The use of *Bacteroides* Genetic Markers to Identify Microbial Sources in Natural Water

by

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ABSTRACT

Water quality in surface water is frequently degraded by fecal contamination from human and animal sources, imposing negative implications for recreational water use and public safety. For this reason it is critical to identify the source of fecal contamination in bodies of water in order to take proper corrective actions for controlling fecal pollution.

Bacteroides genetic markers have been widely used to differentiate human from other sources of fecal bacteria in water. The results of this study indicate that many assays currently used to detect human-specific *Bacteroides* produce false positive results in the presence of freshwater fish. To further characterize *Bacteroides* from fish and human, the fecal samples were cultured, speciated, and identified. As a result, forty six new *Bacteroides* 16S rRNA gene sequences have been deposited to the NCBI database. These sequences, along with selected animal fecal sample *Bacteroides*, were aligned against human *B. volgatus*, *B. fragilis,* and *B. dorei* to identify multi-segmented variable regions within the 16S rRNA gene sequence. The collected sequences were truncated and used to construct a cladogram, showing a clear separation between human *B. dorei* and *Bacteroides* from other sources. A proposed strategy for source tracking was field tested by collecting water samples from central AZ source water and three different recreational ponds. PCR using HF134 and HF183 primer sets were performed and sequences for positive reactions were then aligned against human *Bacteroides* to identify the source of contamination.

For the samples testing positive using the HF183 primer set (8/13), fecal contamination was determined to be from human sources. To confirm the results, PCR products were sequenced and aligned against the four variable regions and incorporated within the truncated cladogram. As expected, the sequences from water samples with human fecal contamination grouped within the human clade.

As an outcome of this study, a tool box strategy for *Bacteroides* source identification relying on PCR amplification, variable region analysis, human-specific *Bacteroides* PCR assays, and subsequent truncated cladogram grouping analysis has been developed. The proposed strategy offers a new method for microbial source tracking and provides step-wise methodology essential for identifying sources of fecal pollution.

DEDICATION

This dissertation is dedicated to my parents, who supported me every step of the way, and for their endless patience, encouragement, and sacrifices.

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BACKGROUND

1.1. Introduction

Monitoring microbial quality of water sources used for drinking, irrigation, aquaculture, and recreational activities is a critical task. The majority of watersheds used as sources of drinking water and for recreational activities are contaminated by microbial pollution. According to an U.S. EPA report, 45% of streams and rivers, 47% of lakes and ponds, and 32% of bays and estuaries are not clean enough for their final use (USEPA, 2002a). The microbial quality of water can be degraded by fecal contamination from point sources (e.g., effluent from wastewater treatment plants, raw sewage from sewer overflows, and storm water) or from non-point sources (e.g., wildlife or runoff from farms) (Kim et al., 2005). Maintenance and management of the microbiological quality of water is very important, as contamination of water can cause high risks to human health and economic losses due to closure of beaches and shellfish harvesting areas. Fecal pollution from both human and animal impose risks to human health from exposure to pathogenic bacteria (Baker and Herson, 1999), viruses (Pina et al., 1998), and protozoa such as *Cryptosporidium* (Lefay et al., 2000). Waters contaminated with human feces are generally considered as a greater risk to human health, as they are more likely to contain human enteric pathogens (Guzewich and Morse, 1986). Knowing the source of contamination is critical to develop effective resource management and, ultimately, solve the problem.

Over the past several years scientists have been using members of the genus *Bacteroides* to identify and quantify level of fecal contamination from non-point sources. They are preferred microbial indicator of fecal pollution due to their high abundance in feces (Madigan et al., 2003), low potential to grow in the environment (Salyers, 1984; Sghir et al., 2000), and high degree of host specificity that likely reflects differences in source animal digestive systems (Bernhard and Field, 2000a; Simpson et al., 2004; Dick et al., 2005a). Recently, several studies have proposed the existence of human-specific *Bacteroides* genetic markers and developed methods for their detection by conventional and quantitative PCR (Bernhard and Field, 2000b; Seurinck et al., 2005; Layton et al., 2006; Reischer et al., 2007). Since the use of *Bacteroides* as a fecal source identification tool is relatively new, extensive field testing is ongoing to determine the specificity of the published assays. Studies that have identified human-specific *Bacteroides* 16S rRNA genetic markers have also reported cross-reactivity of the PCR-primers with canine and swine fecal strains (Kreader, 1995; Layton et al., 2006; Ahmed et al., 2009).

My recent work has shown cross-amplification of several published protocols for the identification of human-specific *Bacteroides* 16S rRNA genetic markers with DNA extracted from fecal samples of fish species (tilapia, catfish, trout, and salmon). Although *Bacteroides* have long been identified as indigenous in the intestine of freshwater fish, to date, no fish species has been tested for cross-amplification with the published human *Bacteroides* markers (McLain et al., 2009). Non-specificity of the designed human-specific *Bacteroides* genetic

markers and their cross-amplification with fish can lead to false identification of the source of fecal contamination in surface water. Hence, to eliminate such a problem it is critical to propose a new Microbial Source Tracking (MST) strategy by following step-wise methodology.

1.2. Objectives

The main objective of this study is to develop a tool box strategy for *Bacteroides* source identification. Specific objectives covered by each chapter are as follows:

- Investigating the specificity of five sets of published human-specific *Bacteroides* primers with fecal DNA from four freshwater fish species.
    - Performing literature review to produce a list of published primer sets specific for amplifying human *Bacteroides* 16S rRNA gene.
    - Selecting standard PCR primers from published literature to evaluate their specificity.
    - Collecting human and fish (tilapia, catfish, trout, and salmon) fecal samples and performing DNA extraction, purification, and PCR amplification.
    - Performing cloning and DNA sequencing to confirm PCR results.
- Isolation of *Bacteroides* from fish and human fecal samples for identification of unique genetic markers.
    - Culturing *Bacteroides* isolates from human and fish (tilapia, grass carp, blue catfish, channel catfish, and trout) fecal samples on *Bacteroides* Bile Esculin agar selective media.
    - Identifying *Bacteroides* species by using biochemical tests such as API strips and molecular techniques.

- Constructing clone libraries of *Bacteroides* 16S rRNA genes of the identified species.

- Proposing a new MST strategy using *Bacteroides* 16S rRNA signatures in water sources

  - *Bacteroides* 16S rRNA gene sequence alignment of fish and selected animal against human *Bacteroides* for identification of variable regions.

  - Developing a tool box strategy based on sequence alignment analysis, human-specific PCR assays, and cladogram grouping analysis.

  - Field testing the strategy by collecting water samples from Central AZ source water, ponds, and lakes.

Chapter 2

LITERATURE REVIEW

2.1. *Bacteroides* Background Information

The majority of the bacterial community in the mammalian colon is comprised of anaerobes and one of the most predominant of these are of the genus *Bacteroides*. This section will go over some background information regarding *Bacteroides* such as their taxonomy, characteristics, species, genome, and occurrence.

2.1.1. Taxonomy

Bacteroides were originally described by Veillon and Zuber (1898) and for almost 30 years they were a collection of heterogeneous bacteria which were grouped based on a common host and physiological similarities such as being obligate anaerobes and gram-negative rods. Soon the physiological heterogeneity of this group started to supersede their vague physiological similarities and it led to the first scientific description of this group by Castellani and Chalmers (1919). Over the years different approaches have been used to reorganize this group including: physiological characteristics (Holdeman et al., 1984), serotyping (Lambe, 1974), bacteriophage typing (Booth, et al 1979), lipid analysis (Miyagawa, 1979), oligonucleotide cataloging (Paster et al., 1985), and 5S - 16S rRNA sequence comparisons (Johnson, 1978; Paster et al., 1994; Van den Eynde et al., 1989; Weisburg et al., 1985).

Bacteria belonging to phyla Bacteroidetes and Firmicutes are the two major groups in the human clone and together make up 95-99% of the gut

microbiota (Karlsson et al., 2010). Placement of the genus *Bacteroides* in the phylum Bacteroidetes is shown in Fig 1. In 1986 three newly isolated *Bacteroides* species derived from human feces were described and named as *B. caccae, B. merdae* and *B. stercoris* (Johnson et al., 1986). In 1989 the *Bacteroides* genus underwent a major revision and was restricted to the type species *Bacteroides fragilis* and closely related organisms based on biochemical and genomic GC content (Shah and Collins, 1989). These closely related organisms include *B. vulgatus, B. thetaiotaomicorn, B. distasonis, B. caccae, B. eggerthii, B. merdae, B. ovatus, B. stercoris,* and *B. uniformis*. The restriction of the *Bacteroides* genus to the *B. fragilis* group resulted in movement of several species from this genus to new genera such as *Prevotella* and *Porphyromonas* (Shah and Collins, 1990; Shah and Collins, 1988). In 1995, *B. gracilis* was moved from the *Bacteroides* genus to *Campylobacter* and was renamed as *Campylobacter gracilis* (Vandamme et al., 1995). In 2003, *B. putredinis* was reclassified and was changed to the new genus *Alistipes* (Rautio et al., 2003). More recently, *B. goldsteinii, B. distasonis,* and *B. merdae* have been excluded from the *Bacteroides* genus and moved to the new genus *Parabacteroides* (Sakamoto and Benno, 2006). With the advent of molecular techniques and 16S rRNA gene sequencing a variety of new species have been added to the genus *Bacteroides* such as: *B. nordii, B. salyersai, B. plebeius, B. coprocola,* and *B. massiliensis* (Wexler, 2007). Recently, based on *Bacteroides* 16S rRNA gene analysis and protein families and their functional content, it has been suggested that *Bacteroides pectinophilus* and *B. capillosus*

should be removed from Bacteroidetes and placed under the Firmicutes phylum (Karlsson et al., 2010).

Despite improvement in classification over the last several decades, *Bacteroides* species are still known as being heterogeneous and some of the species are known to have different protein families and functional content, resulting in their displacement from the genus (Karlsson et al., 2010). The placement of many of these species into the genus *Bacteroides* is usually based on 16S rRNA gene analysis. However, a recent study has suggested that for a more detailed and comprehensive view of the phylogenetic relationship between species, they should be clustered based on distribution of their protein families (Karlsson et al., 2010).

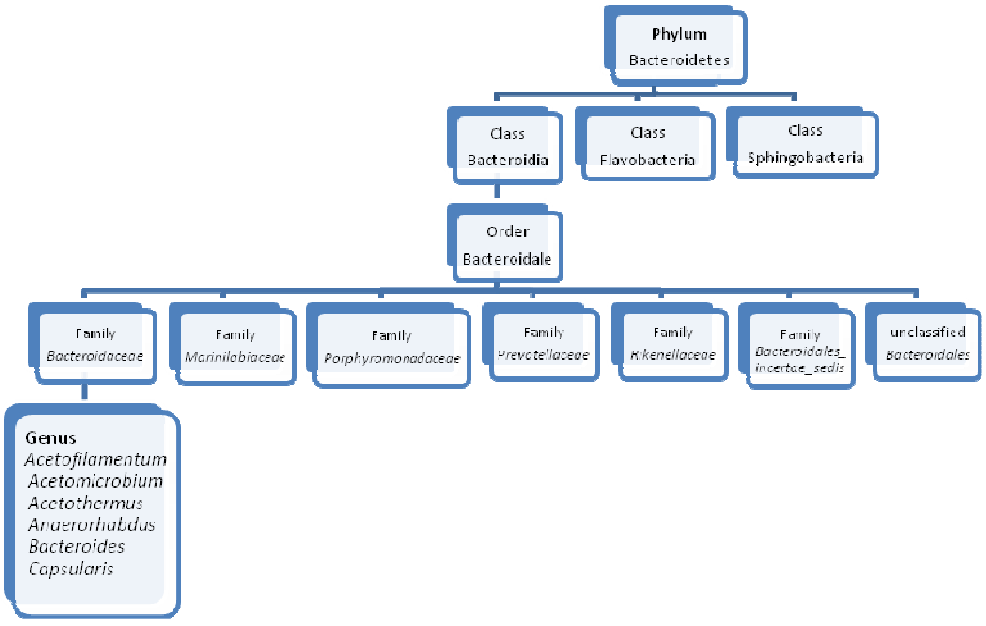


Fig. 1 - Placement of *Bacteroides* genus in Bacteroidetes phylum.

2.1.2. Characteristics

Bacteroides are gram-negative, non-spore forming, saccharolytic, obligate anaerobes, and rod-shaped bacteria that play a fundamental role in the processing of complex molecules to simpler ones in the host intestine (Madigan et al., 2003). These microorganisms are found primarily in the intestinal tracts and mucous membranes of warm-blooded animals (Mitsuoka et al., 1965) and certain cold-blooded animals such as fish (Trust and Sparrow, 1974; McLain et al., 2009). Under special circumstances, *Bacteroides* may be an opportunistic pathogen for host species causing intra-abdominal infections, abscess, or even bacteremia (Bernhard and Field, 2000a). While *B. fragilis* makes up only 1-2% of the normal flora, it is the most notable pathogen isolated from 81% of anaerobic clinical infections (Werner, 1974).

Like many other bacteria, members of the genus *Bacteroides* use glucose for their energy needs. *Bacteroides* produce acetate and succinate as the major metabolic end products. The glucose fermentation pathway is shown in Fig 2.

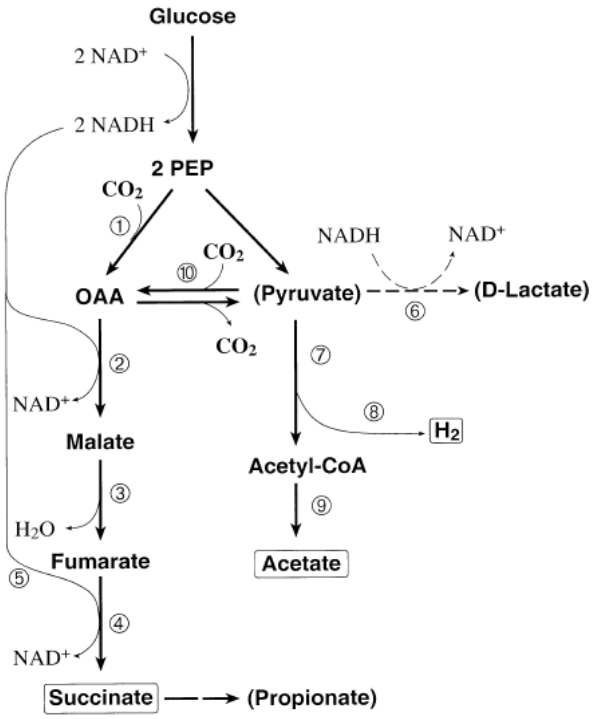


Fig. 2 - Glucose fermentation pathway of *B. thetaiotaomicron*. Enzymes; 1, PEP carboxykinase; 2, malate dehydrogenase; 3, fumarase; 4, fumarase reductase; 5, NADH dehydrogenase; 6, lactate dehydrogenase; 7, pyruvate: ferredoxin:oxidoredutase; 8, hydrogenase; 9, phosphotransacetylase and acetate kinase; 10, pyruvate carboxylase. PEP, phosphoenolpyruvate; OAA, oxaloacetate. Major excreted products are boxed; minor products are in parentheses (Pan and Imlay, 2001).

Bacteroides species require exogenous heme and non-heme iron for their growth. This is because they lack genes for the heme biosynthetic pathway and cannot synthesize tetrapyrrole macrocycle (Holdeman et al., 1984). Although dependency on exogenous heme seems disadvantageous to microbes, it is interesting that heme-dependent microbes number higher compared to heme-independent microbes in the lower intestinal tract (Cornelis and Simon, 2010). This suggests that heme biosynthesis is not critical for colonization of the colonic environment. Under anaerobic conditions in the presence of heme, *B. fragilis* can

generate nearly the double amount of ATP than *Escherichia coli* per mole of glucose (Cornelis and Simon, 2010). This high energy yield is linked to a rudimentary heme-induced fumarate reductase and cytochrome *b*-dependent electron transport energy metabolism pathway which uses fumarate as the terminal electron acceptor (Fig 2).

The members of the genus *Bacteroides* carry a typical gram-negative cell envelope that consists of an inner membrane, the periplasmic space, and an outer membrane. The outer membrane contains lipopolysaccharide (Fig 3). *Bacteroides* membranes also contain a certain lipid known as sphingolipids as well as a mixture of long-chain fatty acids, mainly straight chain saturated, anteisomethyl, and iso-methyl branched acids.

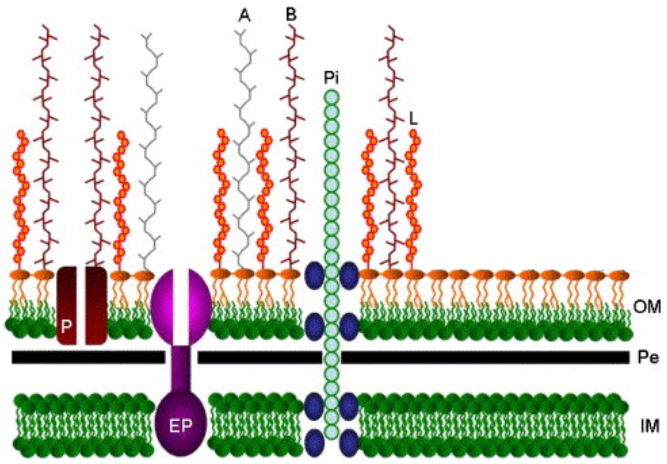


Fig. 3 - A schematic model of the *B. fragilis* cell envelope showing: an inner membrane (IM) or cell membrane, the periplasmic space (Pe), and an outer membrane (OM) containing lipopolysaccharide (orange) that may possess side-chains (L). Also shown is a thin-type (2 nm diameter) pilus (Pi) with export proteins (blue), an outer membrane porin (P) and an efflux pump for expelling antibiotics, heavy metal ions, and other noxious products (EP). On capsular strains, the large polysaccharide capsule consists of two distinct polysaccharides: A and B (Pumbwe et al., 2006).

One of the simple methods that provide useful information on bacterial morphology is the direct microscopic examination of gram stained smears of sample. The typical *Bacteroides* cellular morphology contains pleomorphic, pale, gram negative forms with round ends occurring singularly or in pairs, with vacuoles (Holdeman et al., 1977). Fig 4 shows scanning electron micro-graphs of *B. fragilis*.

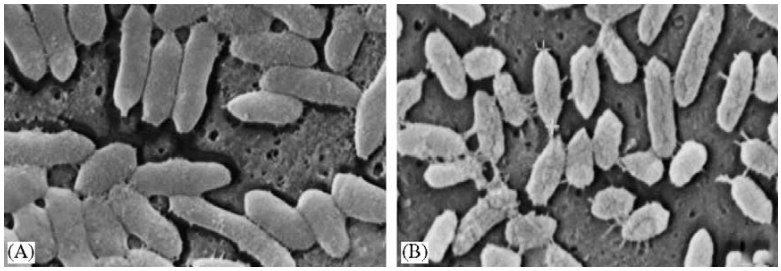


Fig. 4 - Scanning electron micrograph of *B. fragilis* NCTC 9343. (A) Uncultured cells and (B) Cells treated with 0.15% bile salts which resulted in production of pili-like appendages (Pumbwe et al., 2006).

One important characteristic of the *Bacteroides* species is their resistance to a variety of antibiotics such as β-lactams, aminoglycosides, erythromycin, and tetracycline (Salyers et al., 2004). The resistance of *Bacteroides* to any of these antibiotics can occur due to altered target binding affinity, decreased permeability of antibiotics to the microbe cell, or the presence of an inactivating enzyme (Rasmussen et al., 1997). “Tetracycline resistance in the *Bacteroides* is attributable, almost exclusively, to the presence of the *tet*Q gene, which encodes a protein that is believed to alter the ribosome target site for the antibiotic” (Fletcher et al., 1991). The high level of *Bacteroides* resistance to antibiotics has

raised concerns since there is a possibility that *Bacteroides* could transmit antibiotic resistance genes to other pathogenic bacteria (Salyers et al., 2004).

Bacteroides have simple nutrient requirements that reflect their environment. Most of the *Bacteroides* species can grow on a medium containing fermentable carbohydrates, hemin, vitamin B_{12}, ammonia, carbon dioxide, and sulfide, all of which are plentiful in the human colon (Holdeman et al., 1984).

2.1.3. Species

Among the scientific community there is discrepancy regarding the taxonomy of *Bacteroides*. The Integrated Taxonomic Information System (ITIS) (http://www.itis.gov), a partnership of federal agencies and other organizations from the United States, Canada, and Mexico with data stewards and experts from around the world, has accepted 30 species of genus *Bacteroides* by 2010 (Table 1). The German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)) has three times more species of *Bacteroides* than the ITIS (Table 2).

Table 1 - Species of genus *Bacteroides* according to ITIS

B. acidifaciens	*B. fragilis*	*B. polypragmatus*
B. caccae	*B. galacturonicus*	*B. pyogenes*
B. capillosus	*B. goldsteinii*	*B. salyersiae*
B. capillus	*B. helcogenes*	*B. splanchnicus*
B. cellulosolvens	*B. intestinalis*	*B. stercoris*
B. coagulans	*B. massiliensis*	*B. suis*
B. coprocola	*B. nordii*	*B. tectus*
B. coprosuis	*B. ovatus*	*B. thetaiotaomicron*
B. dorei	*B. pectinophilus*	*B. uniformis*
B. eggerthii	*B. pentosaceus*	*B. ureolyticus*
B. finegoldii	*B. plebeius*	*B. vulgates* *B. xylanolyticus*

Source: http://www.catalogueoflife.org/annual-checklist/2010/browse/tree

Table 2 - Species of genus *Bacteroides* according to DSMZ

B. acidifaciens	*B. faecis*	*B. microfusus*	*B. salanitronis*
B. amylophilus	*B. finegoldii*	*B. multiacidus*	*B. salivosus*
B. asaccharolyticus	*B. fluxus*	*B. nodosus*	*B. salyersiae*
B. barnesiae	*B. forsythus*	*B. nordii*	*B. sartorii*
B. bivius	*B. fragilis*	*B. ochraceus*	*B. splanchnicus*
B. buccae	*B. furcosus*	*B. oleiciplenus*	*B. stercoris*
B. buccalis	*B. galacturonicus*	*B. oralis*	*B. succinogenes*
B. caccae	*B.gallinarum*	*B. oris*	*B. suis*
B. capillosus	*B. gingivalis*	*B. oulorum*	*B. tectum*
B. capillus	*B. gracilis*	*B. ovatus*	*B. tectus*
B. cellulosilyticus	*B. graminisolvens*	*B. pectinophilus*	*B. termitidis*
B. cellulosolvens	*B. helcogenes*	*B. pentosaceus*	*B. thetaiotaomicron*
B. clarus	*B. heparinolyticus*	*B. plebeius*	*B. uniformis*
B. coagulans	*B. hypermegas*	*B. pneumosintes*	*B. ureolyticus*
B. coprocola	*B. intermedius*	*B. polypragmatus*	*B. veroralis*
B. coprophilus	*B. intestinalis*	*B. praeacutus*	*B. vulgatus*
B. coprosuis	*B. levii*	*B. propionicifaciens*	*B. xylanisolvens*
B. corporis	*B. loescheii*	*B. putredinis*	*B. xylanolyticus*
B. denticola	*B. macacae*	*B. pyogenes*	*B. zoogleoformans*
B. disiens	*B. massiliensis*	*B.ruminicola*	
B. distasonis	*B. melaninogenicus*	*B. ruminicola sub-spp. ruminicola brevis*	
B. dorei	*B. melaninogenicus sub-spp. Intermedius Macacae*		
B. eggerthii	*B. melaninogenicus*		
B. endodontalis			

Source:http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Bacteroides&show_all_details=1

Most *Bacteroides* species strains identified to date belong to ten cultivated species including *Bacteroides vulgatus, B. thetaiotaomicorn, B. distasonis, B. caccae, B. eggerthii, B. merdae, B. ovatus, B. stercoris, B. uniformis,* and *B. fragilis* (Shah and Colins, 1989). The proportions of *Bacteroides* species seen

clinically (disease causing) are shown in Fig 5. As shown, *B. fragilis* is the most common clinically isolated species among *Bacteroides.*

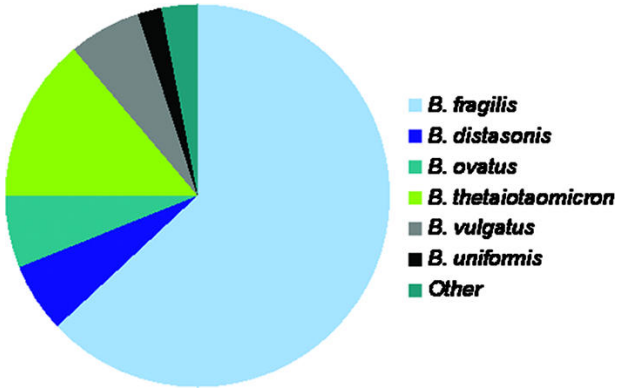


Fig. 5 - Proportions of *Bacteroides* species seen clinically (Wexler, 2007)

Bacteroides species are very diverse, having saccharolytic and proteolytic activities. The classification of *Bacteroides* species can be performed based on analysis of acid end products, DNA composition, cell walls, enzymes, and lipids (Shah and Collins, 1983). Table 3 shows the DNA composition of the *Bacteroides* species. As shown, *Bacteroides fragilis* contains Guanine (G) and Cytosine (C) in the range of 41 to 44 mol%. Other *Bacteroides* species belonging to the *B. fragilis* group also contain G+C in the range of 41 to 44 mol%. Some of the species belonging to the *B. fragilis* group have remarkably different DNA composition compared to other species within this group and it has been suggested that they should be excluded from this genus. Examples are *B. praeacutus* and *B. ureolyticus* which contain very low G+C (28 to 30 mol%) and *B. capillosus* and

B. mirofusus which contain very high G+C (60 to 61 mol%) (Shah and Collins, 1983).

Table 3 - DNA base composition of *Bacteroides* species (Shah and Collins, 1983)

Species	Mol% G+C	Type Strain
Bacteroides coagulans	37	ATCC[1] 29798
Bacteroides furcosus	34	ATCC 25622
Bacteroides hypermegas	32-34	NCTC[2] 10571
Bacteroides praeacutus	28	ATCC 25539
Bacteroides termitidis	34	NCTC I1300
Bacteroides ureolyticus	28-30	NCTC 10941
Bacteroides asaccharolyticus	50-54	ATCC 25260
Bacteroides amylophilus	40-42	ATCC 29744
Bucteroides bivius	40	ATCC 29303
Bacteroides buccalis	45-46	NCDO[3] 2354
Bacteroides denticola	51	NCDO 2352
Bucteroides disiens	40-42	ATCC 29426
Bacteroides distasonis	43-45	ATCC 8503
Bacteroides eggerthii	44-46	ATCC 27754
Bacteroides fragilis	41-44	ATCC 25285
Bacteroides yingivalis	45-48	ATCC 33277
Bacteroides melaninogenicus (subsp. levii')	45-48	ATCC 29147
Bacteroides loescheii	46	ATCC 15930
Bucteroides macacae	43-44	ATCC 33141
Bucteroides melaninogenicus (subsp. Intermedius)	40-44	ATCC 25611
Bacteroides meianinogenicus (subsp. Melaninogenicus)	40-42	ATCC 25845
Bacteroides orulis	45	ATCC 33269
Bacteroides oris	42-46	ATCC 33573
Bacteroides ovatus	39-43	ATCC 8483
Bacteroides pentosaceus	50-5 1	NCDO 2353
Bacleroides ruminicola (subsp. Brevis)	50	ATCC 19188
Bacteroides ruminicola (subsp. Ruminicola)	49	ATCC 19189
Bacteroides splanchnicus	40	ATCC 29572
Bacteroides succinogenes	43-49	ATCC 19169
Bacteroides thetaiotaomicron	40-43	ATCC 29148
Bacteroides uniformis	45-48	ATCC 8492
Bacteroides vulgates	40-42	ATCC 8482
Bacteroides capillosus	60	ATCC 29799
Bacteroides microfusus	60-6 1	ATCC 29728
Bacteroides multiacidus	56-58	ATCC 27723

ATCC[1], American Type Culture Collection

NCTC[2], National Collection of Type Culture

NCDO[3], National Collection of Dairy Organisms

2.1.4. Genome

Studying a bacteria species' genome is important in not only classification, but also understanding the organism's physiology and virulence. The *Bacteroides* genome has been the focus of different research groups in the U.S. and Europe. The Wellcome Trust provided funds to the Sanger Institute for sequencing the genomes of two *Bacteroides fragilis*, strains NCTC9343 and 638R. The project was completed in collaboration with Sheila Patrick (Queen's University of Belfast, UK), Garry Blakely (University of Edinburgh, UK), Val Abratt of the Department of Molecular and Cell Biology at the University of Cape Town (South Africa), Prof. Brian Duerden (University of Wales College of Medicine, UK), and Prof. Ian Poxton (University of Edinburgh Medical School, UK) (http://www.sanger.ac.uk/). So far, a total of three genome projects have been done on two different species of *Bacteroides*. These genome projects were for *Bacteroides thetaiotaomicron* VPI-5482 (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntbt01), *Bacteroides fragilis* YCH46 (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=ntbf03), and *Bacteroides fragilis* NCTC 9343 (http://www.genedb.org/Homepage/Bfragilis_NCTC9343) (Fig. 6).

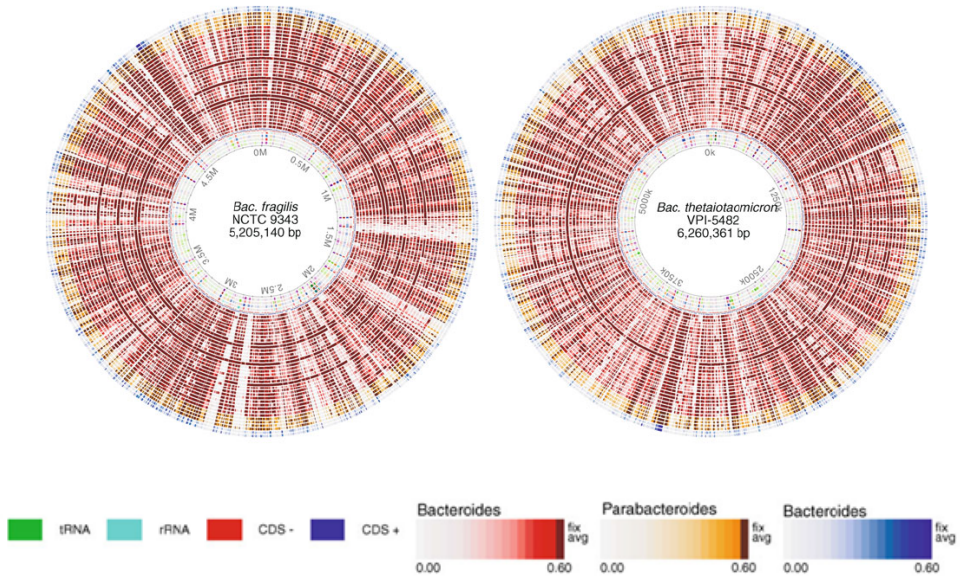


Fig. 6 - Blast atlas of the genome of *B. fragilis* NCTC 9343 and *Bacteroides thetaiotaomicron* VPI-5482 (Karlsson et al., 2010).

In addition to the complete genome, partial genomes of several *Bacteroides* isolates are also available. The Broad Institute (a collaborative effort of MIT and Harvard) has a *Bacteroides* group sequencing project, which has partial genomic information of 30 species/isolates of *Bacteroides* (Table 4).

Table 4 - Genomic characteristics of some *Bacteroides* isolates

Bacteroides Species	Size	%GC	Genes	tRNAs	rRNAs
Bacteroides sp. D1	5.98 Mb	41.91	4,785	61	3
B. sp. D1 Plasmid 1	2.75 Kb	41.64	4	N/A	N/A
Bacteroides sp. 9_1_42FAA	5.58 Mb	42.40	4,824	64	3
B. sp. 9_1_42FAA Plasmid 1	42.91 Kb	31.90	57	N/A	N/A
Bacteroides sp. 2_2_4	7.09 Mb	42.13	5,947	71	3
B. sp. 2_2_4 Plasmid 1	5.59 Kb	39.64	12	N/A	N/A
B. sp. 2_2_4 Plasmid 2	2.74 Kb	41.50	4	N/A	N/A
Bacteroides sp. 2_1_7	5.18 Mb	45.08	4,566	66	2
B. sp 2_1_7 Plasmid 1	2.75 Kb	41.56	5	N/A	N/A
B. fragilis 3_1_12	5.53 Mb	43.63	4,928	63	3
B. fragilis 3_1_12 Plasmid 1	2.78 Kb	41.50	4	N/A	N/A
Bacteroides sp. D2	6.94 Mb	41.71	5,132	65	4
Bacteroides sp. D4	5.53 Mb	41.74	4,431	58	3
Bacteroides sp. 4_3_47FAA	5.45 Mb	42.69	4,615	66	3
Bacteroides sp. 3_2_5	5.16 Mb	43.21	4,505	65	5
Bacteroides sp. 1_1_6	6.86 Mb	43.06	5,594	56	4
Bacteroides sp. 2_1_16	5.24 Mb	43.20	4,609	68	3
Bacteroides sp. 2_1_22	6 Mb	41.89	4,748	62	2
Bacteroides sp. 2_1_33B	4.93 Mb	44.87	3,966	70	2
Bacteroides sp. 3 1 33 FAA	5.42 Mb	42.02	4,667	64	3
Bacteroides sp. D20	4.49 Mb	46.38	3,652	59	4
Bacteroides sp. 1_1_14	6.45 Mb	43.26	5,046	61	7
Bacteroides sp. 3_1_19	5.25 Mb	44.99	4,316	70	10
Bacteroides sp. 3_1_23	6.52 Mb	41.66	5,012	65	8
Bacteroides sp. 20_3	5.75 Mb	45.21	4,889	75	11
Bacteroides sp. D22	6.34 Mb	41.98	5,013	65	8
Bacteroidetes bacterium str. F0058	2.11 Mb	43.05	1,876	44	5
Bacteroides sp. 4_1_36	4.64 Mb	46.37	3,729	57	6
B. eggerthii 1_2_48FAA	4.59 Mb	44.65	3,869	55	7
Bacteroides sp. 3_1_40A	5.51 Mb	42.58	4,570	70	10

Source:http://www.broadinstitute.org/annotation/genome/bacteroides_group/GenomeStats.html

The 16S rRNA gene of *Bacteroides* has been widely used for analysis of the diversity of *Bacteroides* species and differentiating them from neighboring species (Karlsson et al. 2010; Paster et al., 1994). In a recent study a phylogenetic tree was constructed using the 16S rRNA sequence of 33 *Bacteroides,* nine *Prevotella*, eight *Chlorobium*, four *Parabacteroides*, four *Porphyromonas*, and 47 other Genera in the Bacteroidetes/Chlorobi Super phylum (Fig 7). It shows the

Bacteroides genus as one large cluster that includes most *Bacteroides* species; however, *B. pectinophilus* and *B. capillosus* are clustered in a group distant from the other *Bacteroides* species.

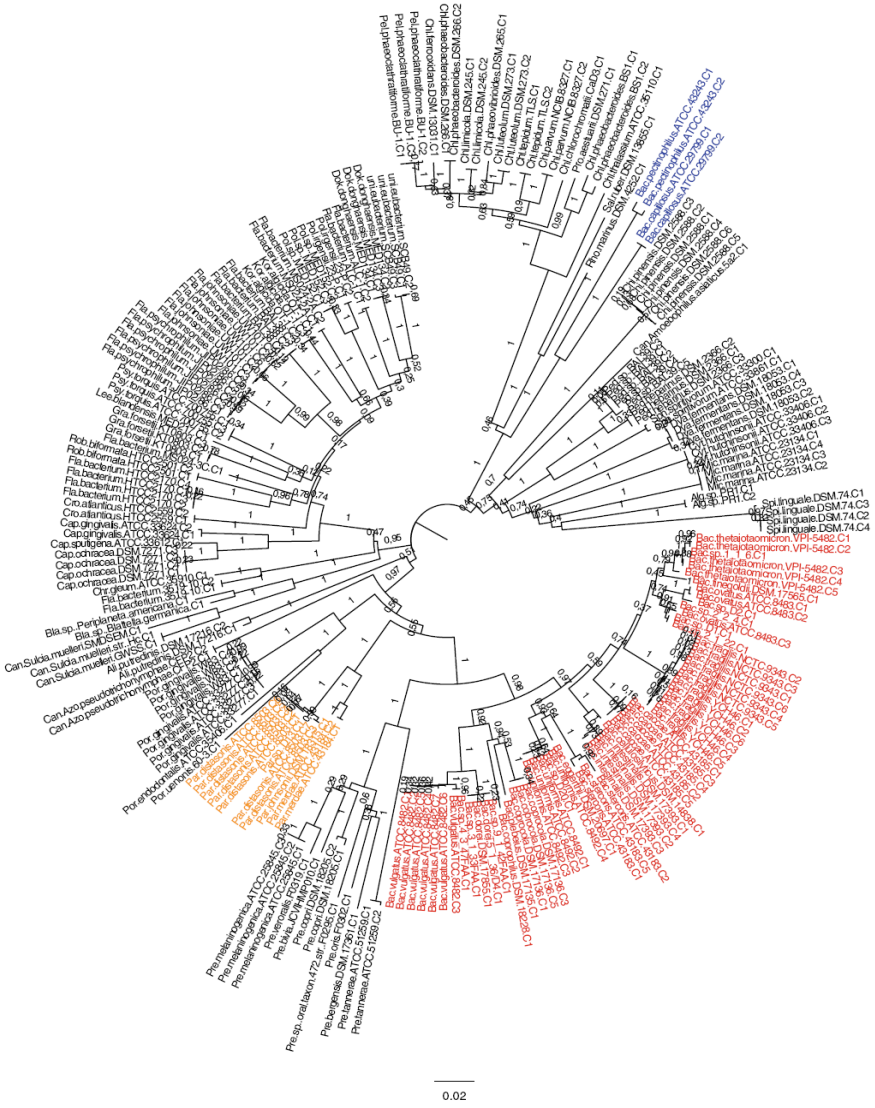


Fig. 7 - Phylogenetic tree based on 16S rRNA sequence. *Bacteroides* sequences are red except for sequences from *B. capillosus* and *B. pectinophilus* which are blue; *Parabacteroides* sequences are orange, and other species are black. Bootstrap values indicate the certainty of each cluster (Karlsson et al., 2010).

Phylogenetic studies have also used Heat-Shock Protein (HSP) genes such as the Hsp60 gene for identification and characterization of *Bacteroides* species. According to a recent study, an analysis based on the *Bacteroides* Hsp60 gene sequence may provide accurate results regarding *Bacteroides* taxonomy. This study suggests that because of the high variability that exists in the Hsp60 gene of *Bacteroides* compared to the 16S rRNA gene, they can be used in the design of more specific primers for PCR for the rapid identification of the *Bacteroides* species (Sakamoto et al., 2010). There are also studies in progress using other types of genes such as dnaJ, gyrB, recA, and rpoB for *Bacteroides* characterization.

2.1.5. Occurrence

Based on culture and culture-independent methods it has been shown that *Bacteroides* species account for 20 to 52% of the human fecal flora (Duerden, 1980; Franks et al., 1998; Hold et al., 2002; Hopkins et al., 2001; Sghir, 2000; Suau et al., 1999). Analyses of the 16S rRNA gene clone libraries suggest *Bacteroides* are slightly less abundant in nonhuman fecal flora. "According to a study about 11.2% of the phylotypes in a pig fecal clone library were related to *Bacteroides* or *Prevotella*" (Leser et al., 2001). Another study has found that about 18% of the recovered sequences in a clone library of horse fecal DNA belong to representatives of the Bacteroidales order (Daly et al., 2001). By analyzing 16S rRNA gene sequences derived from the feces of cattle it has been found that there are large contributions from Bacteroidales in cattle (Bernhard and Field, 2000a; Wood et al., 1998). According to a study, two different obligate

anaerobes from the intestinal tracts of freshwater fish species were identified and based on morphological, biochemical, and physiological characteristics of isolates, as *Bacteroides* types A and B (Sakta et al., 1981). Although many members of the phylum Bacteroidetes have been recovered from the hindgut of termites, no true *Bacteroides* species have been detected thus far from the environment (Ohkuma et al., 2002).

2.2. *Bacteroides* Isolation and Identification Methods

This section will go over different techniques for *Bacteroides* isolation and identification using culture-based methods, biochemical methods, and molecular techniques.

2.2.1. Identification Using Culture-based Methods

Preliminary identification and inoculation of *Bacteroides* can be achieved by using an appropriate combination of enriched, nonselective, selective, and/or differential media. The non-selective isolation of *Bacteroides* can be accomplished on various basal media such as Brucella agar, Brain-heart infusion agar, Colombia agar, and Wilkins-Chalgren agar supplemented with 5-10% horse or sheep blood and hemin (5 µg/ml) (Summanen et al., 1993). The selective isolation of *Bacteroides* can be achieved on either Kanamycin-Vancomycin Laked blood Brucella (KVLB) agar (Summanen et al., 1993) or bile-containing media such as *Bacteroides* Bile Esculin (BBE) agar (Livingston et al., 1978). KVLB agar inhibits growth of most facultative anaerobic bacteria while allowing selective isolation of mainly *Prevotella* species and *Bacteroides* species (kanamycin inhibits growth of facultative gram-negatives while vancomycin

inhibits gram-positives and *Porphyromonas*). BBE is selective for isolation of most of *Bacteroides* species including the *Bacteroides fragilis* group.

Utilizing selective media allows rapid presumptive identification of *Bacteroides* species. Samples cultured on BBE plates and incubated anaerobically can be examined after 18-36 hours. *Bacteroides* species usually form colonies larger than one millimeter in diameter and surrounded by a brown-black zone due to esculin hydrolysis. The *B. fragilis* group hydrolyze esculin to form dextrose and esculetin. This compound reacts with the ferric ions contained within the medium, turning the medium around the colonies a dark brown to black color. The tolerance to bile and hydrolysis of esculin aids in presumptively identifying the *B. fragilis* group. Esculin can be hydrolyzed by some other microorganisms that are bile-resistant and not members of the *B. fragilis* group. Some examples of such microorganisms are *Bacteroides splanchnicus, Fusobacterium mortiferum, Klebsiella pneumoniae, Enterococcus* species, and yeasts (Livingston et al., 1978). In general, *B. fragilis* group colonies are two to three millimeters in size, while the organisms mentioned above are less than one millimeter in diameter. Further examinations such as biochemical tests must be performed to ensure the cultured microorganisms are *Bacteroides* (Finegold et al., 1986; Lennette et al., 1985). Fig 8 shows cultured *Bacteroides* on both blood agar and BBE agar.

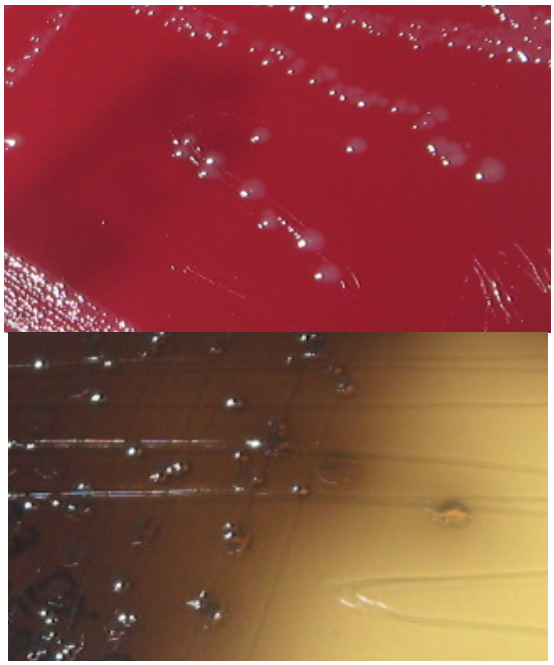


Fig. 8 - Top picture: *B. fragilis* pure culture (ATCC #23745) cultured on Blood agar. Bottom picture: *B. fragilis* pure culture (ATCC #23745) cultured on *Bacteroides* Bile Esculin agar (BBE). Photos: ASU Environmental Microbiology lab.

2.2.2. Identification Using Biochemical Methods

Definite identification and differentiation of *Bacteroides* species can be achieved by assays including fermentation of an array of sugars, biochemical tests, and the detection of fermentative end products by gas/liquid chromatography. “A simplified and rapid scheme for biochemical and fermentative identification of bile resistant *Bacteroides* allows speciation of the group within 24 hours after obtaining a pure culture” (Citron et al., 1990). Various rapid identification systems for anaerobic bacteria such as *Bacteroides* also are available, such as API 20A, Rapid ID 32A and API ZYM (Biomerieux, Durham, NC, USA), Minitek (MT; BBL Microbiology Systems, Cockeysville, Md, USA), and An-Ident (Analytab Products Inc, Plainview, NY, USA) systems. Most of

these systems work by utilizing several chromogenic substrates for the detection of constitutive enzymes. Generally, *Bacteroides* species can be identified using these systems, however, performing additional assays is often recommended since some species of *Bacteroides* cannot easily be distinguished by these phenotypic tests.

2.2.3. Molecular Techniques

Molecular techniques such as restriction endonuclease digestion, DNA or RNA hybridization, PCR, and a combination of these techniques have been widely practiced for the identification of *Bacteroides* species. In the hybridization technique, the chromosomal DNA of *Bacteroides* is extracted and transferred to hybridization membrane pre-fixed by species specific DNA fragment probes. Using the DNA hybridization approach, accurate identification of *B. fragilis* in experimental blood cultures and quantification of *B. thetaiotaomicron, B. uniformis, B. distasonis, B. ovatus,* and *B. volgatus* from fecal samples have been reported (Groves and Clark, 1987). False positive results due to non-specific binding are one of the limitations of this technique (Kuritza and Salyers, 1985).

Restriction Endonuclease Analysis (REA) of chromosomes of many bacteria, such as *Bacteroides* has been performed in determining their genetic relationship. REA is highly reproducible and accurate. For generating a fingerprint of the chromosome, an appropriate restriction endonuclease is utilized to digest total genomic DNA, then the resulting digested DNA is electrophoresed on an agarose gel. The patterns obtained are used to relate species via Restriction Fragment Length Polymorphism (RFLP). One of the limitations of this technique

is that occasionally the generated RFLP patterns are too complicated to be analyzed. To simplify this, they can be coupled to hybridization techniques that use specific DNA probes. “Using this approach, distinct patterns were observed for all of the *Bacteroides* species and each could be easily differentiated” (Smith and Callihan, 1992).

Analysis of the complex REA of chromosomal DNA profiles is tedious due to generation of hundreds of fragments by conventional methods. To resolve this complexity, *Bacteroides* can be differentiated by pulse filed gel electrophoresis (PFGE). This technique separates large DNA fragments that are generated with rare-cutting restriction endonucleases (Shaheduzzaman et al., 1997). The only limitation of this technique is that it cannot resolve bands of nearly identical size (Davis et al., 2003).

The use of PCR has revolutionized *Bacteroides* identification by targeting their 16S rRNA gene. *Bacteroides* species can be reclassified based on sequences of 16S rRNA. This can be achieved by using several published *Bacteroides* 16S rRNA gene molecular markers. The species specific molecular markers for *B. eggerthii*, *B. fragilis*, *B. ovatus*, *B. uniformis*, *B. thetaiotaomicron*, and *B. volgatus* have been used for identification of predominant *Bacteroides* species in the human intestine (Miyamoto et al., 2002).

Bacteroides species can also be identified by restriction endonuclease analysis of PCR amplified DNA. “Analysis of the RFLP patterns generated from PCR-amplified 16S rRNA digested with *Hpa*II and *Taq*I produced profiles that allowed identification of all *Bacteroides* species type strains” (Stubbs et al.,

2000). *Bacteroides* speciation by 16S rDNA PCR-RFLP is a rapid and accurate approach. A similar methodology was used to examine the 16S rRNA internal transcribed spacer region among *Bacteroides* species (Kuwahara et al., 2001). Using this technique, distinctive species-specific RFLP patterns were produced and 90 isolates of *Bacteroides* were differentiated to species level including *B. fragilis, B. distasonis, B. ovatus, B. eggerthii, B. thetaiotaomicron, B. uniformis,* and *B. volgatus*.

Limitations of molecular techniques for bacteria identification include a lack of sensitivity and specificity of assays, high cost, and high level of expertise necessary to perform the techniques (Richards, 1999).

2.3. Microbial Source Tracking

Microbial Source Tracking (MST) is a widely used approach for identification of sources responsible for the fecal pollution of water systems (Stoeckel, 2005). There are numerous MST techniques available which vary in many ways, including costs, level of training, and experience required to implement. MST methods can be divided in two groups: Library-dependent methods or Library-independent methods (Griffith et al., 2003). Library-dependent methods begin with making a database by collection and characterization of a large number of bacteria from known sources (e.g. humans, cows, and wildfowl) and then comparing and contrasting isolates from other sources to the database. There are some limitations associated with the library-dependent technique such as being time consuming and labor intensive. Also, it is not known how large the size of the library must be or how similar libraries from

different geographical areas are (USEPA, 2005). Library-independent methods identify the source of fecal contamination based on detecting host-specific markers.

2.3.1. Library-dependent Methods

Library-dependent methods consist mainly of two types: phenotypic (non-molecular) and genotypic (molecular) methods. Phenotypic analysis is based on cellular or physiological comparisons between the isolates while genotypic analysis is based on comparison of DNA sequence between the isolates. The two most common phenotypic methods used for MST are antibiotic resistance profiles and carbon source utilization. Genotype methods, also referred as fingerprinting methods, rely on molecular techniques that isolate the DNA from the microbes and characterize differences in the nucleic acid sequence. The genotypic approach can target the DNA of the entire organism, particular genes, or a specific DNA sequence. The four most common library based genotypic methods are repetitive extragenic palindromic (rep)-PCR, randomly amplified polymorphic DNA (RAPD) analysis, pulsed field gel electrophoresis (PFGE), and ribotyping.

2.3.2. Library-independent Methods

Library-independent methods are based on the presence or absence of the target organism or gene which does not require a source library. The common library independent methods are examining the ratio of fecal coliform to fecal streptococci, detection of bifidobacteria, detection of *Bacteroides fragilis* bacteriophages and F^+ coliphages, and identification of gene-specific and host specific markers. The application of *Bacteroides* for MST studies is mostly

related to *Bacteroides fragilis* bacteriophage detection and host specific identification, which is further discussed in the following section.

2.3.2.1. *Bacteroides fragilis* Bacteriophages Detection for MST

Bacteriophages that infect *Bacteroides fragilis* strain HSP40 and other strains such as RYC2056 are only found in human feces and hence have been used as specific indicators of human fecal pollution (Tartera and Jofre, 1987). According to a MST study that was performed in Barcelona, Spain, bacteriophages that infect *Bacteroides fragilis* strain HSP40 were detected in water contaminated with the fecal matter of humans, and it was revealed that the CFU concentration was $5.3x10^3$ per 100 ml of water. Moreover, they discovered no *Bacterioides fragilis* bacteriophages were present in water contaminated with fecal matter of wildlife only. Although *Bacterioides fragilis* bacteriophages were present in the water contaminated with human fecal matter, their concentrations were low relative to the number of detected coliphages (Tartera et at., 1989).

An advantage of using *Bacteroides fragilis* bacteriophages as an indicator of fecal contamination is that they do not replicate in the environment (Scott et al., 2002), minimizing the possibility of false positive results. Additionally, their presence in the environment has been found to significantly correlate with the presence of human enteric viruses (Jofre et al., 1989). However, the application of *Bacteroides fragilis* bacteriophages to MST studies is limited, since they are present in low numbers which cannot be detected (Scott et al., 2002). “It is also well documented that *Bacteroides* host strains vary in their ability to discriminate between phages of different sources but also that phage detection by a given host

strain varies geographically" (Payan et al., 2005). This means an assay based on phage detection would be limited geographically.

2.3.2.2. Host Specific Identification for MST

Host specific PCR assay is one of the commonly used techniques for differentiating the source of fecal contamination in environmental samples. The application of these target-specific PCR-based assays is culture-independent and they require host-specific primers for an array of microbes such as *Bacteroides*, *Bifidobacterium*, *Enterococcus*, and viruses. Molecular markers for human and animal associated *Bacteroides* (Bernhard and Field, 2000a,b), human and animal associated *Bifidobacterium* (Nebra et al., 2003), human-specific *Enterococci faecium* species (Scott et al., 2005), and human and animal associated viruses (Fong et al., 2005) have been used in MST studies.

Among different host specific PCR assays, *Bacteroides* targeted PCR assays is the most commonly used for MST studies. The use of *Bacteroides* as a potential fecal indicator was suggested by Fiksdal (1985). The reason that *Bacteroides* was suggested as an indicator for fecal contamination is because they have a short survival rate in the external environment due to their strict growth requirement and occupy a large portion of fecal bacteria compared to fecal coliforms and enterococci (Sghir et al., 2000).

Environmental factors such as temperature, predators in the water, sunlight, and salinity have been tested for their effect on the survival rate of *Bacteroides* species and detection of their molecular markers. Among these different factors, temperature has the highest effect (Table 5). A high rate of

nucleic acid degradation and *Bacteroides* die-off has been correlated to higher temperatures (Schulz and Childers, 2011., Bell et al., 2009; Kreader, 1998; Okabe and Shimazu, 2007; Savichtcheva et al., 2005). Also, there are reports on the effect of predators in the environment on *Bacteroides* die-off (Bell et al., 2009; Kreader, 1998; Okabe and Shimazu, 2007). The effect of sunlight on *Bacteroides* survival is still controversial (Bae and Wuertz, 2009; Walters and Field, 2009; Walters et al., 2009). Bae and Wuertz (2009) found small decay rates due to light at salinity of 33%. Walters and Field (2009) also found small decay rates in freshwater microcosms however the reported decay rates were significantly different. Salinity has not shown a major effect on the survival of *Bacteroides* in the environment (Okabe and Shimazy, 2007).

Table 5 - Effect of temperature on *Bacteroides* decay rate

Temperature (C)	Salinity (%)	Decay Rate* Ln (Ct/Co)d^{-1}
10	0	-0.856
20	0	-1.221
30	0	-1.310
10	5	-0.759
20	5	-0.864
30	5	-0.714
10	30	-0.182
20	30	-0.438
30	30	-0.261

* Log linear decay rates estimated in Ln (Ct/Co)d^{-1} where C_t is the threshold cycle and C_o is the concentration of cells at time zero (Schulz and Childers, 2011).

Several recent studies have proposed the existence of human-specific genetic markers in *Bacteroides-Prevotella* 16S rRNA gene and have developed a method for their detection by conventional and quantitative PCR (Bernhard and Field, 2000a,b; Seurinck et al., 2005; Layton et al., 2006; Reischer et al., 2007). Additionally, PCR assays targeting the *Bacteroides* 16S rRNA gene in organisms from different hosts such as pig, horse, dog, elk, bovine, and cow have been developed as listed in Table 6.

Table 6 - List of some of the host specific PCR assays targeting 16S rRNA gene of *Bacteroides*

Host	References for Previously Designed Host Specific PCR Assays Targeting 16S rRNA Gene of *Bacteroides*
Human	Bernhard and Field, 2000a,b; Seurinck* et al., 2005; Layton* et al., 2006; Reischer* et al., 2007; Okabe* et al., 2007; Kildare* et al., 2007
Pig	Dick et al., 2005a and Okabe* et al., 2007
Horse	Dick et al., 2005a
Dog	Dick et al., 2005b and Kildare* et al., 2007
Elk	Dick et al., 2005a
Bovine	Layton* et al., 2006; Okabe* et al., 2007; and Reischer* et al., 2007
Cow	Kildare* et al., 2007; Bernhard and Field, 2000a,b

* Real time PCR assays

Since the use of *Bacteroides* as a fecal source tracking tool is relatively new, extensive field testing is ongoing to determine specificity of the published assays. Cross-amplification of human-specific *Bacteroides* PCR molecular markers with canine and swine fecal samples has been reported (Layton et al.,

2006; Kildare et al., 2007; Ahmed et al., 2009). Additionally, cross-amplification of human-specific *Bacteroides* genetic markers with fish fecal DNA was reported in my recent work (McLain et al., 2009). The aim of this study is to eliminate the problem of cross-amplification and to develop a strategy for the identification of a human-specific *Bacteroides* using molecular techniques.

Chapter 3

INVESTIGATING SPECIFICITY OF FIVE SETS OF PUBLISHED HUMAN-SPECIFIC *BACTEROIDES* PRIMERS WITH FECAL DNA FROM FOUR FRESHWATER FISH SPECIES

3.1. Abstract

Understanding the origin of fecal pollution of water is paramount in assessing associated health risks and developing effective pollution control strategies. *Bacteroides* genetic markers have been widely used to differentiate human sources of fecal bacteria from other sources in natural water. This study documents cross-amplification of the *Bacteroides* 16S rRNA gene from both freshwater fish and human fecal samples when using human-specific PCR primers. Four out of five protocols previously reported as human-specific assays amplified fecal DNA from at least one fish species. Sequencing of PCR products from fish fecal DNA using Layton's primers (HuBac566F and HuBac692R) revealed no mismatches to human-specific primers. However, the nucleotide sequences of fish fecal clones differed markedly from those of human feces, displaying more than 10% mismatches outside of the primer regions, suggesting that the fish-related bacteria may consist of different strains. The results indicate cross-amplification of current human-specific PCR assays with fish feces, which can call into question the use of the published *Bacteroides* primers in source tracking studies of water where fish contribute to fecal load.

3.2. Introduction

Host specific *Bacteroides* genetic markers have been widely used in Microbial Source Tracking (MST) studies for identifying and quantifying sources of fecal pollution in environmental water. The recent advances in PCR technology have enabled the development of PCR assays to identify host specific *Bacteroides* 16S rRNA gene markers in human and animals (Bernhard and Field, 2000a,b; Kildare et al., 2007; Layton et al., 2006; Reischer et al., 2007). Standard PCR assays (Bernhard et al., 2000a,b; Dick et al., 2005a,b) and quantitative PCR (qPCR) assays (Seurinck et al., 2005; Layton et al., 2006; Kildare et al., 2007; Reischer et al., 2007) have been widely used to determine the relative amounts of host specific fecal contributions to water samples.

Because of the utilization of the molecular *Bacteroides* methodology, extensive testing of potential sources of fecal contamination in surface water for cross-amplification with markers identified as human-specific has resulted in both enhanced specificity and increased scientific acceptance of the MST tool. Several recent studies have evaluated specificity of human-specific *Bacteroides* genetic markers by testing human markers against feces from domestic animals, livestock, and bird and mammal wildlife (Kildare et al., 2007; Ahmed et al., 2009; Layton et al., 2006) (Table 7).

Table 7 - List of human-specific *Bacteroides* genetic markers cross amplified with *Bacteroides* of other animals

Human-Specific *Bacteroides* Primer Sets	Host Species *Bacteroides* DNA Amplified	References reporting cross amplification
HuBac[1]	Cat, Cow, Dog, Duck , Horse, Pig, and Sheep	Kildare et al., 2007; Layton et al., 2006
BacHum[2]	Dog, Horse, Pig, and Sheep	Ahmed et al., 2009
BacH[3]	Dog, Goat, and Sheep	Ahmed et al., 2009
HF183[4]	Cat and Dog	Kildare et al., 2007
HF134[5]	Dog	Ahmed et al., 2008

HuBac[1]: Designed by Layton et al., 2006

BacHum[2]: Designed by Kildare et al., 2007

BacH[3]: Designed by Reischer et al., 2007

HF183[4]: Designed by Seurinck et al., 2005

HF134[5]: Designed by Bernhard and Filed., 2000a

For several decades, *Bacteroides* have been identified as indigenous microflora in the intestine of freshwater fish (Trust and Sparrow, 1974). Obviously, fish contribute to the fecal load in many natural water systems, but to date no fish species has been tested for cross-amplification with published human *Bacteroides* markers. The primary objective of this portion of the study is to evaluate the specificity of five sets of published human-specific *Bacteroides* primers with fecal DNA extracted from four freshwater fish species (tilapia, catfish, trout, and salmon).

3.3. Materials and Method

3.3.1. Fish Fecal Sample Collection

Fresh fecal samples were collected from human and fish sources. The Nile tilapia (*Oreochromis niloticus*) fecal sample was collected from an aquaculture laboratory at the University of Arizona Maricopa Agricultural Center (Maricopa, AZ, USA). Fecal pellets were manually collected from the water of the main aquaculture tank and placed into sterile centrifuge tubes having 20 ml sterile 1X phosphate-buffered saline (PBS).The collection of fecal samples from North American Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) involved removing individual fish from tanks and manually expressing feces into centrifuge tubes containing 20 ml sterile PBS. Collected feces samples were shipped immediately on ice. The source of salmon and trout feces were the USDA-ARS National Cold Water Marine Aquaculture Center, Franklin, ME and the USDA-ARS National Center for Cool and Cold Water Aquaculture, Leetown, WV, respectively. For fecal collection from channel catfish (*Ictalurus punctatus*), one fish was placed into a clean 30-gallon cooler filled with sterile water at the University of Arizona Environmental Research Laboratory (Tucson, AZ, USA). The fish was removed after several hours and the water was filtered to collect feces.

3.3.2. DNA Extraction and PCR

Approximately 0.5 g wet fecal sample was subjected to DNA extraction using the Zymo Fecal DNA Kit (ZymoResearch, Orange, CA) according to manufacturer's protocol. Following the DNA extraction, standard PCR was

performed on extracted DNA samples using five primer sets (Table 8). All PCR reactions were performed using a Promega GoTaq® Green Master Mix (Promega Corp., Madison, WI, USA) with primer concentrations shown in Table 8 and 45-60 ng extracted target DNA in 25 µl reaction mixture. PCR was performed using a Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA) and the reaction mixtures were incubated for 5 min at 95^{o} C and then subjected to 35 cycles consisting of 30 s at 95^{o} C, 45 s at 60^{o} C, and 30 s at 72^{o} C ending with a final extension time of 5 min at 72^{o} C. The reaction mixtures were stored at 4^{o} C until they were analyzed by agarose gel electrophoresis. Agarose gel electrophoresis was performed in1.5% agarose gels containing 0.5µl of SYBR safe DNA gel stain (Invitrogen) per ml. The gels were electrophoresed for 1 h at constant voltage of 100V and were analyzed by a Kodak Gel Logic 112 Digital Imaging System (Carestream Molecular Imaging, New Haven, CT, USA).

Table 8 - Assays used to test for PCR amplification of human and fish fecal DNA samples in this study

Assay	Primer Concentration (nM)	Expected Product Size (bp)	References
HuBacF & HuBacR	600	123	Layton et al., 2006
BacHumF & BacHumR	40	81	Kildare et al., 2007
BacHF & BacHR	200	118	Reischer et al., 2007
HF183F & HF264R	200	82	Seurinck et al., 2005
HF134F & HF654R	400	521	Bernhard & Field., 2000b

3.3.3. Cloning and Sequencing

To identify primer sites within amplified DNA, amplicons generated from human and fish DNA samples using HuBac primers were subjected to cloning and DNA sequencing analysis. Ampilicons were first purified with the QIAquick PCR purification kit (Qiagen Sciences. Calencia, CA, USA) and ligated into TOPO® plasmid vectors (Invitrogen Life Technologies, Carlsbad, CA, USA) and screened for inserts using standard PCR and the methods of (Chung et al., 2006). Positive clones using HuBac primers produced a 203-bp PCR product containing the 123-bp DNA insert and the 80-bp flanking regions of the plasmid vector. Once plasmid inserts were confirmed they were directly sequenced using an automated ABI Prism 3730 DNA Analyzer (Applied Biosystems). Obtained sequences were aligned with ClustalX software (Thompson et al., 1994) and their identity was verified using the Blast program of the Gene Bank database (NCBI).

3.4. Results and Discussion

3.4.1. Cross-amplification testing of human-specific assays

Five sets of primers previously reported as human-specific *Bacteroides* were evaluated for cross-reactivity against *Bacteroides* in fishes' feces and they are presented in Table 9. Results of PCR amplification using HuBac primers on Nile tilapia and human samples are shown in Fig 9.

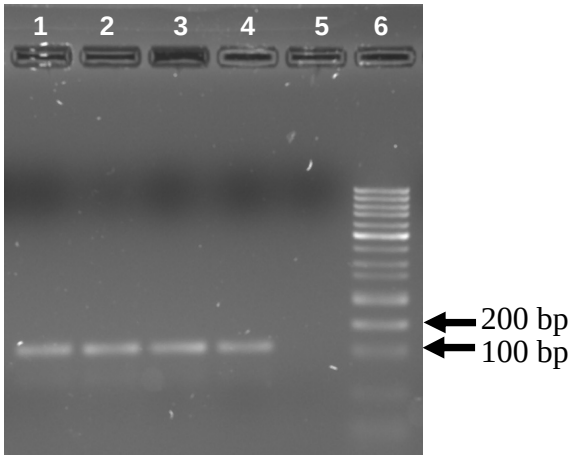


Fig. 9 - Gel photograph of PCR amplification using HuBac primers on extracted fecal DNA of Nile tilapia (Lanes 1and 2) and humans (Lanes 3 and 4). Both fecal sources show expected 123-bp PCR products. Lane 5: negative control (water template); Lane 6: 50-bp DNA ladder.

Table 9 - Evaluation of amplification results of the primer sets specific to human *Bacteroides* on DNA extracted from human and fish samples

	Source of Fecal DNA				
Assay	Human	Tilapia	Catfish	Trout	Salmon
HuBac	+	+	+	+	+
BacHum	+	-	-	+	-
BacH	+	+	+	+	-
HF183	+	-	-	+	-
HF134	+	-	-	-	-

The + symbol denotes generation of PCR product of expected size, while – symbol shows samples without expected sized PCR product.

PCR amplicons of DNA extracted from human feces using the noted primer sets resulted in bands of the expected size. These results were taken as presumptive for successful performance of published protocols. When each of these protocols were tested on DNA extracted from fish fecal samples, HuBac primers amplified all fish samples tested and BacHum, BacH, and HF183 primer sets amplified at least one of the *Bacteroides* samples collected from fish species (Table 9). The HF134 primer set did not amplify DNA samples from fish species. Performed assays suggest that HF134 may provide accurate results in fish-laden water. However, there are reports of cross-amplification of this primer set with fecal DNA from dogs (Ahmed et al., 2009). Therefore, it is highly recommended to also include fish fecal samples in screening potential cross-amplification in MST studies.

3.4.2. Initial alignment of clone sequences from fish fecal bacteria

To identify primer sites within the amplified region of DNA template, the amplicons generated using the HuBac primers were cloned and sequenced. Alignment results of DNA sequences (123-bp) amplified with HuBac primers from human and fish (tilapia) fecal DNA are shown in Fig 10. As illustrated in Fig 10, HuBac primer sequences in the amplified regions are 100% homologous to forward and reverse primers. Also shown in Fig 10, nucleotide sequences of clones from fish fecal samples differed markedly from those of human feces, displaying >10% mismatches outside of the homologous primer sites. Although random misincorporations of nucleotides, resulting in nucleotide substitution, can happen by Taq DNA polymerase, the existence of more than eight mismatches

within the 55 nucleotide region, excluding the primer, suggest that the fish related fecal bacteria are different strains than those of humans. Observed cross reactivity of the HuBac primers with fish feces in PCR did not result from contamination of the fish fecal samples with human feces.

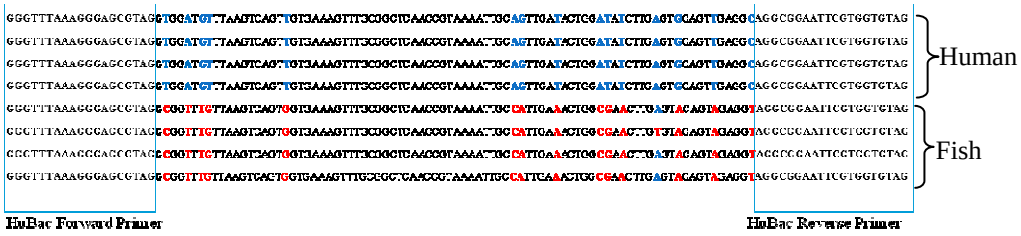


Fig. 10 - DNA sequence alignment of PCR amplicons generated using the HuBac assay on human and fish fecal samples. Highlighted regions indicate mismatches between human and fish *Bacteroides*.

3.4.3. Impact on source-tracking studies for water quality

Accurate identification of the source of fecal pollution in surface water is critical for corrective actions to control fecal pollution. To date, many human-specific *Bacteroides* genetic markers are published in literature, and they only have been examined for cross-reactivity against DNA extracted from feces of animals such as canines, swine, cats, and horses. Cross-amplification of HuBac primers with cattle and swine have been reported (Layton et al., 2006; Kildare et al., 2007; Ahmed et al., 2009). Additionally there are reports of significant cross-amplification of BacHum, HF183, BacH, and HF134 human-specific primers with dog feces (Kildare et al., 2007; Ahmed et al., 2009). Cross-amplification of human-specific assays in dog and cat feces has been reported in several additional studies (Ahmed et al., 2008; Kildare et al., 2007).

Although bacteria of the genus *Bacteroides* have long been identified as indigenous in the intestine of freshwater fish, to date, no fish species have been tested for cross-amplification with any published human *Bacteroides* markers. Fish contribute to fecal load in many natural water systems. Non-specificity of designed human-specific *Bacteroides* molecular markers and their cross-amplification with fish fecal microbes can lead to falsely identifying the source of fecal contamination in surface water; hence, to enhance current microbial source MST, the accuracy of existing human-specific *Bacteroides* assays must be improved. For the first time, the findings of this study documents the importance of including fish samples for cross-evaluation studies, which will help to eliminate a major potential source of error in MST studies (McLain et al., 2009)

3.5. Conclusions

- This study showed cross-amplification of published human-specific *Bacteroides* 16S rRNA genetic markers with fecal DNA from a freshwater fish species.
    - HuBac primers amplified DNA from all fish tested.
    - BacHum, BacH and HF183 amplified fecal DNA from at least one fish species.
    - The HF134 human-specific assay did not amplify fecal DNA extracted from any of the fish. However there are reports of cross-amplification of this human-specific *Bacteroides* primer with dog fecal samples.

- The results indicate that fish samples must be included in screening of potential fecal sources in MST studies utilizing human-specific assays.

Chapter 4

ISOLATION OF *BACTEROIDES* FROM FISH AND HUMAN FECAL SAMPLES FOR IDENTIFICATION OF UNIQUE GENETIC MARKERS

4.1. Abstract

Bacteroides genetic markers have been used to identify human fecal contamination in natural water, but recent work (Chapter 3) confirmed cross-amplification of several human-specific *Bacteroides* assays with fecal DNA from fish. Fish are often natural inhabitants of water bodies under study and thus, these results highlight the need for identification of genetic markers that are unique to human *Bacteroides*. *Bacteroides* Bile Esculin (BBE) agar was used to isolate *Bacteroides* from fish and human fecal samples. The isolated colonies were identified to species level using Rapid ID 32A API® strips. For each of the identified isolates, 16S rDNA was amplified and sequenced to aid in identification of unique markers to be utilized for development of more stringent human-specific assays. In human feces, *B. vulgatus* was dominant, comprising 75% of isolates, whereas in tilapia feces, *B. eggerthii* was dominant (66%). *Bacteroides* from grass carp, channel catfish and blue catfish may include *B. uniformis, B. ovatus,* or *B. sterocoris*. Phylogenic analyses of the 16S rRNA gene sequences showed distinct *Bacteroides* groupings from each fish species, while human sequences clustered with known *B. vulgatus*. None of the fish isolates showed significant similarity to *Bacteroides* sequences currently deposited in NCBI. As a result, this study expands the current sequence database from cultured fish

Bacteroides. Such data are essential for identification of unique genetic markers in fish *Bacteroides* that can be utilized in differentiating fish and human fecal contamination in water samples.

4.2. Introduction

Bacteroides are frequently used as microbial indicators for tracking the sources of fecal pollution using genotypic methods. They are preferred because of their limited potential for growth in the environment, which means that identified genetic markers are proportional to actual fecal contamination. In addition, *Bacteroides* make up a significant portion of the fecal bacterial population and have high degree of host specificity (Bernhard and Field, 2000a).

Bacteroides is the predominant bacterial group in the complex intestinal flora of almost all warm-blooded animals (Mitsuoka et al., 1965) and have also been identified in the intestine of cold-blooded animals such as fish (Trust and Sparrow, 1974; McLain et al., 2009). Recent research efforts have shed some light on the taxonomy and epidemiology of *Bacteroides* (Falagas and Siakavellas, 2000), but due to their strict requirement for anaerobic conditions and abstruse nutritional needs, *Bacteroides* have been difficult to culture under laboratory conditions. Therefore, little is known about the ecology of this bacterial group and its distribution in the intestines of humans and animals.

Isolation and culture of *Bacteroides* from intestinal material can be achieved under anaerobic conditions using variety of selective media such as *Bacteroides* Bile Esculin (BBE) agar (Livingston et al., 1978) and non-selective media such as blood agar with hemin and vitamin K (Summanen et al., 1993). To

aid in characterization of cultured isolates to the genus or species level, biochemical tests such as API® strips can be used (Jenkins et al., 1991**)**. Molecular techniques including Polymerase Chain Reaction (PCR) have also aided in the study of *Bacteroides*, and sequence analysis of their 16S rRNA gene has enabled researchers to characterize previously unknown isolates and increase current understanding of their distribution in the intestinal flora of different animals (Wood et al., 1998; Bernhard and Field, 2000a,b; Leser et al., 2001).

To date, approximately 90 species of *Bacteroides* have been reported; however, the majority of isolated *Bacteroides* belong to following cultivated species: *B. vulgatus, B. thetaiotaomicorn, B. caccae, B. eggerthii, B. fragilis, B. ovatus, B. stercoris,* and *B. uniformis* (Shah and Collins, 1989). Of these known species, three (*B. coprocola, B. uniformis* and *B. vulgatus*) have been shown to be dominant in human feces (Li et al., 2009). *Bacteroides* in animal guts have not been well-characterized, and more so in fish feces. Trust and Sparrow (1974) identified *Bacteroides* in the intestine of freshwater fish, and Sakta et al. (1981) reported the isolation of two obligate anaerobes from fish intestines and classified them as *Bacteroides* type A and B based on morphological, biochemical and physiological characteristics. However, *Bacteroides* type A has since been reclassified as *Cetobacterium somerae* based on the work of Tsuchiya et al. (2008).

Recent work (Chapter 3) showed cross-amplification of several published assays targeting human-specific *Bacteroides* 16S rDNA molecular markers with fecal DNA from four fish species (tilapia, catfish, trout and salmon) (McLain et

al., 2009), suggesting the possibility that fish fecal *Bacteroides* are phylogenetically closely related to *Bacteroides* in human feces. Since fish may inhabit environmental water often tested for the presence of human fecal contamination, it is critical to address the potential for phylogenetic relatedness of fish and human *Bacteroides*. This information will contribute to the current database of *Bacteroides* phylogenetics and may allow identification of unique genetic markers in human and/or fish fecal DNA to eliminate misidentification of sources of fecal contamination.

To investigate this possibility, *Bacteroides* isolates from human and fish fecal samples were cultured. Following biochemical testing to classify the *Bacteroides* isolates, the 16S rDNA gene was amplified, and the amplicons were sequenced to determine genetic relatedness of *Bacteroides* isolates from human and fish feces. Ultimately, this work will aid in the development of molecular assays that will differentiate human and fish *Bacteroides*.

4.3. Materials and Methods

4.3.1. Fish Sample Collection

Fresh fecal samples were collected from fish and human subjects. Nile tilapia (*Oreochromis niloticus*) feces were collected from aquaculture laboratory at the University of Arizona, Maricopa Agricultural Center (Maricopa, AZ, USA). Fecal pellets were collected manually from the water in the main aquaculture tank and placed into sterile centrifuge tubes containing 20 ml sterile 1X phosphate-buffered saline (PBS). The fecal sample of Grass carp (*Ctenophyaryn godonidella*), blue catfish (*Ictalurus furcatus*), and channel catfish (*I. punctatus*)

were provided by the USDA-ARS Catfish Genetic Research Lab (Stoneville, MS, USA). These samples were collected by removing individual fish from tanks and manually expressing feces into centrifuge tubes containing 20 ml sterile PBS. The tubes containing fecal samples were shipped on ice to the environmental microbiology laboratory at ASU. Rainbow trout (*Oncorhynchus mykiss*) fecal samples were also collected, using manual fecal expression, from the Page Springs Fish Hatchery, Arizona Game and Fish Department (Phoenix, AZ, USA).

4.3.2. Isolation of *Bacteroides* using culture-based techniques

In the laboratory, isolation and identification of *Bacteroides* proceeded as described in Wadsworth-KTL Anaerobic Bacteriology Manual (Jousimies-Somer et al., 2002) and the Manual of Clinical Microbiology (Jousimies-Somer et al., 2003) with little modifications. First, fecal samples (from 20 ml sterile PBS) were streaked onto two types of media: blood agar with hemin and vitamin K and BBE agar (Hardy Diagnostics, Santa Maria, CA, USA). The streaked culture plates were placed in Bio-Bag^{TM} Type A systems (BD-Diagnostic Systems, Franklin Lakes, NJ, USA) to maintain an anaerobic atmosphere and incubated at 37° C. Each Bio-Bag contained aresazurin oxygen reduction indicator to ensure anaerobicity during incubation. BBE plates were examined after three days and colonies with representative morphology and color to *Bacteroides* (gray, circular and raised colonies; approximately 1 mm in diameter), were randomly selected and subcultured onto chopped meat medium (Hardy Diagnostics) and incubated under anaerobic conditions at 37° C for 24 h to obtain high cell counts. Fig 11 shows an example of the chopped medium and the biobag used in this experiment.

Fig. 11 - Anaerobic bag, inoculated (right) and uninoculated (left) chopped meat medium tubes. The tubes were inoculated using colonies from BBE plate to obtain culture for API strip procedure.

4.3.3. *Bacteroides* Identification using Biochemical Methods

Bacteroides isolates were subcultured in chopped meat medium to obtain higher number of cells required for the biochemical identification procedure using API® Rapid ID 32A strips (Biomerieux, Durham, NC, USA) to further identify the isolates to the species level. Rapid ID 32A is a standardized system for identification of anaerobic bacteria utilizing 29 miniaturized enzymatic tests. Each enzymatic reaction is identified via a colorimetric assay, and patterns of color development are compared to a database of patterns from known *Bacteroides* species, resulting in "percentage relatedness" of the unknown isolate to the known *Bacteroides* species. The API® test was performed according to manufacturer's instructions, with the exception that instead of harvesting colonies directly from blood agar and suspending it in the API® suspension buffer, higher cell density was achieved by centrifugation of the *Bacteroides* grown in the chopped meat medium and suspension of the resulting pellet in API® buffer. Fifty

µl of suspended cells were loaded into miniaturized wells (cupules) on the API® strip, each containing a different substrate used to determine the metabolic capabilities of the isolates. Inoculated strip were incubated at 37° C for 4 h followed by addition of specific reagents resulting in color formation. “Percentage relatedness” to known *Bacteroides* species was determined using color coding in accordance to manufacturer instructions and APIweb™ identification software. *B. fragilis* (ATCC# 23745) served as a positive control strain for both culturing and the API® test. An example of the API strip used in this experiment is shown in Fig 12. The API® strips were selected as an identification tool since it is a practical and easy to use, uses direct bacterial cells and offers a large and robust database which is accessible through the Internet-based APIweb™ service.

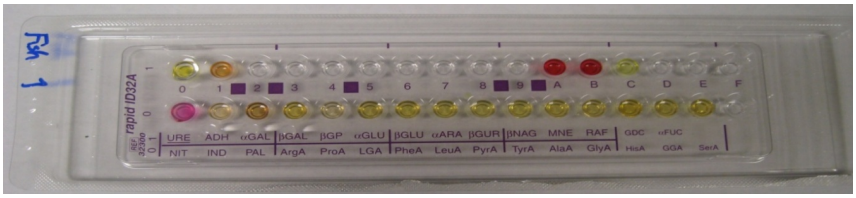


Fig. 12 - API test strip (Rapid ID 32A) used to speciate *Bacteroides* harvested from fish fecal sample

4.3.4. DNA Extraction and PCR Amplification

To compare the 16S rDNA sequences from the *Bacteroides* cultured from human and fish feces, PCR, cloning and sequencing were performed. Isolates identified as *Bacteroides* using API® strips were grown in chopped meat medium to obtain a high cell density, and DNA was extracted from 1 ml of the culture using the Zymo Fecal DNA Kit (Zymo Research, Orange, CA, USA). Standard

PCR was performed on all culture-identified *Bacteroides* species from humans and fish. *Bacteroides*16S rDNA from human isolates were amplified using the primers of Bernhard and Field (2000b) (Table 10). This PCR assay produces a longer product size (~534 bp) compared to other human-specific *Bacteroides* PCR assays, and has also been shown not to amplify fish *Bacteroides* (McLain et al., 2009). For fish fecal DNA, a combination of a universal forward primer (Kane et al., 1993) and a reverse primer shown to amplify fish *Bacteroides* (McLain et al., 2009) were used to generate longer sequences of the 16S rRNA gene (~755 bp). To align longer segments of 16S rDNA from the *Bacteroides* isolates, universal primers (Kane et al., 1993) were used to obtain product size of ~1381bp.

Table 10 - Assays used to amplify 16S rRNA gene of *Bacteroides* from human and fish samples

Assay	Expected Amplicon Size (bp)	Reference
HF134F:5′-GCCGTCTACTCTTGGCC-3′ HF654R:5′-CCTGCCTCTACTGTACTC-3′	534	Bernhard and Field, 2000b
11f: 5′-GTTTGATCCTGGCTCAG-3′ HuBac692r:5′CTACACCACGAATTCCGCCT-3′	755	Kane et al., 1993; Layton et al., 2006
11f[1]: 5′-GTTTGATCCTGGCTCAG-3′ 1392r[1]:5′-ACGGGCGGTGTGTAC-3′	1381	Kane et al.,1993

[1]Universal Primers

All PCR reactions were performed using a Promega GoTaq® Green Master Mix (Promega Corp., Madison, WI, USA) with either 600 nM (11f and HuBac692r and11f and 1392r) or 400 nM (HF134F and HF654R) primer concentrations in 25 µl final volume. PCR was performed using a Gene Amp

PCR System 9700 (PE Applied Biosystems, Foster City, CA) with a temperature profile: 5 min at 95^{o} C; 35 cycles of 30s at 95^{o} C, 45s at 60^{o} C and 30s at 72^{o} C; and a final extension of 5 min at 72^{o} C. PCR products were visualized using electrophoresis on 1.5% agarose gels containing 0.5µl/ml of SYBR safe DNA gel stain (Invitrogen Life Technologies, Carlsbad, CA, USA) for 1 h at 100V and were analyzed by a Kodak Gel Logic 112 Digital Imaging System (Carestream Molecular Imaging, New Haven, CT, USA).

4.3.5. Molecular Techniques: Cloning, DNA Sequencing and Phylogenetic Tree Construction

The 16S gene amplicons from the identified species were cloned and sequenced. The amplicons were purified using a QIAquick PCR purification kit (Qiagen Sciences. Calencia, CA, USA) and ligated into TOPO® plasmid vectors (Invitrogen) following the manufacturer's instructions. Clones were screened for inserts using the standard PCR methods of Chung et al. (2006). Screening targeted the expected product size, plus the 80 bp flanking regions of the plasmid vector.

Plasmid inserts were directly sequenced using an automated ABI Prism 3730 DNA Analyzer (Applied Biosystems). The 16S rDNA obtained sequences were blasted using the NCBI Gene Bank (http://www.ncbi.nlm.nih.gov/) to identify *Bacteroides* spp. with homologues sequences currently available in NCBI database (Table 11). The BLAST search resulted in the identification of *B. vulgates*, *B. eggerthii*, *B. graminisolvens* and *Cetobacterium somerae* as a possible match. Thereafter, a thorough literature review identified the following *Bacteroides* species to be dominant in animal feces: *B. vulgatus*, *B.*

thetaiotaomicorn, B. caccae, B. eggerthii, B. fragilis, B. ovatus, B. stercoris, and *B. uniformis*. The accession numbers for these species reported in the literature were used to retrieve sequence data from the Gene Bank. The sequence data from this study and the sequence data retrieved from the Gene Bank were used to generate phylogenetic tree for identifying the relatedness of *Bacteroides* isolates from this study with the isolates reported in the literature.

Table 11 - Bacterial species in NCBI Gene Bank homologues to 16S rDNA sequences obtained for this study.

NCBI available sequences for cultured isolates	NCBI available sequences for uncultured isolates
Bacteroides vulgates ATCC (8482) (CP000139.1) (99%)	Bacterium from human fecal sample (EF404383) (99%)
Bacteroides eggerthii (AB510700.1) (89%)	Bacterium from yellow catfish (*Pelteobagrus fulvidraco*) (GQ360025.1) (99%)
Bacteroides graminisolvens (AB547643) (94%)	Bacterium from Chinese mitten crab (DQ856503.1) (98%)
Cetobacterium somerae from common carp (AB353124) (99%)	Bacterium from zebra fish (HM778680) (99%)

4.4. Results and Discussion

4.4.1. *Bacteroides* culturing and API® test Results

After three days of incubation under anaerobic conditions, most of the bacterial cultures from human and fish fecal samples appeared as gray, entire (unbroken), circular, raised colonies. Those displaying this morphology and greater than 1mm in diameter were presumptively identified as *Bacteroides* (Livingston et al., 1978). Media surrounding the colonies displayed a brown to

black color resulting from the hydrolysis of esculin within the media and the production of insoluble iron salts (Fig 13).

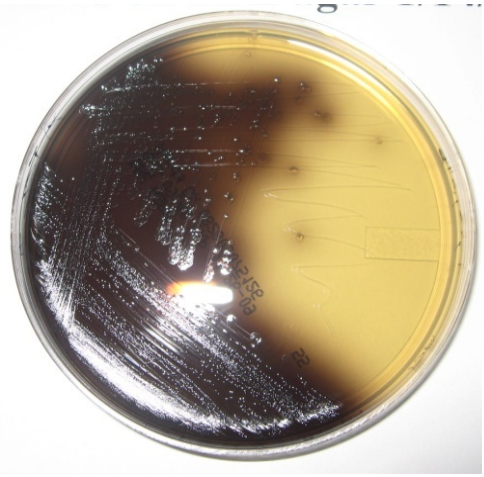


Fig.13 - *Bacteroides* colonies cultured from fish fecal sample on BBE plate

The colonies from each BBE plate resulted in positive identification of 33-75% of *Bacteroides* species (Table 12). Of the *Bacteroides* isolates from human feces 75% were identified as *B. vulgatus*. Moore and Holdman (1974) and Benno et al. (1989) also demonstrated that *B. vulgatus* is one of the dominant *Bacteroides* species in human fecal flora.

In the case of Nile tilapia, 66% of isolated colonies were identified as *B. eggerthii*. Tentative *Bacteroides* isolates from grass carp, channel catfish and blue catfish did not correspond to previously identified *Bacteroides* spp. using API® strip analysis; however, they were successfully identified to the genus level and were narrowed down to three most probable *Bacteroides* species (*B. uniformis*; *B. ovatus*; and *B. sterocoris*).

Though bacterial isolates from trout exhibited *Bacteroides* specific characteristics on the BBE plate (Livingston et al., 1978), the API® strip analyses identified them as *Clostridium perfringens*. Alignment of the 16S gene amplicon indicated that the isolate is closely related to *Cetobacterium somerae* isolated from common carp (AB353124). This was in agreement with Tsuchiya et al. (2008) who concluded that *Bacteroides* type A recovered from freshwater fish intestines are closely related to *Cetobacterium somerae*. Studies have shown that *Bacteroides* species are divergent. For example, a recent study analyzing the genomic information of all the *Bacteroides* species available in genomic databases reported that some of the *Bacteroides* strains have characteristics similar to *Clostridium* and should be reclassified (Karlsson et al., 2010). According to this study, the 16S rRNA gene sequences of *B. capillosus* share 96-98% similarity with sequences of *Clostridium orbiscindens* and should be reclassified to genus *Pseudo flavonifractor*.

The API® strip was originally developed for clinical applications and since the clinical samples contain less diversity of *Bacteroides* population, the analysis is only based on 29 substrates spectrum of metabolic diversity, therefore it provides limited applicability for environmental samples. However, for environmental isolates of *Bacteroides*, enzymatic tests based on broader range of substrates (up to 133) can provide appropriate bases for proper identification of *Bacteroides* species (Karlsson et al., 2010). Misidentification of divergent *Bacteroides* species in environmental samples is highly likely using API® strips, which are primarily designed for clinical applications. However, API® strips are

one step closer to the identification of the cultures, which can be further characterized using sequencing.

After API® identification, one *Bacteroides* isolate from each representative fish and human was sequenced and blasted against cultured and uncultured 16S rRNA gene sequences in the NCBI Gene Bank Database (Table 12). The fact that the 16S rRNA gene sequences from cultured fish *Bacteroides* did not match closely to any 16S rRNA gene sequences from cultured *Bacteroides* in the NCBI database is not surprising. Despite reports of isolates of obligate and facultative anaerobes from fish (Sakata et al., 1981), the sequences deposited in NCBI are most frequently from mammals, and to the best of my knowledge no study has reported the cultured isolates of *Bacteroides* species in the feces of fish prevalent in the U.S. water. In addition to the preference for mammalian sequences in the NCBI database, fish isolates are extremely rare. This might be due to the fact that culturing techniques for isolation of *Bacteroides* are a tedious task to perform. Studies have shown that 60 to 80% of human intestinal microflora are difficult to culture and these numbers are even greater for other animal hosts (Hold et al., 2002; Leser et al., 2001).This can explain why most bacterial 16S rRNA gene sequences from environmental samples deposited in NCBI are based on metagenomics studies and not from isolated colonies. Despite these limitations, the genomic samples contained in the NCBI database did aid in *Bacteroides* identification, as isolates from tilapia and trout closely matched an uncultured bacterium from yellow catfish and zebra fish (Table 12), providing

evidence of the value of culturing techniques to the strengthening of the NCBI gene bank database.

Table 12 - Characterization of *Bacteroides* species isolated from human and fish feces using culture-based and molecular techniques

Sample Source	Growth		Colony Appearance on BBE Agar	API® Strips[1] (Rapid ID 32A)	% Match of 16S sequences from *Bacteroides* isolated in this study with the16S sequences in NCBI[2] (reported from cultured samples)	% Match of 16S sequences from *Bacteroides* isolated in this study with the 16S sequences in NCBI (reported from uncultured samples / metagenomic data)
	Blood Agar	BBE Agar				
***Bacteroides fragilis* (ATCC#23745)**	+	+	Gray, Raised, Circle, Blackish coloration in the media	*Bacteroides fragilis*	ND (Not Determined)	ND
Human	+	+	Gray, Raised, Circle, Blackish coloration in the media	75% of colonies identified on BBE agar were *Bacteroides vulgates* (%ID=99.4, T = 0.2)	99% match to *B. vulgates* ATCC (8482) (CP000139.1)	99% match to uncultured bacterium from human fecal sample (EF404383)
Nile tilapia	+	+	Gray, Raised, Circle, Blackish coloration in the media	66% of colonies identified on BBE agar were *Bacteroides eggerthii* (%ID =99.8, T=0.41)	89% match to *B. eggerthii* (AB510700.1)	99% match to Uncultured bacterium from yellow catfish (Pelteobagrus fulvidraco) (GQ360025.1)
Grass carp	+	+	Gray, Raised, Circle, Blackish coloration in the media	33% of colonies identified on BBE agar were either *B. uniformis* (%ID =59, T=0.5), *B. ovatus* (%ID = 32.3, T=0.51), or *B. stercoris* (%ID = 5.7, T=0.34)	94% match to *B. graminisolvens* (AB547643)	98% match to uncultured bacterium from Chinese mitten crab (DQ856503.1)
Blue catfish	+	+	Gray, Raised, Circle, Blackish coloration in the media	66% of colonies identified on BBE agar were either *B. uniformis* (%ID =59, T=0.5), *B. ovatus* (%ID = 32.3, T=0.51), or *B. stercoris* (%ID = 5.7, T=0.34)	93% match to *B. graminisolvens* (AB547643)	98% match to uncultured bacterium from Chinese mitten crab (DQ856503.1)
Channel catfish	+	+	Gray, Raised, Circle, Blackish coloration in the media	33% of colonies identified on BBE agar were either *B. uniformis* (%ID =59, T=0.5), *B. ovatus* (%ID = 32.3, T=0.51), or *B. stercoris* (%ID = 5.7, T=0.34)	94% match to *B. graminisolvens* (AB547643)	98% match to uncultured bacterium from Chinese mitten crab (DQ856503.1)
Trout	+	+	Gray, Raised, Circle, Blackish coloration in the media	66% of colonies identified on BBE agar were *Clostridium perfringens* (%ID = 99.9, T = 0.33)	99% match to *Cetobacterium somerae* from common carp (AB353124)	99% match to Uncultured bacterium from zebra fish (HM778680) 99% match to Uncultured bacterium from yellow catfish (Pelteobagrus fulvidraco) (GU293182)

API® Strips[1]:'T' denotes reliability of identification test results. For *Bacteroides*, the API® %ID of 80 or higher it would be valid for species identification, whereas below 80% is accurate for the genus level according to manufacturer manual.
Excellent Identification up to species level (%ID >= 99.9 & T > = 0.75), Very Good Identification to species level (%ID >= 99.0 & T > = 0.5), Good Identification (%ID >= 90.0 & T > = 0.25), Acceptable Identification (%ID >= 80.0 & T > = 0).T: Reliability of identification test result.

NCBI[2]: National Center for Biotechnology Information, Gene Bank data base.

4.4.2. Phylogenetic analysis of partial *Bacteroides* 16S rDNA sequence from human and fish

For phylogenetic investigation, a clone library was constructed by amplifying (~600bp and ~ 1381bp) the 16S rDNA from *Bacteroides* isolates from human and fish feces. The phylogenetic analyses showed sequences of *Bacteroides* isolates clustered into four clade representing species from human and fish samples (Fig 14). Interestingly, it was noted that *Bacteroides* from different fish species were grouped differently. The observed diversity among *Bacteroides* isolates from different fish species can be due to the variation in ecological zone from where the fish samples were collected. There are reports supporting that microbial diversity in the fish intestinal tract may vary due to different environmental conditions such as diet and the type and quality of water (Yoshimizu et al., 1980; Austin and Al-Zahrani, 1988; Bergh et al., 1994).

The phylogenetic analysis shows the sequences from human *Bacteroides* and *B. vulgatus* in the same clade. It was expected based on the results obtained by API® strip tests. This is also in agreement with the literature since *B. vulgatus* is one of the dominant *Bacteroides* species in human feces (Li et al., 2009). Tilapia sequences as well as uncultured *Bacteroides* from trout clustered together with NCBI uncultured bacterium from yellow catfish. Grass carp, blue catfish and channel catfish all branched into two separate groups. It was also observed that human isolates are closely related to *Bacteroides* strains from the NCBI Gene Bank (*B. vulgatus, B. uniformis, B. eggerthii, B. stercoris, B. ovatus, B. caccae, B. fragilis, and B. thetaiotaomicron*) compared to fish *Bacteroides*. Fish *Bacteroides*

showed no close match to cultured *Bacteroides* sequences, illustrating the need for expanding the genomic data base for fish *Bacteroides* in NCBI.

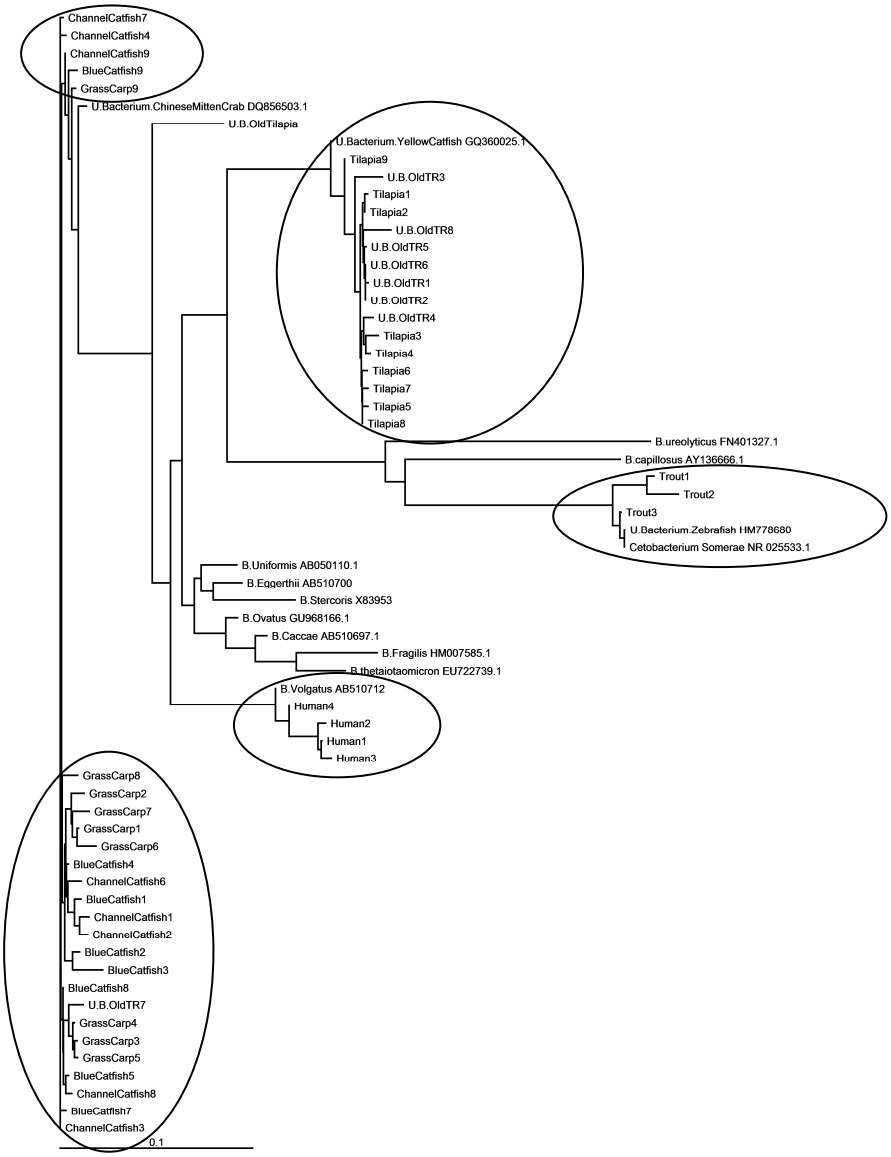


Fig. 14 - Phylogenetic relationship of *Bacteroides* 16S gene sequences (~600 and ~1381bp) of clones recovered in this study. Sequences presented are from cultured *Bacteroides* isolated in this study from human and fish species (tilapia, blue catfish, channel catfish and grass carp) and uncultured *Bacteroides* obtained in this study from fish species (tilapia, catfish and trout) and 10 strains of *Bacteroides* and one strain of *Cetobacterium somerae* (only designated as cultured) obtained from NCBI Gene Back. The NCBI strains in the tree are listed with their accession numbers. The numbers next to fish names and human are referred to the individual clone within the library. In addition, three uncultured *Bacteroides* (U.B.) from yellow catfish, Chinese mitten crab and zebra fish were included from NCBI in this phylogenetic analysis. Uncultured *Bacteroides* obtained in the lab are listed in the tree and starts with (U.B Old). ClustalX software was used for generation of this phylogenic tree.

To accomplish the objective of this study it was essential to first culture *Bacteroides* from human and fish fecal samples to obtain pure cultures and then identify at the species level using API® test strips. In addition, the 16S rDNA gene analysis was also a necessary step. Knowing the phylogenetic placement of bacterial isolates is a critical step in deciphering molecular markers for their specific identification (Spanggaard et al., 1999).

Despite the fact that cultured-based identification of *Bacteroides* is labor intensive procedure, the above strategy is valuable for generating pure culture isolates and for presumptive speciation of bacterial isolates. It is acknowledged that PCR and subsequent sequencing is an alternative for definitive identification of the species of the tested microorganisms, in addition, the amplicons from the isolated colonies can be used as a confirmatory tool for identifying environmental isolates. Such confirmation is an essential part of quality control and quality assurance for unknown samples. The proposed strategy offers a platform to identify environmental samples/isolates at a higher confidence level and allows subsequent cloning and sequencing to increase the utility of molecular techniques by selecting better molecular bio-marker. The use of culture based methods in conjunction with molecular techniques such as cloning and sequencing allows identifying variable regions and provides a basis for developing a new Microbial Source Tracking (MST) method using human-specific *Bacteroides* molecular marker.

Nucleotide sequence accession numbers. All of the sequences determined in this study have been deposited in the Gene Bank database under accession numbers JQ317220 to JQ317269.

4.5. Conclusions

- Human and fish *Bacteroides* were successfully cultured on BBE agar and identified using API® Strip.
- API® test results indicated that dominant *Bacteroides* species are as follows:
    - *Bacteroides vulgatus* in human sample
    - *Bacteroides eggerthii* in Nile tilapia sample
    - *Bacteroides uniformis, Bacteroides ovatus, and Bacteroides sterocoris* in either grass carp, channel catfish or blue catfish
- From phylogenetic tree analysis, it can be observed that fish sequences from this study did not group with sequences of NCBI "cultured" species.
    - Limited available "cultured" sequences in NCBI may be due to the difficulties of *Bacteroides* cultivation.
    - This study will help to fill the gap of the differences between fish and human *Bacteroides* for the characterization of specific genetic markers.
- The strategy of using combined cultured isolates and sequencing in this study will provide a basis for developing a new MST method using human-specific *Bacteroides* genetic marker. This is essential since most of

the genomic information deposited in NCBI gene bank is based on metagenomics.

- This study expands culture-based sequence database for fish *Bacteroides* which is essential for characterizing the genetic markers of fish *Bacteroides* and separating them from human *Bacteroides* for future MST studies.

Chapter 5

A NEW MICROBIAL SOURCE TRAKING STRATEGY USING *BACTEROIDES* 16S rRNA SIGNATURES IN WATER SOURCES

5.1. Abstract

Bacteroides genetic markers have been widely used to differentiate human from other sources of fecal bacteria contamination in water. The work presented in Chapter 3 indicates that many assays currently used to detect human-specific *Bacteroides* produce false positive results in the presence of fish fecal contamination. *Bacteroides* 16S rRNA gene sequences obtained from fish and other selected animals were used to achieve the objective of this study: to develop a new Microbial Source Tracking (MST) strategy relying on a human-specific *Bacteroides* genetic marker.

Bacteroides 16S rRNA gene sequences from fish and selected animals were aligned against human *B. volgatus*, *B. fragilis*, and *B. dorei* to identify variable regions: region 1 (71-101), region 2 (142-271), region 3 (451-511) and region 4 (581-701), within the *Bacteroides* 16S rRNA gene sequence. Conserved sequences between regions were deleted to develop a truncated sequence. The non-truncated and truncated sequences were used to construct cladograms. The cladogram constructed from truncated sequences show a clear separation of human *B. dorei* from *Bacteroides* of other sources. The proposed strategy was field tested by collecting water samples from central AZ source water and three different recreational ponds. PCR using HF134 and HF183 primer sets was

performed and sequences from positive reactions were aligned against human *Bacteroides* sequences to identify the source of contamination.

Based on PCR results, the source of fecal contamination was presumptively identified. For the samples tested positive using HF183 primer set (8/13), fecal contamination was determined to be from human sources. To confirm the results, PCR products were sequenced and aligned against the four variable regions and then incorporated within the truncated cladogram. As expected, the sequences from water samples with human fecal contamination were grouped within the human clade.

A variability matrix developed after exclusion of conserved sequences amongst the four regions was utilized to establish clear groupings for sequences within the truncated cladogram, sufficiently differentiating *Bacteroides* isolates from varying host animals. The proposed strategy offers a new method for MST and a step-wise methodology essential for identifying sources of fecal pollution is provided.

5.2. Introduction

Water quality in rivers, ponds, and lakes is frequently degraded by fecal contamination from human and animal sources, imposing negative implications for recreational uses and public safety (Bernhard et al., 2003). According to the U.S Environmental Protection Agency (EPA), the standard method for identification of fecal pollution in water is based on cultivation of fecal indicator bacteria (FIB) such as *Escherichia coli* and *Enterococci*. Although this method identifies incidents of fecal pollution in water, it does not identify the source of

contamination, hence making fecal pollution control efforts ineffective in many situations (Walters and Field, 2009).

Over the past several years scientists have been using members of the genus *Bacteroides* to identify and quantify the source of fecal pollution via molecular techniques (Bernhard and Field., 2000a,b; Seurinck et al., 2005; Layton et al., 2006; Reischer et al., 2007). *Bacteroides* is potentially an ideal target organism for MST studies due to their high numbers in the gut and fecal microbiota, inability to survive in oxygenated water, and high degree of host specificity (Bernhard and Field, 2000a). Additionally, "*Bacteroides* has shown to correlate with the presence of fecal pathogens such as *E. coli* O157:H7, *Salmonella*, and *Campylobacter*" (Walters and Field, 2009).

Waters contaminated with human feces are generally considered a greater risk to human health, as they are more likely to contain human enteric pathogens (Guzewich and Morse, 1986). For this reason, identification of human fecal pollution in water bodies is critical. Several assays using human-specific *Bacteroides* genetic markers have been developed to identify fecal sources and quantify *Bacteroides* in water (Bernhard et al., 2000a; Seurinck et al., 2005; Layton et al., 2006; Kildare et al., 2007; Reischer et al., 2007). The majority of assays used are designed to target *B. volgatus* from human fecal samples. It is known that some members of *Bacteroides* species such as *B. fragilis*, *B. thetaiomicron*, and *B. volgatus* are 100 to 1000 fold more abundant in human feces than in animal feces, suggesting that markers from these species are human-specific (Pei-Ying et al., 2008). In contrast to this idea, according to a study

performed by Dick et al. (2005a), many *Bacteroides* species such as *B. volgatus*, *B. uniformis*, *B. thetaiotaomicron,* and *B. stercoris* may not be useful as targets for identification of human fecal contamination. Their assertion/conclusion was based on the fact that these *Bacteroides* species are detected in the feces of non human hosts such as dogs, cats, gulls, and pigs when using human-specific *Bacteroides* genetic markers, leading to false positive results.

In order to eliminate false positive results, the *Bacteroides* species used for human source identification must either not be present or, if present, exist in very low numbers within non-human sources. *B. dorei* is a novel *Bacteroides* species isolated from human feces with 96% similarity to *B. volgatus* and 93% similarity to *B. massiliensis* based on the 16S rRNA gene (Bakir et al., 2006). To detect *B. dorei*, the HF183 forward primer designed by Bernhard and Field (2000b) can be used for presumptive identification; however, it is not reliable to do so without verifying the identity of amplified PCR products via sequencing.

In addition to relying on host-specific *Bacteroides* genetic markers, source tracking can potentially be achieved by performing sequence alignment analysis to identify variable regions. Many bacterial 16S rRNA genes contain nine variable regions. Based on these variable regions, an assay can be designed to detect the sequence of the target organism (Van de Peer et al., 1996). By performing *Bacteroides* 16S rRNA gene sequence alignment from different sources and comparing them with human-specific *Bacteroides*, it is possible to look for unique regions within the gene and develop a strategy for source tracking.

The cross-amplification of human-specific *Bacteroides* markers with the DNA extracted from fish feces was an unexpected finding (McLain et al., 2009). This led to closer examination of fish *Bacteroides* to avoid false positive results when using the proposed strategy. The objective of this study was to develop a tool box strategy for *Bacteroides* source identification relying on PCR amplification, variable region analysis, human-specific *Bacteroides* PCR assays, and subsequent truncated cladogram grouping analysis. By following the proposed strategy, accurate source identification can be achieved by eliminating problems of cross amplification with *Bacteroides* from non-human hosts, particularly with fish and dog.

5.3. Materials and Methods

This section is divided in two parts: 1) development of a tool box strategy for *Bacteroides* source identification, and 2) field testing of the proposed strategy for source identification.

5.3.1. Developing a Tool Box Strategy for Source Identification

5.3.1.1. Fecal Sample Collection

Human fecal samples were collected from five healthy adults. Prior to the sample collection, the objectives of study were explained to subjects and their participation was voluntary. Animal feces were collected from apparently healthy animals (4 dogs, 2 cows, 2 horses, 1 cat, 1 pig and 1 duck). Dog samples were collected from four different house pets (Golden Retriever and Pomeranian, in Tempe, AZ, German Shepherd and English Mastiff in Maricopa, AZ). Cow samples (dairy and beef) were collected from two farms located in Maricopa, AZ.

Horses (Arabian and Thoroughbred) and pig (York) samples were collected from a farm in Tolleson, AZ. A cat sample from a mixed breed animal was collected from a house pet in Maricopa, AZ. A duck fecal sample was collected near a pond located in Layton lake community in Gilbert, AZ. All the fecal samples were placed into 50 ml sterile centrifuge tubes having 20 ml sterile 1X phosphate-buffered saline (PBS) at the time of collection and transferred on ice to the environmental microbiology laboratory at Arizona State University.

5.3.1.2. *Bacteroides* Isolation from Fecal Samples using Culture-based Techniques

To strengthen the data set for cultured *Bacteroides* from different sources, additional human and animal fecal samples were cultured on blood agar containing hemin and vitamin K and BBE agar (Hardy Diagnostics, Santa Maria, CA, USA) as previously described in Chapter 4. Briefly, colonies with *Bacteroides* characteristics were subcultured in chopped meat medium broth (Hardy Diagnostics). Culture samples were subjected to DNA extraction, PCR amplification using universal primers (11f and 1392r), cloning, and DNA sequencing. Sequences from each sample were blasted against 16S rRNA gene sequences from cultured and un-cultured samples in the NCBI Gene Bank database. The procedures followed for these analyses are described in Chapter 4.

5.3.1.3. *Bacteroides* Isolation from Fecal Samples using Molecular Techniques

To strengthen the data base of 16S rRNA sequences from un-cultured *Bacteroides*, the fecal samples were subjected to direct DNA extraction and PCR amplification. Briefly, PCR assays were performed using a Promega GoTaq®

Green Master Mix (Promega Corp., Madison, WI, USA) with primer concentration of 10µM (Bac32F and Bac708R) in 25 µl final volume, using a Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA) with the following temperature profile: 35 cycles of 30s at 94^{o}C, 1 min at 53^{o}C, and 2 min at 72^{o}C, with a final extension of 6 min at 72^{o}C (Bernhard and field, 2000a). Visualization of PCR products, cloning, and DNA sequencing was performed as described in Chapter 4.

5.3.1.4. *Bacteroides* 16S rRNA Gene Sequence Alignment and Variable Regions Identification

The 16S rRNA gene sequences of cultured and un-cultured *Bacteroides* from human and animal fecal samples obtained for this study, fish samples sequences (Chapter 4), and selected sequences from the NCBI were aligned against human *B. volgatus*, *B. fragilis* and *B. dorei* using multi-align interface software to identify variable regions within the 16S rRNA gene (Corpet, 1988). The variable regions were selected based on having nucleotide differences, which was ranged between 2.5 to 78%, when compared to human *Bacteroides*. The sequence accession numbers used for alignment are shown in Table 13. It is important to note that some *Bacteroides* 16S rRNA gene sequences isolated from human and animal fecal samples in this study are yet to be submitted to the NCBI database and their accession numbers will be included in at a later time.

Table 13 - Accession numbers for NCBI sequences and study samples aligned to identify four variable regions

Source	Accession numbers
Human	JQ317269, EU722737, and X*
Cat	AY695706 to AY695711, AY859646 to AY859650, X
Cow	AY695667 to AY695669, AY859651.1 to AY859655.1, EU573790, EU573797, EU573795.1, EU573800.1, EU573803.1, 1,EU573820.1, EU573825.1, EU573830.1, EU573832.1, EU573833.1, HM754529.1, HM754520, HM754519, AB237845 to AB237858, X
Dog/ Canine	AY695698 to AY695705, AY859657.1 to AY859661.1, DQ113673, DQ113675.1, EU772969, FJ221359.1, FJ221344.1, FJ221365.1, FJ221364.1, FJ221363.1, FJ221361.1, FJ221360.1, FJ221300.1, FJ221310.1, FJ221320.1, FJ221330.1, FJ221350.1, X
Duck	AB666116 , AB666125, AB666136 to AB666138, AB666144, AB666152, AB666153
Fish	AB592585.1, AB591886.1, AY682067.1, AY682055.1, GQ360025.1, GQ360021.1, GQ360021.1, GQ360025.1, JQ317220 to JQ317265, X
Gull	AY695712 to AY695716, AY859665.1 to AY859667.1, FJ221200, FJ220311.1, FJ221036.1, FJ221000.1, FJ221100.1,FJ221110.1, FJ221176.1, FJ221169.1, FJ221178.1, FJ221166.1, FJ221190.1, FJ221200.1
Pig	AY695689 to AY695697, AY859674.1, AB237860, AB237861, AB237865.1, AB237868.1, X

*Accession numbers will be provided once submission process to NCBI is completed.

5.3.1.5. Cladogram Construction using Non-truncated and Truncated Sequences

Initially, 4 cladograms were constructed: 1) cultured, non-truncated, 2) cultured, truncated, 3) uncultured, non-truncated, and 4) uncultured, truncated (Table 14). For truncation, conserved sequences between the four variable regions were deleted and variable segments joined, resulting in a DNA segment better representative of the genetic variability of *Bacteroides* from different sources. In addition to the four cladograms listed above, a fifth was generated using uncultured truncated sequences from human and non-human *Bacteroides*, with the addition of field sample sequences. *Bacteroides* 16S rRNA gene sequences from different sources were used to construct the cladograms as listed in Table 15.

Table 14 - Number of cladograms constructed

Number of Cladograms	Type of Cladogram
1	Cultured, non-truncated (Human and non human sources)
2	Cultured, truncated (Human and non human sources)
3	Uncultured, non-truncated (Human and non human sources)
4	Uncultured, truncated (Human and non human sources)
5	Uncultured, non-truncated (Human, non human and field samples)

Table 15 - Sources for *Bacteroides* 16S rRNA gene sequences used in cladogram construction

Cultured *Bacteroides* used for cladogram construction	Un-cultured *Bacteroides* used for cladogram construction
2 Human sources 5 Fish sources: Tilapia, Trout, Grass carp, Blue catfish, and Channel catfish 2 Dog sources: Pomeranian and Golden Retriever 1 Cow source: Beef cow	3 Human sources 2 Fish sources: Tilapia and Catfish 4 Dog sources: Pomeranian, Golden Retriever, German Shepherd, and English Mastiff 2 Cow sources: Dairy and Beef cows 2 Horse sources: Arabian and Thoroughbred 1 Cat source: Mixed breed 1 Pig source: York

To examine the validity of the developed truncated cladogram, blind samples of *Bacteroides* 16S rRNA gene sequences in NCBI (Table 13) were analyzed using scenario 4, Table 14 for identifying human versus non-human sources. The strategy clearly identified the sources listed in the NCBI.

5.3.2. Field Testing the Tool Box Strategy

A tool box strategy for *Bacteroides* source identification was developed including variable region analysis, PCR amplification, and subsequent truncated

cladogram grouping analysis. This section outlines the steps that were followed to field test the proposed strategy.

5.3.2.1. Site Selection Criteria and Field Sampling

To test the field applicability of the proposed tool box strategy for *Bacteroides* source identification, a variety of samples from central AZ source water, ponds, and fishing lakes filled with reclaimed water were collected. Samples from the Salt River, the Verde River (upstream of Granite Reef Dam which is located 22 miles Northeast of Phoenix, AZ, on the Salt River) and the CAP (near Granite Reef Dam) were collected. In addition, samples from the intake of two drinking water treatment plants (South Tempe (AZ) and Chandler (AZ) Drinking Water Treatment Plants) were collected. Samples were collected from a pond in Layton Lake community in Gilbert, AZ, the Riparian Institute in Gilbert AZ (1 sample from a pond and 2 samples from a fishing lake), and a pond in Pacana Park in Maricopa, AZ. Samples were collected in sterile 1-liter polypropylene containers and placed on ice immediately for transport to the laboratory. A list of the samples and a map of sampling locations is provided in Table 16 and Fig 15.

Table 16 - Sites selected for field sampling

Sampling Location	Sampling Date
Salt River[a] (Upstream of Granite Riff Dam), Mesa, AZ	1/9/2012
Verde River[a] (Upstream of Granite Riff Dam), Mesa, AZ	1/9/2012
CAP Canal[a] (Near Granite Riff Dam), Mesa, AZ	1/9/2012
Salt River[b] (Upstream of Granite Riff Dam), Mesa, AZ	1/29/2012
Verde River[b] (Upstream of Granite Riff Dam), Mesa, AZ	1/29/2012
CAP Canal[b] (Near Granite Riff Dam), Mesa, AZ	1/29/2012
South Tempe Treatment Plant (South Canal), Tempe, AZ	1/25/2012
Chandler Treatment Plant (Consolidated Canal), Chandler, AZ	1/24/2012
Layton Lake Pond, Gilbert, AZ	12/19/2011
Riparian Pond, Gilbert, AZ	1/28/2012
Riparian Fishing Lake (South side), Gilbert, AZ	1/28/2012
Riparian Fishing Lake (East Side), Gilbert, AZ	1/28/2012
Pacana Park (North side), Maricopa, AZ	2/1/2012
Pacana Park (East side), Maricopa, AZ	2/1/2012

(a) Hereinafter [a] is referred to the samples collected on 1/9/2012

(b) Hereinafter [b] is referred to the samples collected on 1/29/2012

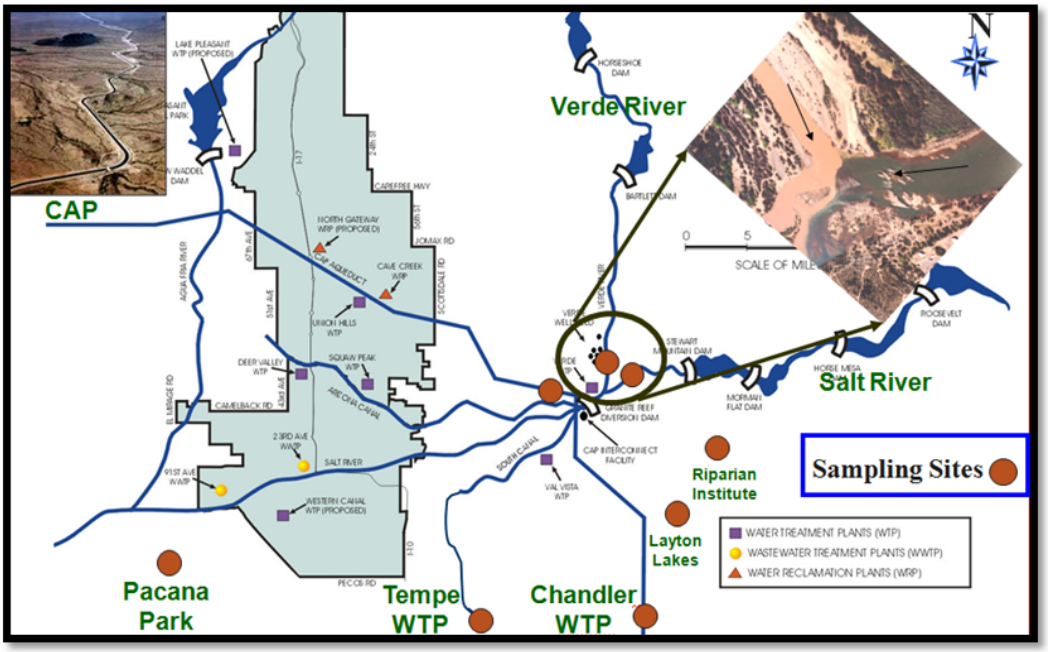


Fig. 15 - Sites selected for field testing

5.3.2.2. Procedural Recovery Efficiency

The first 200 ml of each collected water sample was concentrated using membrane filtration. To examine the consistency of the procedure, following recovery of bacterial cells from filters a recovery efficiency test was performed. For this test, a known concentration of *E. coli* was spiked into 200 ml of sterile 1X PBS and passed through a 0.45 µm pore size membrane filter. The filter was then transferred to a 15 ml centrifuge tube containing 10 ml of 1X PBS and vortexed for 5 minute to elute the bacteria from it. The vortexed filter was then placed on to an m-Endo agar plate to observe how much of *E. coli* remained on the filter. The buffer containing eluted bacteria was then centrifuged for 20 minutes at 3000 RPM (~ 1863 RCF). After centrifugation, the supernatant was discarded and the pellet was suspended in 1 ml of 1X PBS and membrane filtered.

These membranes were then plated onto m-Endo agar. These plates were incubated at 37^{o} C and enumerated after 24 hours. The number of colonies counted on these membranes represented the *E. coli* cells eluted from the initial membrane and these data were used for recovery efficiency calculations. The experiment was performed in triplicate and each sample was analyzed in duplicate. These recovery efficiency experiments were performed using *E. coli* as a surrogate for *Bacteroides* due to the difficulty of culturing the latter.

Due to the satisfactory results from the recovery efficiency test, membrane filtration followed by vortexing was deemed an acceptable method for collection of *Bacteroides* cells from field samples. Field samples did not undergo a second membrane filtration as cells for DNA extraction were taken directly from the solution containing vortexed membranes.

5.3.2.3. Evaluation of Primer Sets to Amplify Human *Bacteroides*

To finalize the selection of the PCR primer sets for the detection of human *Bacteroides* from water samples, the specificity of the two previously known human-specific primers (Table 17) were tested using extracted DNA from fish and animal fecal samples.

For the PCR assay, HF134F and HF183F primers were paired with the general *Bacteroides* reverse primer (Bac708R) for two separate reactions (Bernhard and field, 2000b). PCR was performed as described previously with the following minor changes to primer concentration and temperature profile. 400 nM (HF134F), 200 nM (HF183F), and 10 µM (Bac708R) primer concentrations were used and temperature profile was altered to: 5 min at 95^{o} C, 35 cycles of 30s at

95° C, 45s at 60° C, and 30s at 72° C, and a final extension of 5 min at 72° C for both sets of PCR assays. For further analysis, amplicons were purified, cloned, and sequenced as described previously.

Table 17 - Primers used to amplify *Bacteroides* from field samples

Primers	Expected Amplicon Size (bp)	Reference
HF134F: 5′-GCCGTCTACTCTTGGCC-3′ HF708R: 5′-CAATCGGAGTTCTTCGTG-3′	574	Bernhard and Field, 2000b
HF183F:5′ATCATGAGTTCACATGTCCG-3′ HF708R: 5′-CAATCGGAGTTCTTCGTG-3′	525	

5.3.2.4. Detection of *Bacteroides* from Field Samples by PCR

Once the specificity of the HF134 and HF183 primer sets were tested on extracted DNA from animal fecal samples, they were used on extracted DNA from water samples for *Bacteroides* source identification. DNA was extracted and concentrated from 1 ml of field water samples using a Zymo Fecal DNA Kit (Zymo Research, Orange, CA, USA). Following DNA extraction, PCR was performed as described previously, with the HF183 primer set reactions subjected to semi-nested PCR as suggested by Bernhard et al (2003).

5.3.2.5. Cloning and DNA Sequencing of *Bacteroides* from Field Samples

To verify the identity of PCR products obtained using HF134 and HF183 primer sets, cloning and DNA sequencing were also performed. The amplicons generated using the HF134 and HF183 primer sets with field samples were first purified for cloning purposes. The majority of the amplicons generated using HF134 primer set showed an extra band slightly larger than the expected

amplicon size. For these amplicons with double bands, DNA sequencing was performed on each band separately. Each band was cut and removed from the gel while on a UV transilluminator using a sterile blade. These pieces of gel containing DNA bands were then subjected to purification using a QIAquick gel extraction kit (Qiagen Sciences. Calencia, CA, USA). For amplicons showing a single band at the expected product size on a DNA gel, purification was performed directly on the PCR product using a QIAquick PCR purification kit (Qiagen Sciences. Calencia, CA, USA). Cloning and DNA sequencing were performed as previously described in Chapter 4. The objectives of cloning and DNA sequencing were to verify the identity of amplified sequences and use the sequences to construct truncated cladograms as a tool box strategy for MST.

5.3.2.6. Decision Making Tool Box

A decision making tool box including PCR amplification, variable region analysis, human-specific PCR assay, and subsequent truncated cladogram grouping analysis was generated. The tool box is tabulated and discussed in the results and discussion section.

5.4. Results and Discussion

The results and discussion is divided in two main sections: *Bacteroides* 16S rRNA gene sequence alignments and source tracking results for field samples.

5.4.1. *Bacteroides* 16S rRNA Gene Sequence Alignments

This section presents the results of the characterization of *Bacteroides* flora from different fecal sources using both culture-based and molecular techniques followed by variable region identification and cladogram analysis.

5.4.1.1. Characterization of *Bacteroides* from Different Fecal Sources: Culture-based and Molecular Techniques

Alignment results of the 16S rRNA gene amplicons indicate that *Bacteroides* isolated from human samples are 99% similar to *B. fragilis* (Table 18). Along with *B. volgatus* and other species, *B. fragilis* is common in human fecal samples and a common cause of intestinal infection due to loss of integrity of the bowel mucosa (Busch et al., 1976). The 16S rRNA gene sequence of isolated *Bacteroides* from the cat fecal sample shared 98% sequence identity with *B. stercoris*. These two isolates significantly matched NCBI cultured *Bacteroides,* however, bacteria isolated from other animals did not show this level of similarity to *Bacteroides.* For example, the alignment results from Pomeranian dog feces *Bacteroides* were 99% related to cultured *Bacteroides* from Tilapia (Chapter 4) with the two showing an 89% match to both cultured *B. eggerthii* and cultured *B. uniformis*. On the other hand, isolated *Bacteroides* from Golden Retriever dog feces was 99% similar to isolated *Bacteroides* from grass carp and channel catfish feces (Chapter 4) and all three shared 94% identity with cultured *B. graminisolvens*. With an assigned cut-off value of 99% sequence homology, samples with 89% and 94% similarity to known *Bacteroides* species were not conclusive. For dog fecal samples, culture-based technique identified the isolated

bacteria as *Bacteroides,* however the sequence alignment results cannot be used for speciation.

Bacterial isolates from York pig and beef cow exhibited *Bacteroides* specific characteristics on BBE agar (Livingston et al., 1978), while NCBI BLAST analysis indicated that these isolates show homology of 97% for *Propioni bacterium acnes* and 99% for *Bacillus cereus,* respectively. This can be explained by the fact that *Bacteroides* species could be divergent and may have been reclassified as other bacteria species (Karlsson et al., 2010). For example, *B. merdae* was recently reclassified as *Parabacteroides merdae* (Sakamoto and Benno, 2006). As mentioned previously, among the scientific community there is discrepancy regarding the taxonomy of *Bacteroides*. For example, the Integrated Taxonomic Information System has accepted 30 species into genus *Bacteroides* as of 2010, while the German Collection of Microorganisms and Cell Cultures has three times more species of *Bacteroides*.

For horse samples, growth on BBE plate was observed, but colonies did not exhibit *Bacteroides* morphology and were not further analyzed by sequencing. For dairy cow and duck samples, growth was seen on blood agar, but not on BBE plates. This can be explained by the fact that some species of *Bacteroides* are difficult to culture (Bernhard and Field, 2000b).

These results demonstrate that, based on culture and *Bacteroides* 16S rRNA gene sequence alignment, no clear conclusion can be made for classification of isolated *Bacteroides* to the species level. This is one of the reasons that variable regions were introduced in this study. It is thought that the

conserved sequences within 16S rRNA gene may mask the variation between different bacteria and by eliminating these sequences a more robust classification may be achieved.

Table 18 - Characterization of *Bacteroides* species isolated from human and selected animal feces using culture-based and molecular techniques

Sample Source	Growth		Colony Appearance on BBE Agar	% similarity of 16S sequences	
	Blood Agar	BBE Agar		Between *Bacteroides* isolated in this study and NCBI[1] sequences from cultured samples	Between *Bacteroides* isolated in this study and NCBI sequences reported from uncultured samples/ metagenomic data
Bacteroides fragilis (ATCC#23745)	+	+	Brown colonies with blackened media	ND (Not Determined)	ND
Human	+	+	Dark and light brown colonies with blackened media	99% match to *Bacteroides fragilis* (AB542764)	ND
Cat (mixed breed)	+	+	Dark brown colonies with blackened media	98% match to *Bacteroides stercoris* (AB510708)	98% match to uncultured bacterium from human fecal sample (EF400632) and bush dog fecal sample (EU772969)
Dog (Pomeranian)	+	+	Dark and light brown colonies with blackened media	89% match to *Bacteroides eggerthii* (AB510700.1) and *Bacteroides uniformis* (EU722741)	99% match to Uncultured bacterium from yellow catfish (*Pelteobagrus fulvidraco*) (GQ360025) and Grass Carp (JN032937)
Dog (Golden Retriever	+	+	Dark and light brown colonies with blackened media	94% match to *Bacteroides graminisolvens* (AB547643)	98% match to uncultured bacterium from Chinese mitten crab (DQ856503.1)
Pig (York)	+	+	Slight black coloration on media/ small and very light brown colonies	97% match to *Propioni bacterium acnes* gene (AB573714.1)	97% match to uncultured bacterium from gastrointestinal specimens from human (HQ812348)
Beef Cow (Feed Lot)	+	+	Dark brown colonies with blackened media	99% match to *Bacillus cereus* (JN644555) isolated from midgut of mosquito	ND
Horse (Arabian)	+	+	Slight black coloration on media /pale coloration and star-like shape	ND	ND
Horse (Thoroughbred)	+	+	Slight black coloration on media /big white mucoid colonies	ND	ND
Dairy Cow	+	-	-	ND	ND
Duck	+	-	-	ND	ND

NCBI[1]: National Center for Biotechnology Information, Gene Bank data base.

5.4.1.2. Variable Region Identification and Analysis:

Using the alignment results, four variable regions were identified based on high levels of nucleotide mismatch with three known human *Bacteroides* isolates. The regions are as follows: 1 (nucleotides 71-101), 2 (nucleotides 142-271), 3 (nucleotides 451-511) and 4 (nucleotides 581-701). The alignment results for human isolates of *B. volgatus*, *B. fragilis*, and *B. dorei* are shown in Tables 19, 20, and 21, respectively. It was observed that *Bacteroides* 16S rRNA gene sequences from all the animals follow the same trend when it comes to number of nucleotide differences in the four regions when aligned versus the three human *Bacteroides* isolates.

Table 19 - Alignment analysis of *Bacteroides* 16S rRNA gene sequences from selected animals compared to human *Bacteroides volgatus*

Sources	**Nucleotide regions within 16S rRNA gene of human *B. volgatus***			
Regions of 16S rRNA	Region 1 71-101	Region 2 142-271	Region 3 451-511	Region 4 581-701
Cat	8-18 bp in 21 targets	26-52 bp in 21 targets	15-18 bp in 21 targets	8-39 bp in 21 targets
Cow	7-21 bp in 54 targets	30-62 bp in 54 targets	13-23 bp in 54 targets	13-38 bp in 54 targets
Dog/Canine	5-18 bp in 43/46 targets 0 bp in 3/46	25-51 bp in 42/46 targets 2 bp in 1/49 0 bp in 3/49	15-29 bp in 42/46 targets 1 bp in 1/49 0 bp in 3/49	9-38 bp in 42/46 targets 1 bp in 1/49 0 bp in 3/49
Duck	5-16 bp in 8 targets	28-50 bp in 8 targets	16-21 bp in 8 targets	13-31 bp in 8 targets
Fish	5-16bp in 36 targets	29-60 bp in 36 targets	15-21 bp in 36 targets	8-42 bp in 32 targets
Gull	7-15 bp in 12 targets	24-55 bp in 15/19 targets 0 bp in 4/19	12-20 bp in 16/20 targets 1 bp in 2/20 0 bp in 2/20	8-24 bp in 12/16 targets 1 bp in 1/16 0 bp in 3/16
Pig	6-21 bp in 22/23 targets 1 bp in 1/23	34-57 bp in 23 targets	12-28 bp in 23 targets	13-42 bp in 23 targets

Table 20 - Alignment analysis of *Bacteroides* 16S rRNA gene sequences from selected animals compared to human *Bacteroides fragilis*

Sources	**Nucleotide regions within 16S rRNA gene of human *B. fragilis***			
Regions of 16S rRNA	Region 1 71-101	Region 2 142-271	Region 3 451-511	Region 4 581-701
Cat	9-17 bp in 10 targets	26-43 bp in 10 targets	11-22 bp in 10 targets	13-39 bp in 10 targets
Cow	4-17 bp in 34 targets	22-56 bp in 34 targets	8-31 bp in 34 targets	10-36 bp in 34 targets
Dog/Canine	9-16 bp in 30 targets	19-47 bp in 30 targets	11-47 bp in 30 targets	12-44 bp in 30 targets
Duck	8-15 bp in 8 targets	20-56 bp in 8 targets	15-24 bp in 8 targets	15-34 bp in 8 targets
Fish	5-12 bp in 16 targets	20-51 bp in 16 targets	13-25 bp in 16 targets	3-45 bp in 12 targets
Gull	7-16 bp in 12 targets	17-42 bp in 17/19 targets 1 bp in 1/19 2 bp in 1/19	11-22 bp in 18/20 targets 0 bp in 2/20	5-21 bp in 14/16 targets 2 bp in 2/16
Pig	9-16 bp in 14 targets	26-56 bp in 14 targets	12-30 bp in 14 targets	18-47 bp in 14 targets

Table 21 - Alignment analysis of *Bacteroides* 16S rRNA gene sequences from selected animals compared to human *B. dorei*

Sources	**Nucleotide regions within 16S rRNA gene of human *Bacteroides dorei***			
Regions of 16S rRNA	Region 1 71-101	Region 2 142-271	Region 3 451-511	Region 4 581-701
Cat	8-16 bp in 21 targets	29-47 bp in 21 targets	14-18 bp in 21 targets	8-38 bp in 21 targets
Cow	6-19 bp in 54 targets	33-59 bp in 54 targets	11-22 bp in 54 targets	11-38 bp in 54 targets
Dog/Canine	5-14 bp in 42/46 targets 1 bp in 3/46 2 bp in 1/46	9-53 bp in 46 targets	15-28 bp in 41/46 targets 1 bp in 4/46 0 bp in 1/46	10-44 bp in 41/46 targets 1 bp in 4/46 2 bp in 1/49
Duck	4-14 bp in 8 targets	30-53 bp in 8 targets	16-21 bp in 8 targets	13-30 bp in 8 targets
Fish	9-17 bp in 36 targets	39-67 bp in 36 targets	15-23 bp in 36 targets	8-43 bp in 32 targets
Gull	11-14 bp in 8/12 targets 1 bp in 4/12	10-62 bp in 19 targets	12-20 bp in 16/20 targets 1 bp in 3/20 2 bp in 1/20	8-23 bp in 12/16 targets 1 bp in 3/16 2 bp in 1/16

Pig	6-20 bp in 23 targets	41-59 bp in 23 targets	12-29 bp in 23 targets	13-43 bp in 23 targets

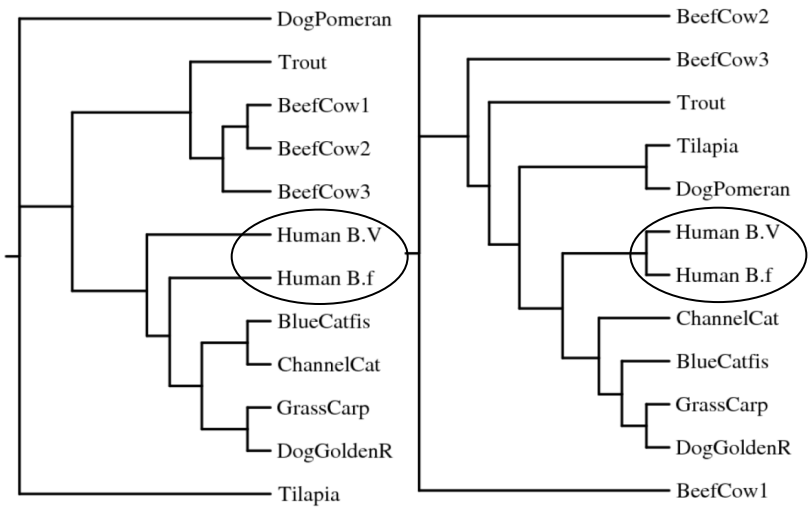
As previously reported, the alignments of several bacterial 16S rRNA gene sequences have shown nine separate hypervariable regions (V1-V9) with the following breakdowns: V1(nucleotides 69-99), V2 (nucleotides 137-242), V3 (nucleotides 433-497), V4 (nucleotide 576-682), V5 (nucleotide 822-879), V6 (nucleotide 986-1043), V7 (nucleotide 1117-1173), V8 (nucleotide 1243-1294), and V9 (nucleotide 1435-1465) (Chakravorty et al., 2007). Interestingly the identified four variable regions in this study fall within the boundaries of V1, V2, V3, and V4. It is known that hypervariable regions V1, V2 and V6 contain the highest heterogeneity and can be used for discriminating different bacterial groups (Chakravorty et al., 2007).

Bacteroides from different animals were aligned against human *B. volgatus* and *B. fragilis* due to their high frequency in human feces (Kreader, 1995). Table 19 shows the *Bacteroides* 16S rRNA gene sequences of all listed animals have nucleotide differences in all four variable regions when compared to human *B. volgatus* except for dog and gull. This is because *Bacteroides* species such as *B. volgatus*, *B. uniformis*, *B. thetaiotaomicron*, and *B. stercoris* have many similar or identical sequences from non-human hosts (Dick et al., 2005a). If the *Bacteroides* 16S rRNA gene sequence from an unknown source is aligned with human *B. volgatus* and the alignment results show there are zero nucleotide differences between the unknown and human sources, then the source of fecal contamination can only be narrowed down to human, dog, and gull.

Alignment results for human isolated *B. fragilis* (Table 20) versus the listed animals indicate that gull *Bacteroides* have only one nucleotide mismatch in region 2, zero in region 3, and two in region 4, indicating that the *Bacteroides* 16S rRNA gene sequence from gull is almost identical to those of human *B. fragilis*. Because of this, it was also necessary to align *Bacteroides* 16S rRNA gene sequences from the selected animals with a *Bacteroides* species not prevalent in other animals, such as *B. dorei* (Haugland et al., 2010). Using the nucleotide differences data in Table 21, identification of *Bacteroides* isolates can be performed for human or non-human sources, however, dog and gull isolates have similar sequences to human *B. dorei* in regions 1, 3, and 4, but not region 2, allowing for human source identification.

5.4.1.3. Cladogram Analysis: Non-truncated vs. Truncated Cladogram

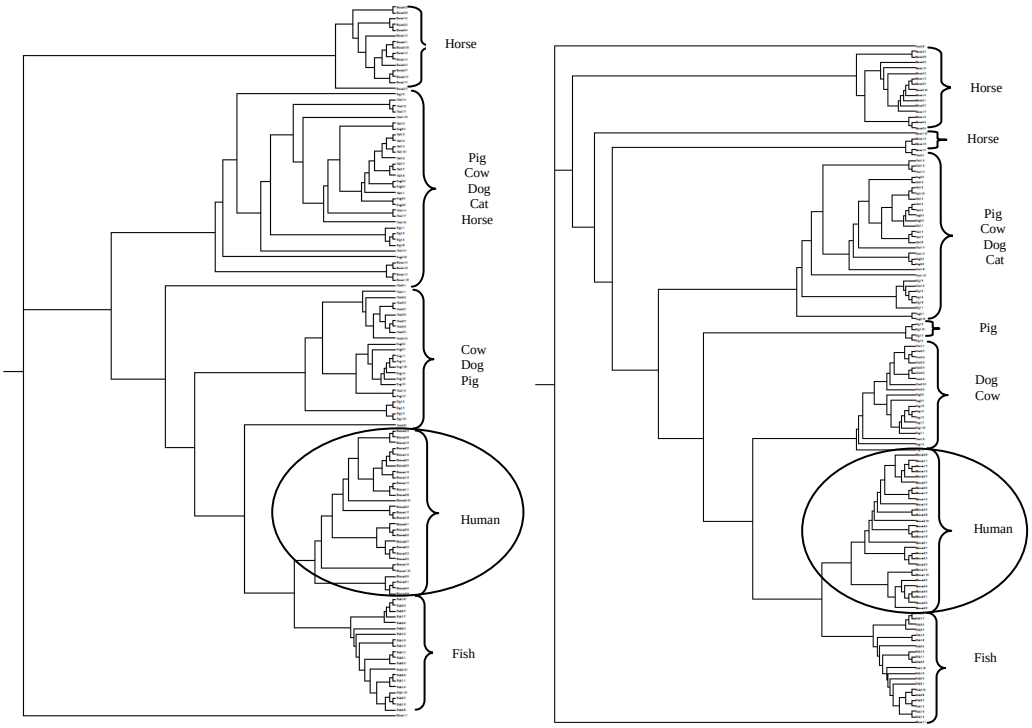
By comparing the cladograms generated for cultured *Bacteroides* (non-truncated vs. truncated, Fig 16a and 16b) it is seen that the sequences are grouped differently. As shown in Fig 16b, human isolates form a separate clade in the truncated cladogram, as opposed to the non-truncated cladogram. This demonstrates that the truncated cladogram shows a higher degree of separation of *Bacteroides* from human and other sources.



(a) (b)

Fig. 16 - Cladograms from cultured *Bacteroides* using a) non-truncated sequences and b) truncated sequences

For uncultured *Bacteroides*, cladograms for non-truncated and truncated sequences were constructed (Fig 17). In summary, the separation of *Bacteroides* from different sources is similar for both non-truncated and truncated sequences, however, it was determined that the clades for truncated sequences (Fig 17b) are more compact by having less sub-clades and shorter distances. Therefore, the cladogram generated from truncated sequences will be used as one of the tools for visually observing the separation of human fecal contamination from other sources.



(a) (b)

Fig. 17 - Cladogram from un-cultured *Bacteroides* a) non-truncated sequences b) truncated sequences

5.4.2. Field Samples Source Identification Results

5.4.2.1. Procedural Recovery Efficiency for Detecting Bacteria from Water Samples

The recovery of the sample processing method utilized for field sample concentration was determined to be between 20-30% (Fig 18). The consistency of the method was deemed satisfactory. Due to issues with *Bacteroides* detection methods, *E. coli* cells were used as a surrogate for this evaluation. It is important to note that the objective of this assay was not to develop a new sample collection, elution, and detection methodology.

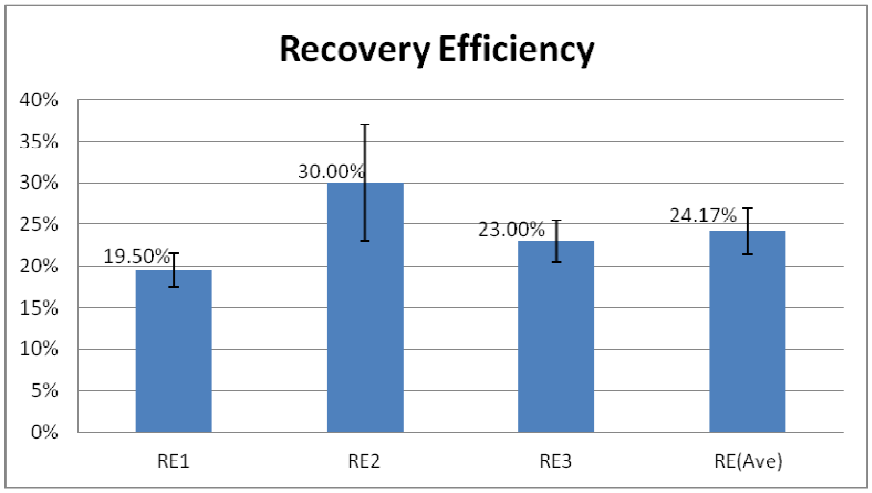


Fig. 18 - Recovery efficiency of *E. coli* from membrane filtration

5.4.2.2. Specificity of HF134 and HF183 Primers for Identifying *Bacteroides* in Animal Fecal Samples

As expected, *Bacteroides* 16S rRNA from human fecal samples were amplified using the HF134 primer set along with *Bacteroides* from dog fecal samples (Table 22). This was not surprising since other studies have also reported cross amplification of HF134 primer with dog fecal samples (Kildare et al., 2007; Ahmed et al., 2008; Ahmed et al., 2009). For further analyses, amplicons from human and the dog fecal samples were sequenced and blasted using the NCBI database to confirm the PCR results and develop cladograms. Interestingly, the sequencing results showed that the HF134 primer set can amplify three different *Bacteroides* species: *B. volgatus*, *B. massiliensis*, and *B. dorei*. According to a study, the 16S rRNA gene sequence of *B. dorei* is 96% similar to *B. volgatus* and 93% similar to *B. massiliensis* (Bakir et al., 2006). The amplification of these

three *Bacteroides* species by the HF134 primer set is possible due to the relative similarity of their 16S rRNA gene sequences.

Sequencing results revealed the presence of *B. dorei* in two and *B. volgatus* in one of the human fecal samples. Sequencing results from the two dog samples indicate the presence of *B. massiliensis* and *B. volgatus.* A variable matrix (Table 19) was used to identify differences in *B. volgatus* sequences obtained from human and dog samples. Based on the alignment results, no differences were observed. This can be explained by the fact that fecal samples from pets such as dogs that share proximity with humans may contain identical or similar *Bacteroides* species such as *B. volgatus* (Dick et al., 2005a). In addition to this, the *B. massiliensis* sequences obtained from dog samples matched 99% to the NCBI *B. massiliensis* 16S rRNA gene sequences isolated from human fecal samples.

PCR results using the HF183 primer set showed that *Bacteroides* from 4/5 human and no animal fecal samples were amplified (Table 22 and Fig 19). These results were in agreement with other published papers demonstrating that the HF183 primer set can be used for human-specific *Bacteroides* assays (Ahmed et al., 2007; Betancourt et al., 2006; Gawler et al., 2007; Griffith et al., 2003; Santoro and Boehm, 2007). An explanation for the lack of amplification in 1/5 human samples could be that *B. dorei* was not present in the sample; this was confirmed by the HF134 primer set resulting in amplification of *B. volgatus*. Since both primers can be used for detecting *B. dorei,* and this species has been

proven to be human-specific, it was decided to utilize both primers from the tool box for the source identification of field samples.

Table 22 - Amplification results for HF134 and HF183 primers on fecal samples

	PCR Results	
Samples	HF134/Bac708R *B. volgatus, B. massiliensis* & *B. dorei*	HF183/Bac708R *B. dorei*
Human 1	+ 8/8 clones → (*B. dorei*)	+
Human 2	+ 8/8 clones → (*B. dorei*)	+
Human 3	+ ND[7]	+
Human 4	+ ND	+
Human 5	+ 7/7 clones → (*B. volgatus*)	-
Fish[1] 1	-	-
Fish 2	-	-
Fish 3	-	-
Fish 4	-	-
Dog[2] 1	-	-
Dog 2	+ 4/5 clones → (*B. massiliensis*) 1/5 clones → (*B. volgatus*)	-
Dog 3	-	-
Dog 4	+ 1/1 clones → (*B. volgatus*)	-
Cow[3] 1	-	-
Cow 2	-	-
Horse[4] 1	-	-
Horse 2	-	-
Cat[5]	-	-
Pig[6]	-	-
Duck	-	-

Fish[1] 1: Cat fish; Fish 2: Tilapia; Fish 3: Grass Carp; Fish 4: Trout

Dog[2] 1: Pomeranian; Dog 2: Golden Retriever; Dog 3: German Shepherd; Dog 4: English Mastiff

Cow[3] 1: Beef Cow; Cow2: Dairy Cow

Horse[4] 1: Arabian Horse; Horse 2: Thoroughbred

Cat[5] 1: Mixed breed

Pig[6]: York

ND[7]: Not Determined (sequencing was not performed)

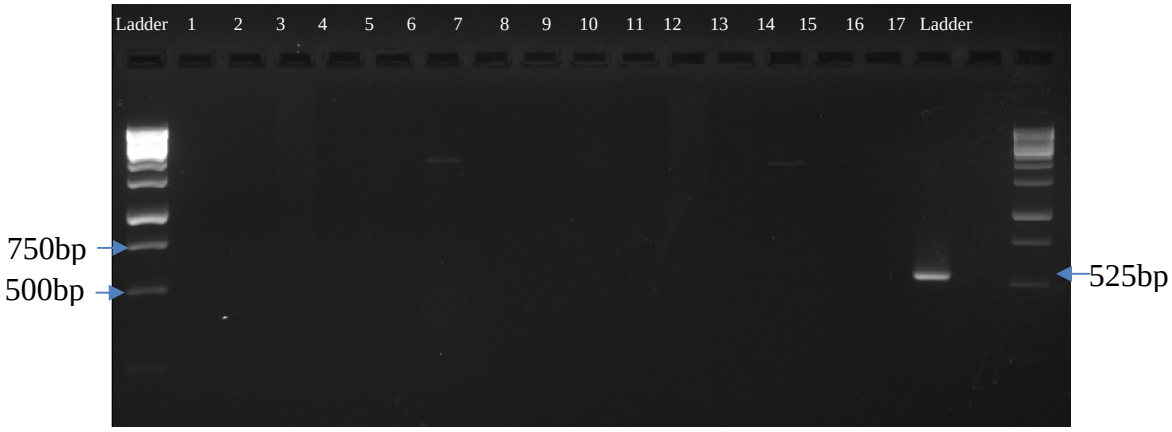


Fig. 19 - DNA Gel photograph of amplicons derived from PCR with HF183 and Bac708R primers on fecal sample DNA from the following animals: Catfish (Lane 1), Tilapia (Lane 2), Grass Carp (Lane 3), Trout (Lane 4), Pomeranian dog (Lane 5), Golden Retriever dog (Lane 6), German Shepherd dog (Lane 7), English Mastiff dog (Lane 8), Beef cow (Lane 9), Dairy cow (Lane 10), Arabian horse (Lane 11), Thoroughbred horse (Lane 12), Cat (Lane 13), Pig (Lane 14), Duck (Lane 15). Lane 16: Positive control (human fecal DNA); Lane 17: Negative control (DNA free water samples); Ladder: 1k bp. The expected product size is 525 bp.

5.4.2.3. Analysis of PCR and Sequencing Data from Field Samples

As shown in Fig 20a, DNA gels of PCR products from reactions using the HF134 primer set with DNA from *Bacteroides* field samples showed bands of the expected product size of ~574 bp. Interestingly, an extra band (~70 bp larger than the expected product size) was also observed for 9 out of 13 samples: Salt River[a] and Salt River[b]; Verde River[a] and Verde River[b]; CAP Canal[a] and CAP Canal[b]; Chandler Treatment Plant; and Riparian Fishing Lake (South and East side).

The PCR assay using the HF183 primer set amplified *Bacteroides* from 8 out of 13 water samples: (Salt River[b], Verde River[b], CAP Canal[b], Layton Lake, Riparian Pond, Riparian Lake: South and East side, and Pacana Park Pond) with the expected product size of 525 bp (Fig 20b). Since the HF183 primer set was

proven to only amplify human-specific *Bacteroides* genetic markers (based on testing results of animal fecal samples and published works), successful amplification using the HF183 primer set identified the source as human. For the confirmation, an HF183 amplicon (Pacana Park) was sequenced and determined to be *B. dorei*, while grouping with the human clade in a cladogram (Fig 22).

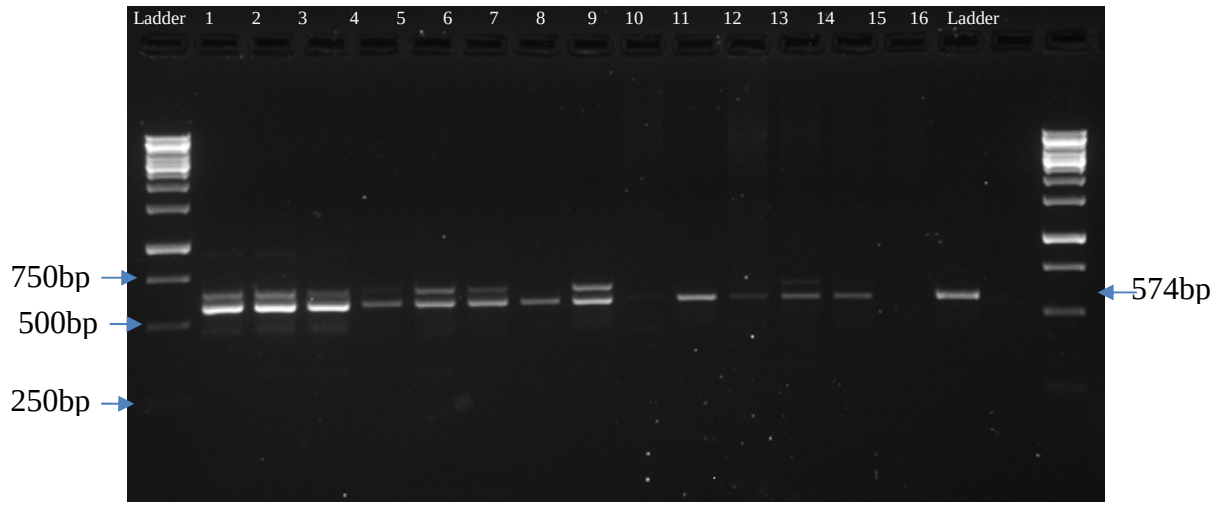
As previously discussed, the HF134 primer can amplify *B. volgatus, B. massiliensis,* and *B. dorei*, therefore, any observed product of the expected size could be the result of any combination of the three species in a fecal sample. To verify the *Bacteroides* species in samples, a sequencing step for either single or double bands was essential. The sequencing results from the following field samples: Salt River[a], Verde River[a], CAP Canal[a], and Chandler Treatment Plant revealed that the top bands were the results of non-specific amplification, determined by blasting the sequences using the NCBI database. The bottom bands were confirmed to be both *B. volgatus* and *B. massiliensis* by sequencing. This finding is in agreement with the results obtained using the HF183 primer set in regard to the absence of *B. dorei* in the above field samples.

A different trend was observed from the sequencing results of the top band for Verde River[b], which indicated the presence of *B. dorei* and *B. massiliensis*. In addition, for CAP Canal[b], *B. dorei*, *B. massiliensis*, *B. volgatus*, and non-specific PCR products were observed. For both field samples, the sequencing results from the bottom bands revealed the amplified product to be *B. volgatus*. It is believed that observed top bands are the result of non-specific amplification and the identified three species may be the results of carryover of

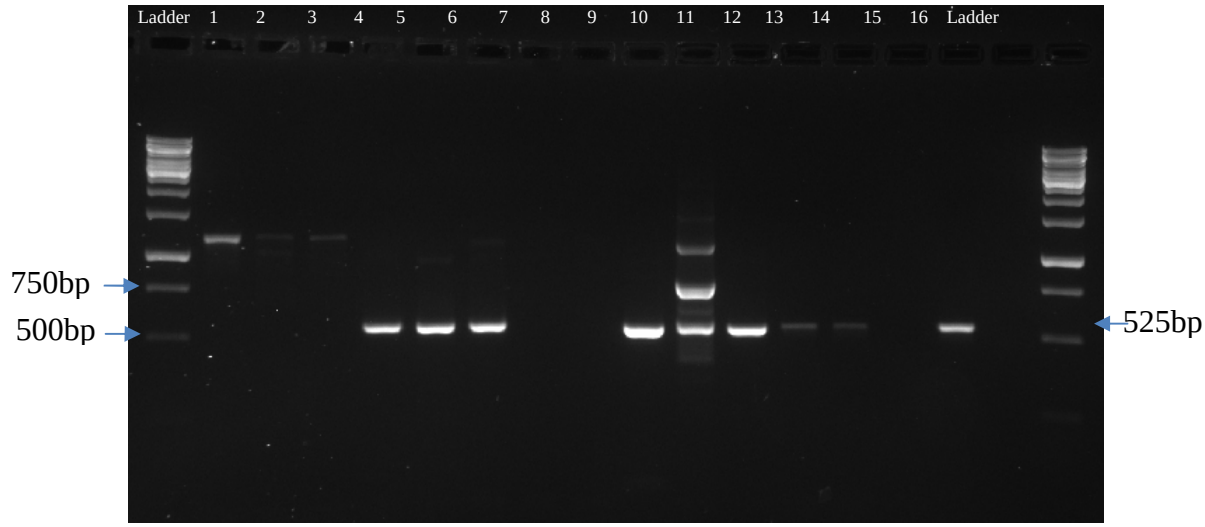
residuals from the bottom band. This artifact has been reported from the sequencing laboratory at ASU (Scott Bingham, personal communication, February 15, 2012). As expected, *B. dorei* was amplified by both HF134 and HF183 primer sets in these samples.

Two sets of samples were collected from the Salt River, Verde River, and CAP Canal, and with only one set having *Bacteroides dorei,* this event can most probably be attributed to human fecal contamination. These samples were taken within a 20 day period. During this time, a rainfall event was reported causing runoff to rivers carrying human fecal matter. It is also possible that a plume of human fecal material was passing through the river at the time of sample collection, an event that can occur after discharge of waste water into rivers. Additionally, human fecal pollution was observed in water samples collected from Layton Lake, Riparian Pond and Fishing Lake, and Pacana Park. The water sources for these sites come from reclaimed water, which includes treated municipal sewage which may contain human fecal bacterial flora. Due to this fact, the detection of human fecal material at these sites was not unexpected.

Table 23 highlights the data shown in Fig 20 by noting the intensities of the DNA gel bands from the latter with positive signs. The final column in Table 23 shows which field samples were sequenced, truncated, and included in a truncated cladogram for confirmation of the source of fecal contamination.



(a)



(b)

Fig 20 - DNA gel photograph of PCR products using (a) HF134 and Bac708R primers and (b) HF183 and Bac708R primers on DNA extracted from field samples from the following sources: Salt River[a], Verde River[a], CAP Canal[a] (Lanes 1 through 3), Salt River[b], Verde River[b], CAP Canal[b] (Lanes 4 through 6), South Tempe Treatment Plant source water (Lane 7), Chandler Treatment Plant source water (Lane 8), Layton Lake (Lane 9), Riparian Pond (Lane 10), Riparian Lake (Lanes 11 and 12), Pacana Pond (Lane 13). All water samples show the expected 574-bp PCR products in (a) and 8/13 show the expected product size of 525 bp in (b). Lane 14: blank; Lane 15: Positive control (human fecal DNA); Lane 16: Negative control (DNA free water samples); Ladder: 1k bp.

Table 23 - Amplification results for HF134 and HF183 primers on field samples

	Primers		**Analysis**
Sampling Location	PCR (HF134) Amplifies *B. volgatus, B. massiliensis, and B. dorei*	PCR[2] (HF183) Amplifies *B. dorei*	Cladogram (Truncated)
Salt[a] River	TB[1] : ++ BB[1] : +++	-	Yes
Verde[a] River	TB: ++ BB: +++	-	Yes
CAP Canal[a]	TB: ++ BB: +++	-	Yes
Salt[b] River	TB: + BB: ++	+++	ND[4]
Verde[b] River	TB: ++ BB: ++	+++	Yes
CAP Canal[b]	TB: ++ BB: ++	+++	Yes
South Tempe Treatment Plant	TB: - BB: ++	-	Yes
Chandler Treatment Plant	TB: +++ BB: ++	-	Yes
Layton Lake Pond	TB: - BB: +	+++	Yes
Riparian Pond	TB: - BB: ++	+++	ND
Riparian Fishing Lake (South side)	TB: + BB: ++	+++	ND
Riparian Fishing Lake (East side)	TB: + BB: ++	+	ND
Pacana Park (North side)	TB: - BB: ++	+	Yes
Pacana Park (East side)[3]	TB: - BB: ++	ND	ND

TB[1] →Top Band, BB[1] →Bottom Band, Positive control is +++

PCR[2] HF183 (Semi Nested PCR) (Positive control is +++)

Pacana Park (East side)[3] →Not loaded on the gel

ND[4] →Not Determined

5.4.2.4. Tool Box and Identification of Sources of Fecal Contamination

A tool box strategy for *Bacteroides* source identification utilizing PCR amplification and variable region analysis, human-specific PCR assays, and subsequent truncated cladogram grouping evaluation was developed. A flow chart outlining the procedure for the tool box strategy is shown in Fig 21 and a list of possible scenarios for the strategy is displayed in Table 24. The first step in source identification is PCR amplification using the HF183 and HF134 primer sets. For water samples with PCR positive results using the HF183 primer set (scenarios 2, 3, and 5 in Table 24), it is assumed that the source of fecal contamination is human. This designation is due to the fact that *B. dorei* was only detected in human and not in any tested animal fecal samples (Table 22). For samples with PCR positive results using the HF134 primer set (scenarios 1 and 4) the source of fecal contamination can be either human or dog. This is due to the fact that *B. volgatus* and *B. massiliensis* are presented in both human and dog fecal samples (Table 22) and *B. dorei* is not detected using the HF183 primer set. For samples such as these, it is assumed that the source of fecal contamination is from a non-human source and sequencing and cladogram analysis are essential for final source identification. In the unlikely situation that *B. dorei* is shown to be present by cladogram analysis (but not detected using the HF183 primer set) the source will be identified as human. Data collected from field samples followed scenarios 1-4, as shown in Table 25.

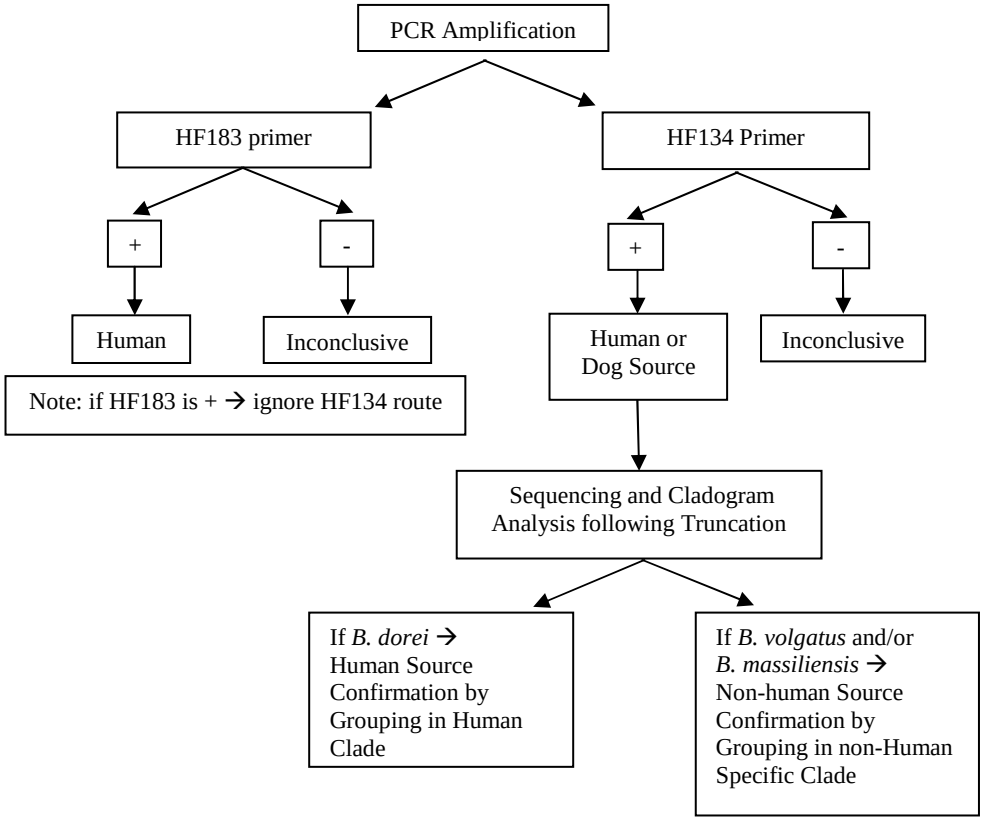


Fig. 21 - Tool box flow chart

Table 24 - Tool box for source identification

	Primers		Analysis		
Scenarios	HF134 Targets *B. volgatus, B. massiliensis* and *B. dorei*	HF183 Targets *B. dorei*	Presumptive Results	Action required	Source
1	+	-	*B. volgatus, B. massiliensis*	Sequencing*	Human or Dog
2	+	+	*B. volgatus, B. massiliensis, B. dorei*	NA	Human
3	+ +	+	*B. volgatus, B. massiliensis, B. dorei* and non-specific PCR product	NA	Human
4	+ +	-	*B. volgatus, B. massiliensis* and non-specific PCR product	Sequencing*	Human or Dog
5	-	+	*B. dorei*	NA	Human
6	-	-	Non *B. volgatus, B. massiliensis,* and *B. dorei*	NA	Non-Human

*PCR product needs to be sequenced, truncated, and analyzed in a truncated cladogram for source identification.

Table 25 - List of water samples corresponding to scenarios listed in Table 23

Scenarios	Water samples
1	Tempe Treatment Plant
2	Layton Lake, Riparian Pond, and Pacana Park
3	Salt River[b], Verde River [b], CAP Canal[b], and Riparian Fishing Lake (South and East sides)
4	Salt River[a], Verde River[a], CAP Canal[a]
5	None
6	None

In scenarios 1 and 4, amplicons are sequenced for truncated cladogram analysis (Fig 22). This confirmatory step is used to detect *B. dorei* with the HF134 primer set. These scenarios did not occur for the field samples collected, however, it is believed that the number of environmental samples tested was sufficient for the proof of concept and additional sample analysis would be required to develop a strategy relying solely on the HF183 primer set. While not included in the described protocol, PCR products from scenarios 2 (only Layton Lake) and 3 (Salt River[b], Verde River[b] and CAP Canal[b]) were sequenced, truncated, and incorporated in a cladogram (Fig 22). This was done to confirm the grouping *B. dorei* sequences derived from PCR amplification with the HF183 primer set in the human only clade.

The cladogram generated from samples including human, animals, and the field samples resulted in seven distinct clades (Fig 22). Four clades were host specific, with 2 containing human, 1 containing fish, and1 containing horse only *Bacteroides*. Two clades grouped several hosts without human and 1 clade grouped human and dog *Bacteroides* together. The two human clades are shown in green and the mixed human and dog clade is shown in blue. As expected, Verde River[b], CAP Canal[b], Layton Lake, and Pacana Park samples contain *Bacteroides* grouped within the human only clade (shown by red arrows within the green human clade). These samples contain *B. dorei*. Conversely Salt River[a], Verde River[a], CAP Canal[a], Chandler Treatment Plant, and Tempe Treatment Plant samples contained *Bacteroides* grouped within the human and dog clade (marked

with red arrows within the blue clade). These samples partially aligned with human samples, however, due to the fact that *B. dorei* was not present in these samples, it was assumed that the source of contamination is non-human. These sequences aligned with dog sequences, conferring the possibility that the source of contamination in these sites came from dog feces.

It is shown that the proposed strategy can differentiate between different sources and offers a new method for MST by providing a step-wise strategy tool box useful for identifying sources of fecal pollution.

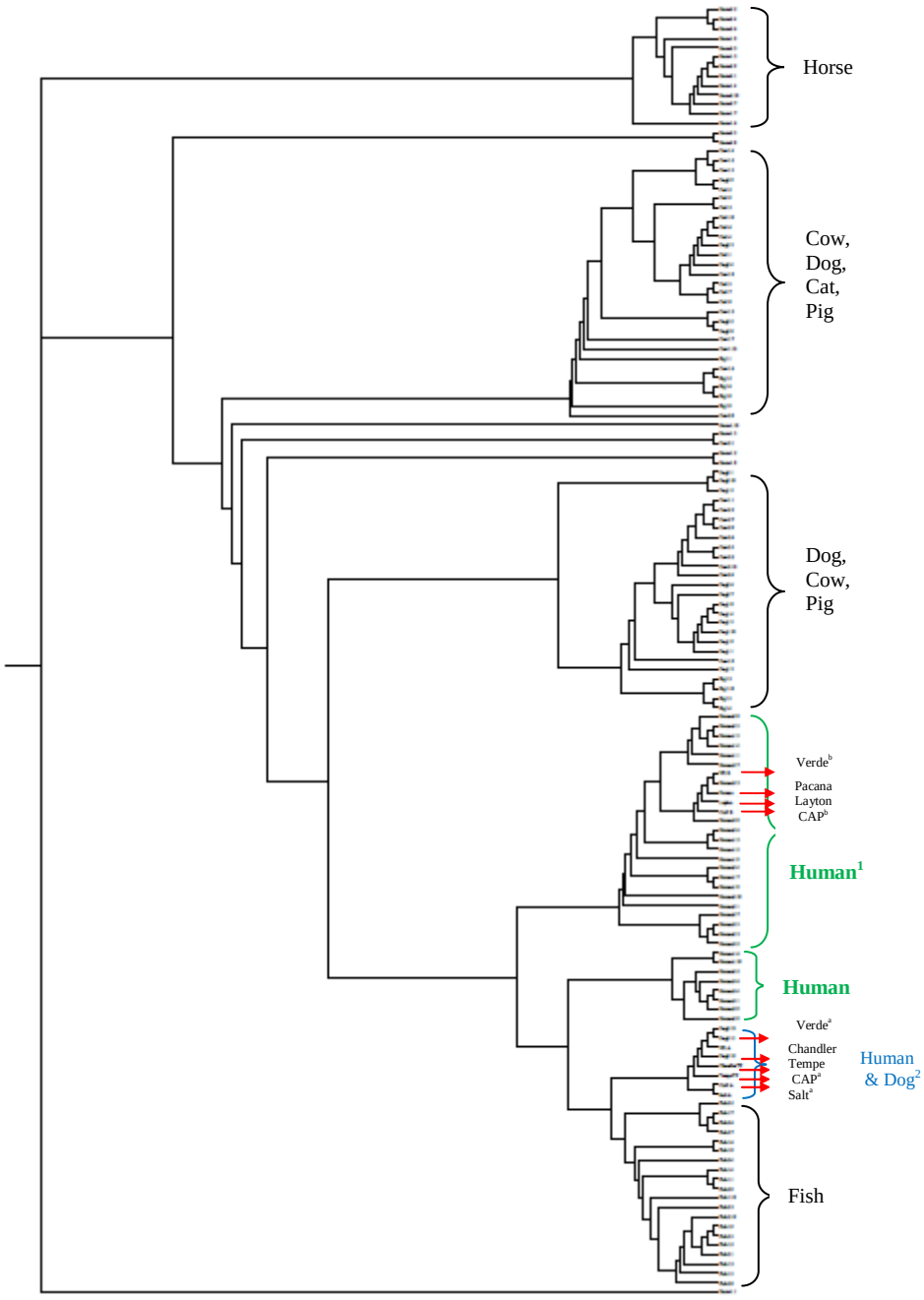


Fig. 22 - Cladogram constructed from truncated 16S rRNA gene sequences from human, animal, and field samples. Field samples (Verde River[b], Pacana Park, Layton Lake, and CAP Canal[b]) group within the Human[1] clade and (Verde River[a], Salt River[a], CAP Canal[a], Tempe Treatment Plant, and Chandler Treatment Plant) group within the Human and Dog[2] clade.

5.5 Conclusion

- A tool box strategy has been developed for *Bacteroides* source identification using PCR amplification and variable region analysis, human-specific PCR assays, and subsequent truncated cladogram grouping.
    - A variability matrix based on the sequence variance in four regions in the *Bacteroides* 16S rRNA gene has been identified via sequence alignment.
    - A cladogram constructed from truncated sequences based on the identified variable regions shows a clear separation of human *B. dorei* from *Bacteroides* of other sources.
    - The presence of *B. dorei* is a good marker for human fecal contamination.
- Field testing results showed incidence of human fecal contamination in Arizona source and reclaimed water.
- The proposed strategy offers a new method for MST and provides a step-wise strategy tool box useful for identifying sources of fecal pollution.

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