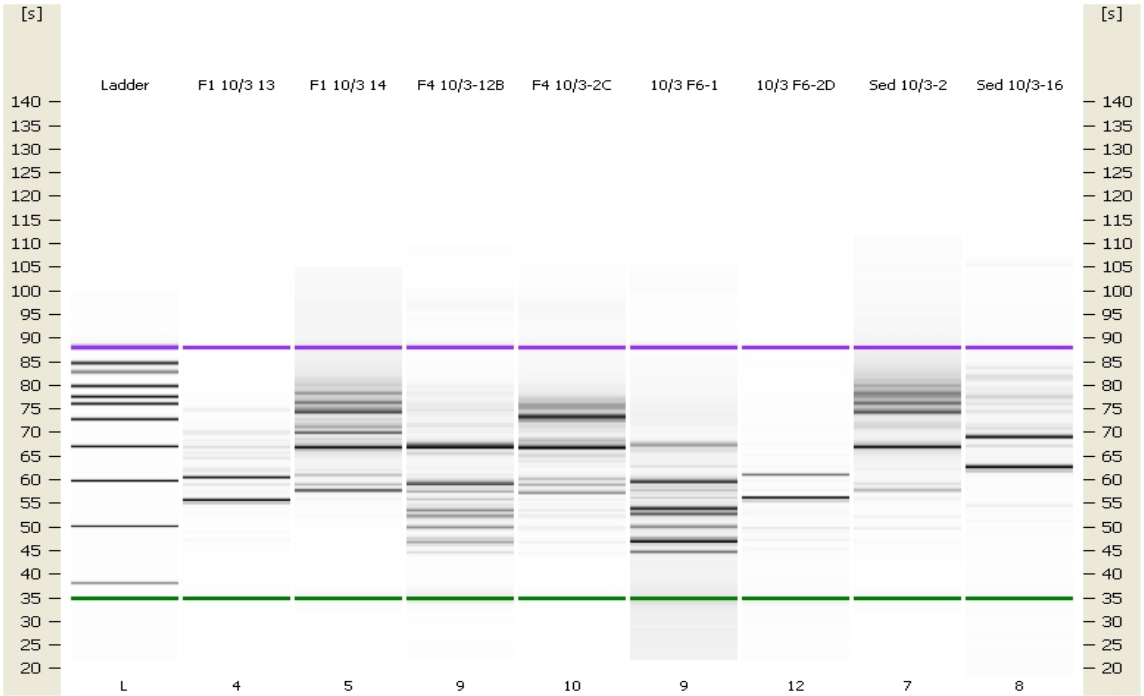

Figure D-8: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* and sediments sampled on October 3, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

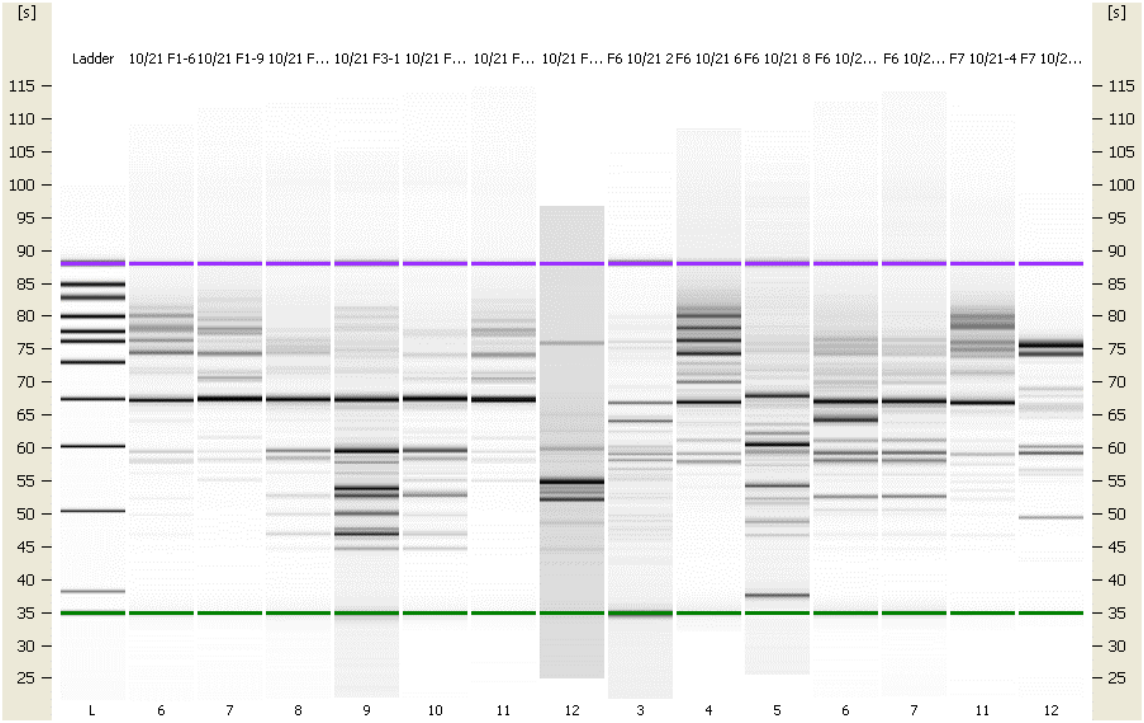

Figure D-9: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* and sampled on October 21, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

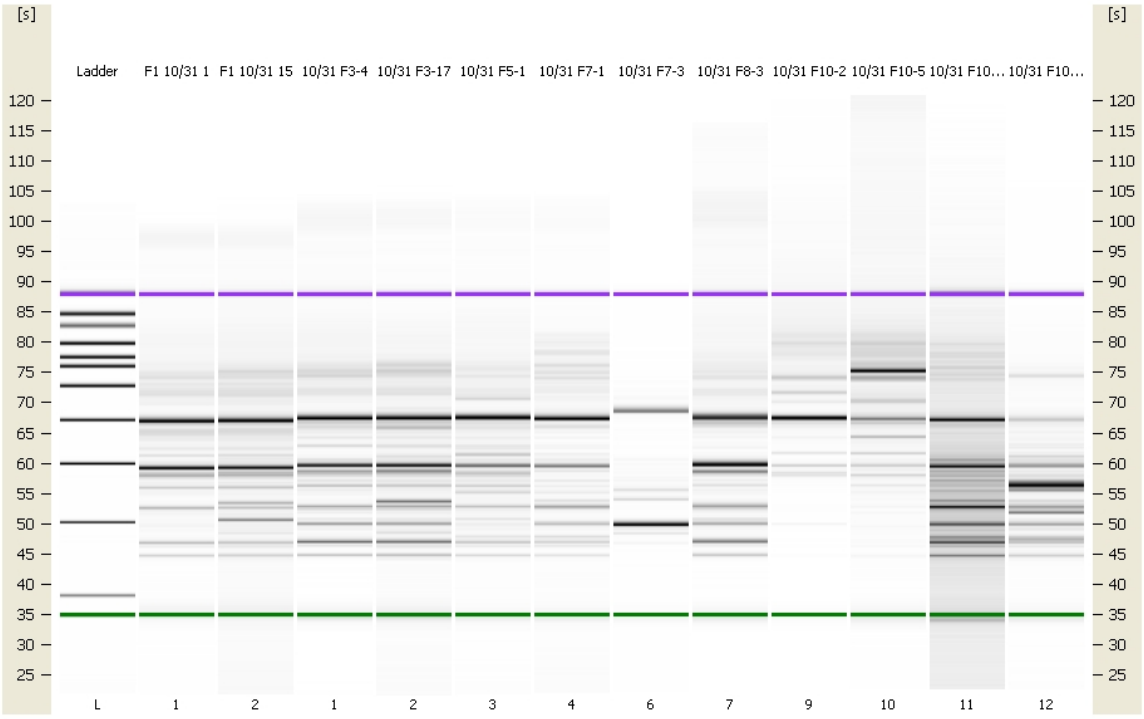

Figure D-10: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on October 31, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

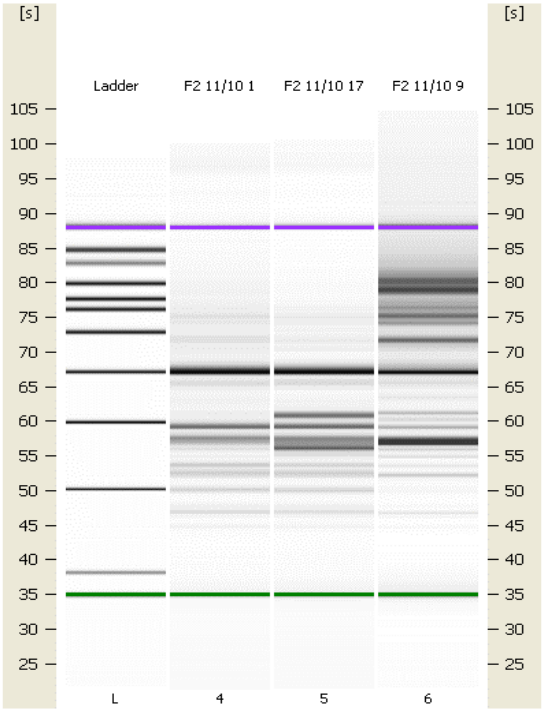

Figure D-11: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on November 10, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

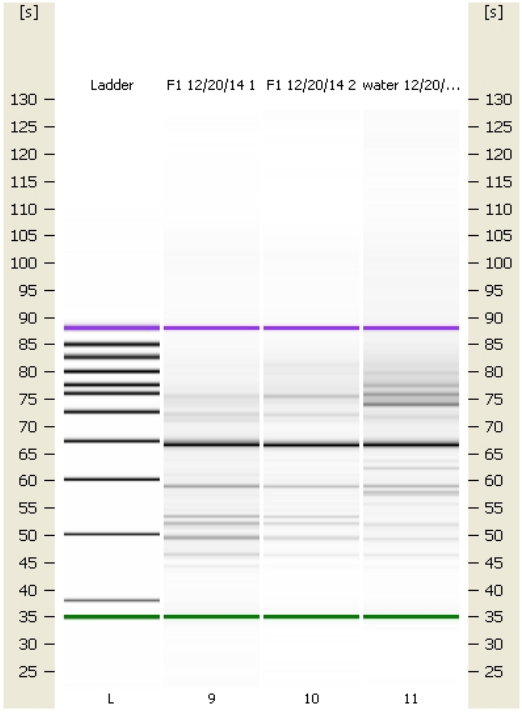

Figure D-12: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* and surface water sampled on December 20, 2014 representative isolates analyzed on the Agilent Bioanalyzer.

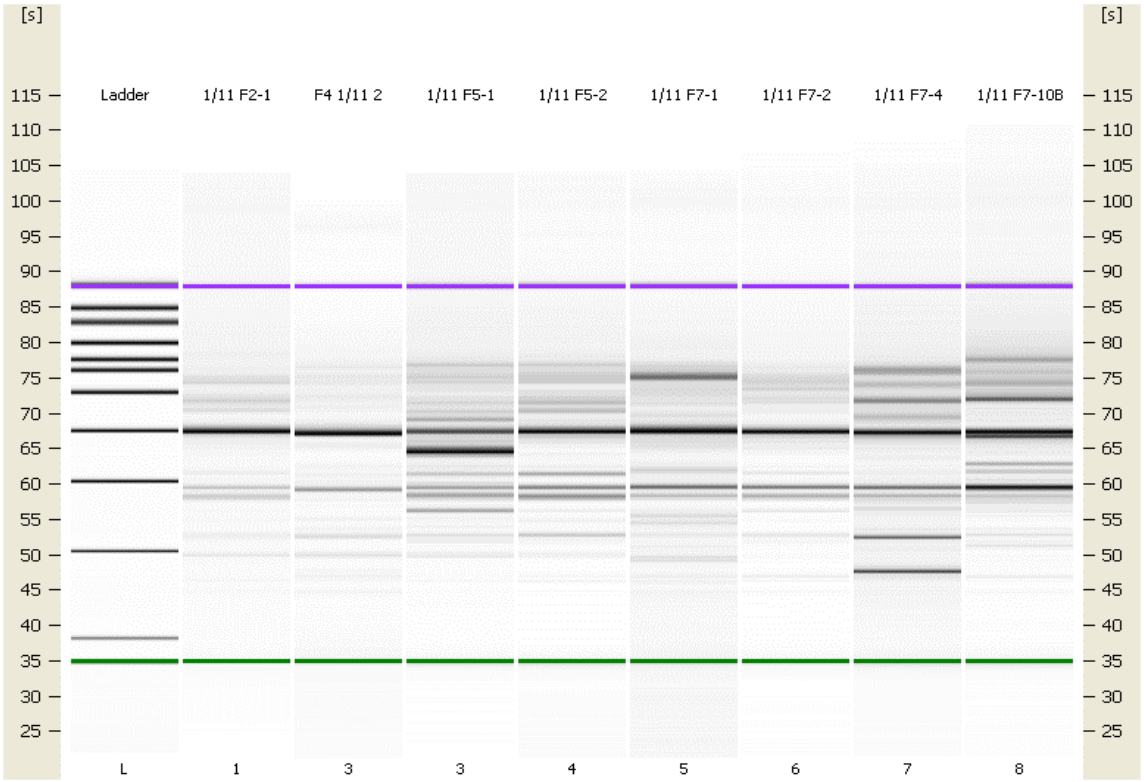

Figure D-13: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on January 11, 2015 representative isolates analyzed on the Agilent Bioanalyzer.

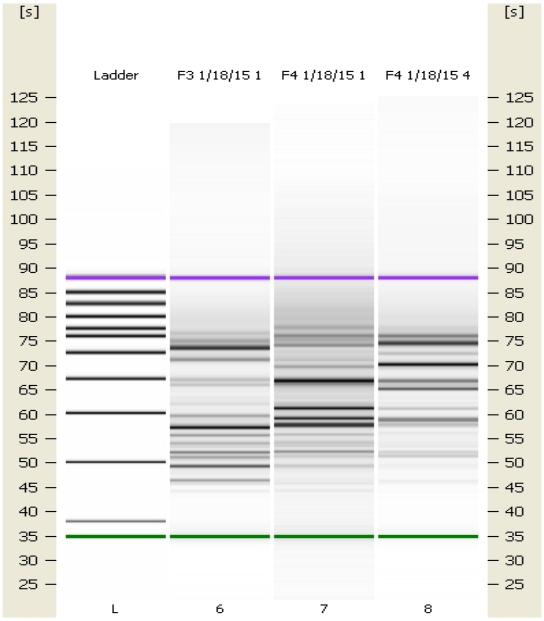

Figure D-14: Rep-PCR characterization of unique salmonellae isolates from *H. plecostomus* sampled on January 18, 2015 representative isolates analyzed on the Agilent Bioanalyzer.

LITERATURE CITED

Aanensen, D. M. and B. G. Spratt. 2005. The multilocus sequence typing network: mlst.net. Nucleic Acids Research 33:W728-W733.

Achtman, M., J. Wain, F. X. Weill, S. Nair, Z. Zhou, V. Sangal, M. G. Krauland, J. L. Hale, H. Harbottle, A. Uesbeck, G. Dougan, L. H. Harrison, S. Brisse, the *S. enterica* MLST study group. 2012. Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. PLoS Pathogens 8(6):e1002776.

Albufera, U., P. Bhugaloo-Vial, M. I. Issack, and Y. Jaufeerally-Fakim. 2009. Molecular characterization of *Salmonella* isolates by REP-PCR and RAPD analysis. Infection, Genetics, and Evolution 9:322-327.

Al-Harbi, A. H. and M. N. Uddin. 2004. Seasonal variation in the intestinal bacteria flora of hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia. Aquaculture 229:37-44.

Amann, R.I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. Journal of Bacteriology 172:762-770.

Andrade, V. M., J. Silva, F. R. Silva, V. D. Heuser, J. F. Dias, M. L. Yoneama, and T. R. O. Freita. 2004. Fish as bioindicators to assess the effects of pollution in two Brazilian rivers using the comet assay and micronucleus test. Environmental and Molecular Mutagenesis 44:459-468.

Arvanitidou, M., K. Kanellou, and D. G. Vagiona. 2005. Diversity of *Salmonella* spp. and fungi in northern Greek rivers and their correlation to fecal pollution indicators. Environmental Research 99(4) 278-284.

Awuor, W. S., O. D. Miruka, and W. N. Eliud. 2011. Characterization of *Salmonella* isolated from Nile Tilapia (*Oreochromis niloricus*) along Lake Victoria Beaches in western Kenya. International Journal of Biomedical Sciences 56:1397-1402.

Balasubramanian, S., M. R. Rajan, and S. P. Raj. 1992. Microbiology of fish grown in a sewage-fed pond. Bioresource Technology 40:63-66.

Barbau-Piednoir, E., S. Bertrand, J. Mahillon, N. H. Roosens, and N. Botteldoorn. 2013. SYBR® Green *q*PCR *Salmonella* detection system allowing discrimination at the genus, species, and subspecies level. Applied Microbiology and Biotechnology 97(22):9811-9824.

Byappanahalli, M. N., R. Sawdey, S. Ishii, D. A. Shively, J. A. Ferguson, R. L. Whitman, and M. J. Sadowsky. 2009. Seasonal stability of *Cladophora* associated *Salmonella* in Lake Michigan watersheds. Water Research 43:806-814.

Cahill, M. M. 1990. Bacterial flora of fishes: A review. Microbial Ecology 19:21-41.

Centers for Disease Control and Prevention. 2013. National *Salmonella* surveillance annual report, 2010. U.S. Department of Health and Human Services, Atlanta, Georgia, USA.

Cherry, W. B., J. B. Hanks, B. M. Thomason, A. M. Murlin, J. W. Biddle, and J. M. Croom. 1972. Salmonellae as an index of pollution of surface waters. Applied Microbiology 24(3):334-340.

Claudon, D. G., D. I. Thompson, E. H. Christenson, G. W. Lawton, and E. C. Dick. 1971. Prolonged *Salmonella* contamination of a recreational lake by runoff waters. Applied Microbiology. 21(5):875-877.

Costerton, J. W., and Z. Lewandowski. 1995. Microbial biofilms. Annual Review of Microbiology 49:711-745.

Curriero, F. C., J. A. Patz, J. B. Rose, and S. Lele. 2001. The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948-1994. American Journal of Public Health 91:1194-1199.

Dione, M., S. Geerts, and M. Antonio. 2012. Characterization of novel strains of multiply antibiotic-resistant *Salmonella* recovered from poultry in Southern Senegal. The Journal of Infections in Developing Countries 6(5):436-442.

Feder, I., J. C. Nietfeild, J. Galland, T. Yeary, J. M. Sargant, R. Oberst, M. L. Tamplin, and J. B. Luchansky. 2001. Comparison of cultivation and PCR-hybridization for detection of *Salmonella* in porcine fecal and water samples. Journal of Clinical Microbiology 39(7):2477-2484.

Gaertner, J., P. E. Wheeler, S. Obafemi, J. Valdez, M. R. J. Forstner, T. H. Bonner, and D. Hahn. 2008a. Detection of salmonellae from fish in a natural river system. Journal of Aquatic Animal Health 20:150-157.

Gaertner, J. P., D. Hahn, F. L. Rose, and M. R. J Forstner. 2008b. Detection of salmonellae in different turtle species within a headwater spring ecosystem. Journal of Wildlife Diseases 44(2):519-526.

Gaertner, J. P., T. Garres, J. C. Becker, M. L. Jimenez, M. J. R. Forstner, and D. Hahn. 2009. Temporal analysis of salmonellae in a headwater spring ecosystem reveals the effects of precipitation and runoff events. Journal of Water and Health 7(1):115-121.

Gaertner, J. P., J. A. Mendoza, M. R. J. Forstner, and D. Hahn. 2011. Recovery of *Salmonella* from biofilms in a headwater spring ecosystem. Journal of Water and Health 9:458-466.

Gaulin, C., C. Vincent, and J. Ismail. 2005. Sporadic infections of *Salmonella* Paratyphi B.,var. Java associated with fish tanks. Canadian Journal of Public Health 96(6):471-474.

Geldreich, E. E., and N. A. Clarke. 1966. Bacterial pollution indicators in the intestinal tract of freshwater fish. Applied Microbiology 14(3):429-437.

Glynn, J. R., and D. J. Bradley. 1992. The relationship between infecting dose and severity of disease in reported outbreaks of *Salmonella* infections. Epidemiology and Infection 109:371-388.

Gorski, L. 2012. Selective enrichment media bias the types of *Salmonella enterica* strains isolated from mixed strain cultures and complex enrichment broths. PLoS One 7(4):e34722.

Greer, A., V. Ng, and D. Fisman. 2008. Climate change and infectious diseases in North America: the road ahead. Canadian Medical Association Journal 178:715-722.

Harbottle, H., D. G. White, P. F. McDermott, R. D. Walker, and S. Zhao. 2006. Comparison of multilocus sequence typing, pulsed-field gel electrophoresis, and antimicrobial susceptibility typing for characterization of *Salmonella enterica* serotype Newport isolates. Journal of Clinical Microbiology 44(7):2449-2457.

Hendricks, C. W. 1971. Increased recovery rate of salmonellae from stream bottom sediments versus surface water. Applied Microbiology 21(2):379-380.

Hernandez, E., J. L. Rodriguez, S. Herrera-Leon, I. Garcia, V. de Castro, and N. Muniozguren. 2012. *Salmonella* Paratyphi B var Java infections associated with exposure to turtles in Bizkaia, Spain, September 2010 to October 2011. European Surveillance and Outbreak Reports 17(25):1-5.

Hunter, P. R. 2003. Climate change and waterborne and vector-borne disease. Journal of Applied Microbiology 94:37S-46S.

Jacobsen, C. S. and T. B. Bech. 2012. Soil survival of *Salmonella* and transfer to freshwater and fresh produce. Food Research International 45(20):557-566.

Jiang, C., K. S. Shaw, C. R. Upperman, D. Blythe, C. Mitchell, R. Murtugude, A. R. Sapkota, and A. Sapkota. 2015. Climate change, extreme events and increased risks of salmonellosis in Maryland, USA: evidence for costal vulnerability. Environment International 83:58-62.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. Mentjies, and A. Drummond. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12):1647-1649.

Kidgell, C., U. Reichard, J. Wain, B. Linz, M. Torpdahl, G. Dougan, and M. Achtman. 2002. *Salmonella typhi*, the causative agent of typhoid fever is approximately 50,000 years old. Infection, Genetics and Evolution 2:39-45.

Kusumaningrum, H. D., Suliantari, and R. Dewanti-Hariyadi. 2012. Multidrug resistance among different serotypes of *Salmonella* isolates from fresh products in Indonesia. International Food Research Journal 19(1):57-66.

Lemarchand, K., and P. Lebaron. 2002. Influence of mutation frequency on the persistence of *Salmonella enterica* serotypes in natural waters. FEMS Microbiology Ecology 41:125-131.

Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M Achtmanm, and B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Science, USA 95:3140-3145.

Malorny, B, J. Hoorfar, C. Bunge, and R. Helmuth. 2003. Multicenter validation of the analytical accuracy of *Salmonella* PCR towards and international standard. Applied and Environmental Microbiology 69(1):290-296.

Malorny, B., E. Paccassoni, P. Fach, C. Bunge, A. Martin, and R. Helmuth. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Applied and Environmental Microbiology 70(12):7046-7052.

McEgan, R., G. Mootian, L. D. Goodridge, D. W. Schaffner, and M. D. Danyluk. 2013. Predicting *Salmonella* populations from biological, chemical, and physical indicators in Florida surface waters. Applied and Environmental Microbiology 79(13):4094-4105.

Moore, B. C., E. Martinez, J. M. Gay, and D. H. Rice. 2003. Survival of *Salmonella enterica* in freshwater and sediments and transmission by aquatic midge *Chironomus tentans* (Chironomidae: Diptera). Applied and Environmental Microbiology 69(8):4556-4560.

Murray, C. J. 1991. Salmonellae in the environment. Scientific and Technical Review of the Office International des Epizooties 10(3):765-785.

Negrel, P., C. Merly, L. Gourcy, O. Cerdan, E. Petelet-Giraud, M. Kralik, G. Klaver, G. van Wirdum, and J. Vegter. 2013. Soil-sediment-river connections: catchment processes delivering pressures to river catchments. Risk-informed Management of European River Basins 29:21-52.

Noda, T., K. Murakami, T. Asai, Y. Etoh, T. Ishihara, T. Kuroki, K. Horikawa, and S. Fujimoto. 2011. Multi-locus sequence typing of *Salmonella enterica* subsp. *enterica* serovar Enteritidis strains in Japan between 1973 and 2004. ACTA Veterinaria Scandinavica 53(38):1-6.

Nordentoft, S., H. Christensen, and H. C. Wegner. 1997. Evaluation of a fluorescence-labelled oligonucleotide probe targeting 23S rRNA for *in situ* detection of *Salmonella* serovars in paraffin-embedded tissue sections and their rapid identification in bacterial smears. Journal of Clinical Microbiology 35(10):2642-2648.

Novotny, L., L. Dvorska, A. Lorencova, V. Beran, and I. Pavlik. 2004. Fish: a potential source of bacterial pathogens for human beings. International Journal for Veterinary and Biomedical Science 49(9):343-358.

O'Toole, G.A., H. B. Kaplan, and R. Kolter. 2000. Biofilm formation as microbial development. Annual Review of Microbiology 54:49-79.

Panaro, N. J., P. K. Yuen, T. Sakazume, P. Fortina, L. J. Kricka, P. Wilding. 2000. Evaluation of DNA fragment sizing and quantification by the Agilent 2100 Bioanalyzer. Clinical Chemistry 46(11):1851-1853.

Parsek, M. R., and E. P. Greenberg. 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. TRENDS in Microbiology 13(1):27-33.

Polo, F., M. J. Figueras, I. Inza, J. Sala, J. M. Fleisher, and J. Guarro. 1998. Relationship between presence of *Salmonella* and indicators of faecal pollution in aquatic habitats. Microbiology Letters 160:253-256.

Pound, K. L., W. H. Nowlin, D. G. Huffman, and T. H. Bonner. 2011. Trophic ecology of a nonnative population of suckermouth catfish (*Hypostomus plecostomus*) in a central Texas spring-fed stream. Environmental Biology of Fishes 90:277-285.

Rahimi, E., A. Shakerian, and A. G. Falavarjani. 2013. Prevalence and antimicrobial resistance of *Salmonella* isolated from fish, shrimp, lobster, and crab in Iran. Comparative Clinical Pathology 22:59-62.

Sha, Q., A. Gunathilake, M. R. J. Forstner, and D. Hahn. 2011. Temporal analysis of the distribution and diversity of *Salmonella* in natural biofilms. Systematic and Applied Microbiology 34:353-359.

Sha, Q., M. R. J. Forstner, T. H. Bonner, and D. Hahn. 2013a. Salmonellae in fish feces analyzed by *in situ* hybridization and quantitative polymerase chain reaction. Journal of Aquatic Animal Health 25:184-190.

Sha, Q., D. A. Vattem, M. J. Forstner, and D. Hahn. 2013b. Quantifying *Salmonella* population dynamics in water and biofilms. Microbial Ecology 65:60-67.

Sha, Q., M. R. J. Forstner, and D. Hahn. 2013c. Diversity of *Salmonella* in biofilms and water in a headwater ecosystem. FEMS Microbiology Ecology 83:642-649.

Thomas, J. L., R. M. Slawson, and W. D. Taylor. 2013. *Salmonella* serotype diversity and seasonality in urban and rural streams. Journal of Applied Microbiology 114:907-922.

Thomason, B. M., J. W. Biddle, and W. B. Cherry. 1975. Detection of salmonellae in the environment. Applied Microbiology 30:764-767.

Tianzhi, W., L. Yunkai, L. Mingchao, Y. Peiling, and B. Zhihui. 2014. Biofilms on the surface of gravels and aquatic plants in river and lakes with reusing reclaimed water. Environmental Earth Sciences 72:743-755.

Traore, O., O. Nyholm, A. Siitonen, I. J. O. Bonkoungou, A. S. Traore, N. Barro, and K. Haukka. 2015. Prevalence and diversity of *Salmonella enterica* in water, fish, lettuce in Ouagadougou, Burkina Faso. BMC Microbiology 15:151-158.

Urwin, R. and M. C. J. Maiden. 2003. Multi-locus sequence typing: a tool for global epidemiology. TRENDS in Microbiology 11(10):479-487.

Versalovic, J., M. Schneider, F. J. De Bruijn, and J. R. Lupski. 1994. Genomic fingerprinting of bacteria using sequence-based polymerase chain reaction. Methods in Molecular and Cellular Biology 5:25-40.

Wattiau, P., C. Boland, and S. Bertrand. 2011. Methodologies for *Salmonella enterica* subsp. *enterica* subtyping: gold standards and alternatives. Applied and Environmental Microbiology 77(22):7877-7885.

Werle, E., C. Schneider, M. Renner, M. Volker, and W. Fiehn. 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Research 22(20):4354-4355.

Winfield, M. D., and E. A. Groisman. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. Applied and Environmental Microbiology 69:3687-3694.

Wise, M. G., G. R. Siragusa, J. Plumblee, M. Healy, P. J. Cray, and B. S. Seal. 2009. Predicting *Salmonella enterica* serotypes by repetitive sequence-based PCR. Journal of Microbiological Methods 76:18-24.