

Investigation into *Bacteroides* Persistence in Drinking Water Distribution  
Systems and Alternative Methods to Detect this Fecal Indicator

by

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## ABSTRACT

*Bacteroides* have been suggested as alternative indicators of fecal pollution since they are highly abundant in feces and are thought to have limited potential to grow in environment. However, recent literature suggests that *Bacteroides* can potentially survive within water distribution systems. The first objective of this study was therefore to investigate the validity of *Bacteroides* as a fecal indicator for drinking water through laboratory experiments and field studies. Experiments were performed using a laboratory scale PVC model water distribution system that was spiked with  $10^9$  *Bacteroides*. Samples were collected over the following four and analyzed by culture and molecular-based techniques. Second, field studies were performed by collecting water meters from two large chlorinated water distribution systems in central Arizona. Upon removal for repair by city personnel, meters were collected and biofilms samples were gathered within two hours. The biofilms were then analyzed using culture and molecular-based assays. The results from these studies support the hypothesis that *Bacteroides* DNA may be found in water distribution systems despite the difficulty of cultivating these bacterial cells. These experiments present the importance of considering biofilm interactions with fecal indicator bacteria when performing molecular assays on environmental samples, as biofilms may provide protection from high oxygen concentrations and grazing protozoa in bulk water that limit the

persistence *Bacteroides* in the environment. Although the significance of biofilm interactions with surface or recreational waters may be small, they are likely important when considering drinking water delivered through distribution systems.

The second objective of this study was to investigate alternative detection methodologies for the fecal indicator *Bacteroides*. In particular, this study focused on using a simplified protocol of Nucleic Acid Sequence Based Amplification (NASBA) and Thermophilic Helicase-Dependent Amplification (tHDA) to amplify the highly conserved 16s rRNA gene in the genomic DNA of fecal indicator *Bacteroides*. The results of this study show that the simplified NASBA procedure was not able to amplify the target, while continuous problems with tHDA exposed the methods lack of reliability. These results suggest higher reliability in the isothermal amplification methods needs to be achieved before application to environmental samples.

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## Chapter 1

### INTRODUCTION

#### **URGENCY OF IMPROVING WATER QUALITY**

The United Nations has stated that at any one time half the world's hospital beds are occupied by people with waterborne disease and that every day 4,000 children die due to unsafe drinking water (UNESCO 2010). Furthermore, the UN has estimated that close to a billion people worldwide still do not have access to safe drinking water and 2.6 billion people lack access to basic sanitation services, such as toilets or latrines. In 2000 the United Nations declared that one of their Millennium Goals was to halve the population of the world without safe water and sanitation (UNESCO 2000).

However, here in the United States issues of water quality are often seen as something that only the developing world is concerned about. The facts, though, reveal otherwise as there were nearly 20 cases of microbial waterborne outbreaks in the United States during the 1990's, resulting in 500,000 illnesses (Craun et al., 2002). Clearly there is urgency in improving water quality both in our country and abroad.

#### **NEED FOR ALTERNATIVE FECAL INDICATORS**

Fecal pollution in water is a serious environmental problem since it constitutes a health risk that is mainly owed to the potential introduction of pathogenic microorganisms that cause waterborne diseases (Crane and

Moore, 1986). Microbial quality of drinking water is thus measured in many parts of the world by testing for the presence of two enteric bacteria groups, coliforms and fecal streptococci, as indicators of fecal contamination (Madema et al., 2003).

However, despite the wide-spread use of *Escherichia coli* and intestinal enterococci as measures of microbial water quality, these fecal indicator organisms do possess limitations. The numbers of *E. coli* and enterococcus bacteria in mammalian feces is low compared to other organisms within the intestinal tract (Harmsen et al., 2002) and they have shown potential for growth in the environment (Rhodes and Kator, 1988; Desmarais et al., 2002).

In the search for alternative fecal indicators, bacterial species that belong to the order *Bacteroidales*, an order that includes the genus *Bacteroides*, have been proposed (Post et al., 1967; Fiksdal 1985). *Bacteroides* is the predominant bacterial group in the complex intestinal flora of almost all mammals, with *Bacteroides coprocola*, *B. uniformis*, *B. dorei*, *B. fragilis*, and *B. volgatus* being the prevailing *Bacteroides* species in human feces (Hong et al., 2008; Bernhard and Field, 2000). *Bacteroides* species have been proposed as alternative indicators of fecal pollution since they are highly abundant in feces (Madigan et al., 2003), are thought to have little potential to grow in environment (Salyers, 1984;

Sghir et al., 2000), and occupy a large portion of fecal bacteria compared to fecal coliforms and enterococci (Sghir et al., 2000).

Moreover, the ability to determine whether the source of fecal contamination is human or other animal is critical in developing effective pollution remediation strategies (McClain et al., 2005). *Bacteroides* species have a high degree of host specificity that likely reflects differences in source animal digestive systems (Bernhard & Field, 2000; Simpson et al., 2004; Dick et al., 2005), further supporting *Bacteroides* as an alternative fecal indicator.

#### **NEED FOR FURTHER INVESTIGATION OF *BACTEROIDES* AS FECAL INDICATORS FOR DRINKING WATER**

Historically, the use of *Bacteroides* species as fecal indicator organisms has been hindered by the difficulty of cultivating these microorganisms. However, in recent decades the popularity of molecular methods has made it possible to detect *Bacteroides* without the difficulties associated with culture-based methods. These advances have led to numerous microbial source tracking studies in the past decade that have focused on *Bacteroidales* species. These microbial source tracking studies have demonstrated that *Bacteroidales* species are able to effectively indicate fecal contamination in recreational waters (Betancourt and Fujioka, 2006; Dick and Field, 2004; Savichtcheva et al., 2007).

The success of *Bacteroidales* species as fecal indicators in recreational waters has led to their suggested use as fecal indicators in drinking water (Saunders et al., 2009). However, van der Wielen and Medema in a recent study showed that *Bacteroides* genetic markers could possibly be found in tap water from nonchlorinated water distribution systems (van der Wielen and Medema, 2010). It was hypothesized that the high number of *Bacteroidales* genetic markers detected in tap water in relation to its source water suggests possible growth of environmental *Bacteroidales* species in nonchlorinated water distribution systems. If true, this would cast into serious question the suitability of *Bacteroidales* species as fecal indicators for drinking water and raise further questions about the persistence of *Bacteroides* in the environment. There is thus need for further investigation of *Bacteroides* as fecal indicators for drinking water.

#### **NEED FOR BETTER DETECTION METHODOLOGIES**

The United States Environmental Protection Agency (EPA) currently measures microbial water quality by using culture-based methods to identify the fecal coliform *E. coli*. However, the use of culture-based methods to detect *E. coli* also has limitations. First, the time required to culture *E. coli* is more than a day, producing a lag in real time water quality data. Second, *E. coli* is present in the intestines of many animals and lacks identifiable host specific strains making it difficult to

distinguish the source of fecal pollution using the standard method. Although molecular techniques such as Polymerase Chain Reaction (PCR) have been developed that are able to more rapidly detect microorganisms, the water utilities have been slow to adopt these newer methods in part due to the high costs involved in training personnel in laboratory intensive techniques and in purchasing the necessary equipment such as thermocyclers for these methods. For these reasons, and because of the sheer volume of assays that water utilities must perform on continuous basis, new methodologies are needed to make molecular amplification techniques cheaper and easier before the benefits of these technologies can be brought to the water community.

### **STUDY OBJECTIVES**

The main objective in this study was to investigate the potential of *Bacteroides* as a fecal indicator for drinking water.

The first specific objective of this study was to (1) investigate the persistence of *Bacteroides* in drinking water distribution systems. In particular, this involved:

- a) Examining the ability of *Bacteroides* to persist in non-chlorinated water distribution systems through laboratory experiments using a pilot scale model water distribution system

- b) Investigating the occurrence of *Bacteroides* in chlorinated water distribution systems through field studies involving two large water distribution systems in central Arizona.

The second specific objective of this study was to develop a simple, rapid, and inexpensive molecular technique for the detection of *Bacteroides* in water samples. In particular, this involved:

- a) Determining the applicability of isothermal molecular amplification methods such as tHDA and a simplified NASBA procedure for water quality testing.
- b) Developing an isothermal amplification method that is able to distinguish between human and non-human sources of *Bacteroides*.

## Chapter 2

### LITERATURE REVIEW

#### **BACTEROIDES BACKGROUND INFORMATION**

The microbiological flora of the mammalian colon is comprised primarily of anaerobic bacteria. One of the most predominant of these anaerobic bacteria are the genus *Bacteroides*. This section will provide background information regarding *Bacteroides* such as their taxonomy, characteristics, species, genome, and use as a fecal indicator.

**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 some physiological similarities: obligately anaerobic 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 that have been used to reorganize this group are: 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).



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 which results in their displacement from this 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).

**Characteristics.** *Bacteroides* are gram-negative, non-spore forming, saccharolytic, anaerobic, and rod-shaped bacteria that play a fundamental role in the processing of complex molecules to simpler ones in the host intestine. These microorganisms are found primarily in the intestinal tracts and mucous membranes of all warm-blooded animals and some cold-blooded animals such as fish and insects. 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 Figure 1.

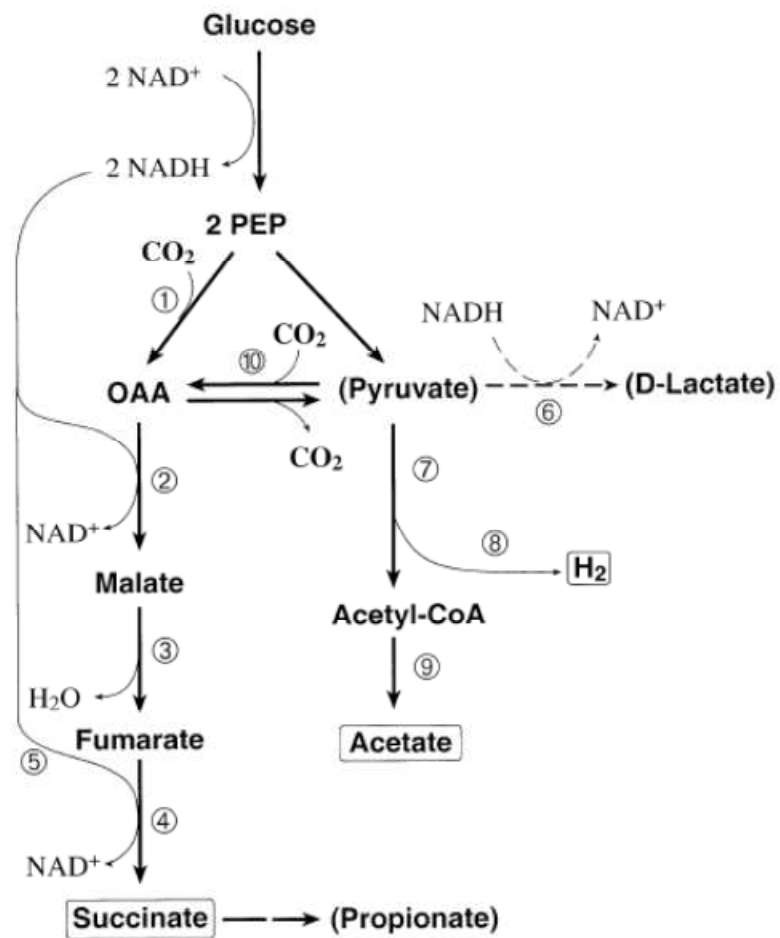


Figure 1- Glucose Fermentation Pathway of *B. thetaiotaomicron*. (Pan and Imlay, 2001)<sup>1</sup>

<sup>1</sup> Enzymes are as follows: 1, PEP carboxykinase; 2, malate dehydrogenase; 3, fumarase; 4, fumarate reductase; 5, NADH dehydrogenase; 6, lactate dehydrogenase; 7, pyruvate:

*Bacteroides* species require exogenous heme and non-heme iron for their growth. This is because they don't have 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. 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 *E. coli* per mol of glucose. 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 (Figure 1).

The members of the genus *Bacteroides* carry a typical gram-negative cell envelope that constitutes an inner membrane, the periplasmic space, and an outer membrane. The outer membrane contains lipopolysaccharide (Figure 2). *Bacteroides* membranes also contain sphingolipids as well as mixture of long-chain fatty acids, mainly straight chain saturated, anteisomethyl, and iso-methyl branched acids.

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ferredoxin:oxidoreductase; 8, hydrogenase; 9, phosphotransacetylase and acetate kinase; 10, pyruvate carboxylase. PEP, phosphoenolpyruvate; OAA, oxaloacetate. Major excreted products are boxed; minor products are in parentheses.

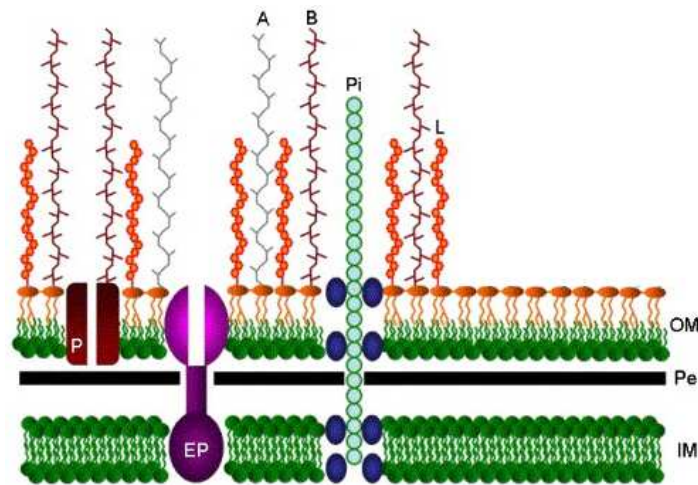


Figure 2- A Schematic Model of the *Bacteroides fragilis* Cell Envelope (Pumbwe et al., 2006)<sup>2</sup>

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).

One of the important characteristics of the *Bacteroides* species is their resistance to a variety of antibiotics such as  $\beta$ -lactams, aminoglycosides, erythromycin and tetracycline. 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

<sup>2</sup> Figure shows 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

the presence of an inactivating enzyme (Rasmussen et al., 1997). In 1950, an antibiotic resistant survey reported that almost all of the *Bacteroides* species are susceptible to tetracycline. However by 1970 it was found that about 80-90% of *Bacteroides* species are resistant to tetracycline.

“Tetracycline resistance in the *Bacteroides* is attributable, almost exclusively, to the presence of the *tetQ* gene, which encodes a protein that is believed to alter the ribosome target site for the antibiotic” (Fletcher et al., 1991).

*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<sub>12</sub>, ammonia, carbon dioxide, and sulfide, all of which are plentiful in the human colon (Holdeman et al., 1984).

**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*

<i>Bacteroides acidifaciens</i>	<i>Bacteroides nordii</i>
<i>Bacteroides caccae</i>	<i>Bacteroides ovatus</i>
<i>Bacteroides capillosus</i>	<i>Bacteroides pectinophilus</i>
<i>Bacteroides capillus</i>	<i>Bacteroides pentosaceus</i>
<i>Bacteroides cellulosolvens</i>	<i>Bacteroides plebeius</i>
<i>Bacteroides coagulans</i>	<i>Bacteroides polypragmatus</i>
<i>Bacteroides coprocola</i>	<i>Bacteroides pyogenes</i>
<i>Bacteroides coprosuis</i>	<i>Bacteroides salyersiae</i>
<i>Bacteroides dorei</i>	<i>Bacteroides splanchnicus</i>
<i>Bacteroides eggerthii</i>	<i>Bacteroides stercoris</i>
<i>Bacteroides finegoldii</i>	<i>Bacteroides suis</i>
<i>Bacteroides fragilis</i>	<i>Bacteroides tectus</i>
<i>Bacteroides galacturonicus</i>	<i>Bacteroides thetaiotaomicron</i>
<i>Bacteroides goldsteinii</i>	<i>Bacteroides uniformis</i>
<i>Bacteroides helcogenes</i>	<i>Bacteroides ureolyticus</i>
<i>Bacteroides intestinalis</i>	<i>Bacteroides vulgatus</i>
<i>Bacteroides massiliensis</i>	<i>Bacteroides xylanolyticus</i>
Source: <a href="http://www.catalogueoflife.org/annual-checklist/2010/browse/tree">http://www.catalogueoflife.org/annual-checklist/2010/browse/tree</a>	

Table 2

Species of genus *Bacteroides* according to DSMZ

<i>Bacteroides acidifaciens</i>	<i>Bacteroides microfusus</i>
<i>Bacteroides amylophilus</i>	<i>Bacteroides multiacidus</i>
<i>Bacteroides asaccharolyticus</i>	<i>Bacteroides nodosus</i>
<i>Bacteroides barnesiae</i>	<i>Bacteroides nordii</i>
<i>Bacteroides bivius</i>	<i>Bacteroides ochraceus</i>
<i>Bacteroides buccae</i>	<i>Bacteroides oleiciplenus</i>
<i>Bacteroides buccalis</i>	<i>Bacteroides oralis</i>
<i>Bacteroides caccae</i>	<i>Bacteroides oris</i>
<i>Bacteroides capillosus</i>	<i>Bacteroides oulorum</i>
<i>Bacteroides capillus</i>	<i>Bacteroides ovatus</i>
<i>Bacteroides cellulosityticus</i>	<i>Bacteroides pectinophilus</i>
<i>Bacteroides cellulosolvans</i>	<i>Bacteroides pentosaceus</i>
<i>Bacteroides clarus</i>	<i>Bacteroides plebeius</i>
<i>Bacteroides coagulans</i>	<i>Bacteroides pneumosintes</i>
<i>Bacteroides coprocola</i>	<i>Bacteroides polypragmatus</i>
<i>Bacteroides coprophilus</i>	<i>Bacteroides praeacutus</i>
<i>Bacteroides coprosuis</i>	<i>Bacteroides propionificiens</i>
<i>Bacteroides corporis</i>	<i>Bacteroides putredinis</i>
<i>Bacteroides denticola</i>	<i>Bacteroides pyogenes</i>
<i>Bacteroides disiens</i>	<i>Bacteroides ruminicola</i>
<i>Bacteroides distasonis</i>	<i>Bacteroides ruminicola sub-spp; Ruminicola brevis</i>
<i>Bacteroides dorei</i>	<i>Bacteroides endodontalis</i>
<i>Bacteroides eggerthii</i>	<i>Bacteroides melaninogenicus</i>
<i>Bacteroides faecis</i>	<i>Bacteroides salanitronis</i>
<i>Bacteroides fingoldii</i>	<i>Bacteroides salivovus</i>
<i>Bacteroides fluxus</i>	<i>Bacteroides salyersiae</i>
<i>Bacteroides forsythus</i>	<i>Bacteroides sartorii</i>
<i>Bacteroides fragilis</i>	<i>Bacteroides splanchnicus</i>
<i>Bacteroides furcosus</i>	<i>Bacteroides stercoris</i>
<i>Bacteroides galacturonicus</i>	<i>Bacteroides succinogenes</i>
<i>Bacteroides gallinarum</i>	<i>Bacteroides suis</i>
<i>Bacteroides gingivalis</i>	<i>Bacteroides tectum</i>
<i>Bacteroides gracilis</i>	<i>Bacteroides tectus</i>
<i>Bacteroides graminisolvans</i>	<i>Bacteroides termitidis</i>
<i>Bacteroides helcogenes</i>	<i>Bacteroides thetaiotaomicron</i>
<i>Bacteroides heparinolyticus</i>	<i>Bacteroides uniformis</i>
<i>Bacteroides hypermegas</i>	<i>Bacteroides ureolyticus</i>
<i>Bacteroides intermedius</i>	<i>Bacteroides veroralis</i>
<i>Bacteroides intestinalis</i>	<i>Bacteroides vulgatus</i>
<i>Bacteroides levii</i>	<i>Bacteroides xylanisolvans</i>
<i>Bacteroides loescheii</i>	<i>Bacteroides xylanolyticus</i>
<i>Bacteroides macacae</i>	<i>Bacteroides zooglooformans</i>
<i>Bacteroides massiliensis</i>	
<i>Bacteroides melaninogenicus</i>	
<i>Bacteroides melaninogenicus</i>	
<i>sub-spp; Intermedius macacae</i>	
Source: <a href="http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Bacteroides&amp;show_all_details=1">http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Bacteroides&amp;show_all_details=1</a>	

However, most *Bacteroides* species strains belong to ten cultivated species including *Bacteroides vulgatus*, *B. thetaiotaomicron*, *B. distasonis*, *B. caccae*, *B. eggerthii*, *B. merdae*, *B. ovatus*, *B. stercoris*, *B. uniformis* and *B. fragilis* (Shah and Collins, 1989). The proportions of *Bacteroides* species seen clinically are shown in Figure 3. As shown, *B. fragilis* is the most frequently isolated species among *Bacteroides*.

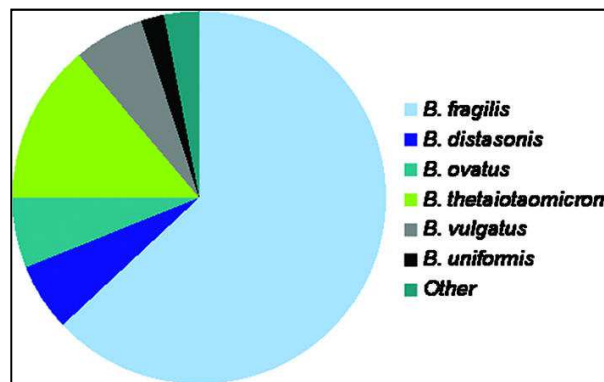


Figure 3- Proportions of *Bacteroides* Species Seen Clinically (Wexler, 2007)

**Genome.** 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 (<http://www.sanger.ac.uk/>). The 16S rRNA gene of *Bacteroides* has been widely used for analysis of diversity of *Bacteroides* species and differentiating it from neighboring species (Karlsson et al. 2010; Paster et al., 1994). However, studies have also used members of the Heat-Shock



Protein (HSP) such as the Hsp60 gene for identification and characterization of the *Bacteroides* species. According to a recent study, an analysis based on *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.

**Use as Fecal Indicators.** Fecal pollution in water is a serious environmental problem since it constitutes a health risk that is mainly owed to the potential introduction of pathogenic microorganisms that cause waterborne diseases (Crane and Moore, 1986). Moreover, the ability to determine whether the source of fecal contamination is human or other animal is critical in developing effective pollution remediation strategies (McClain et al., 2009).

The use of *Bacteroides* as a potential fecal indicator was suggested by Fiksdal (Fiksdal et al., 1985). In addition, recent studies have proposed using members of the *Bacteroides* genus as alternative indicators of fecal pollution since they are highly abundant in feces (Madigan et al., 2003), have little potential to grow in environment (Salyers, 1984; Sghir et al.,

2000), have a high degree of host specificity that likely reflects differences in source animal digestive systems (Bernhard & Field, 2000a; Simpson et al., 2004; Dick et al., 2005a), and occupy a large portion of fecal bacteria compared to fecal coliforms and enterococci (Sghir et al., 2000).

Moreover, work has been done to correlate the presence of *Bacteroides* molecular markers with *E. coli*. A study conducted by Bower and colleagues has shown that *Bacteroides* human specific molecular markers may be present in fresh water lakes when culturable *E. coli* levels are as low as 0-10 CFU/100 ml, far lower than the recommended limit for *E. coli* in recreational waters (Bower et al., 2005). Bowers and colleagues also concluded that *Bacteroides* and *E. coli* have similar longevities in terms of intact cells that can be recovered for analysis (Bower et al., 2005). This information illustrates how *Bacteroides* can perform well as a fecal indicator when compared with the traditional fecal indicator *E. coli*. Moreover, higher correlations between detection and persistence of enteric pathogens exist for *Bacteroides* than for other fecal indicator bacterial groups (Savichtcheva et al., 2005; Walters and Fields, 2006; Walters et al., 2009), supporting the validity of *Bacteroides* as a fecal indicator.

### **BACTEROIDES OCCURRENCE**

*Bacteroidales* bacteria are believed to be found in reservoirs restricted to the body cavities of animals, including humans. Based on

culture and culture-independent methods it has been found 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). Moreover, no strains adapted to aquatic environments are currently known (Walter and Fields 2006). Furthermore, *Bacteroidales* distributions among differing host animals are believed to be governed primarily by diet-digestive system type (Ley et al., 2008). These host distributions can then be utilized as fecal indicators that have that ability to distinguish between fecal pollution sources (i.e. dog versus human).

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 phlotypes 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* (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 the *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,

classified as *Bacteroides* types A and B (Sakta et al., 1981). Although many members of the phylum Bacteroidetes have been recovered from the hindgut of the termite, no true *Bacteroides* have been detected from the natural environment prior to this study (Ohkuma et al., 2002).

### ***BACTEROIDES* SURVIVAL**

Biotic and abiotic environmental factors have been shown to effect the survival and persistence of *Bacteroides* and its DNA in the environment. When utilizing *Bacteroides* as a fecal indicator in conjunction with molecular detection techniques, the survival and persistence of both the cell and its DNA must be considered. Examples of the environmental factors that can affect the persistence of *Bacteroides* and its DNA include dissolved oxygen concentrations, grazing protozoa, temperature (Bell et al., 2009; Okabe and Shimazu, 2007), bacteriophages (Payan et al., 2005), solar irradiation (Bae and Wuertz, 2009; Walters and Field, 2009; Walters et al., 2009), and sedimentation (Carson et al., 2005), among others.

Because *Bacteroidales* have traditionally been thought of as obligate anaerobes, their survival in the extra-intestinal environment has been thought to have been negligible. However, it is now known that at least some bacteria in the *Bacteroidales* group are not obligately anaerobic. While most *Bacteroidales* species can only survive in the presence of low oxygen concentrations for a period of a few hours,

*Bacteroides fragilis*, which was formerly considered an obligate anaerobe, is now known to require nanomolar concentrations of oxygen for growth and possesses an O<sub>2</sub>-dependent cytochrome (Baughn et al., 2004). This is in line with the observation that *Bacteroides fragilis* cells show some degree of oxygen tolerance when they are handled in the laboratory.

Walters and Field (2006) demonstrated that *Bacteroides* can grow in aerobically incubated sewage for up to 24 h and that their molecular marker can persist for at least 24 h under the same conditions (Walters and Fields 2006). This is in agreement with the findings of Kreader (1998), who showed that the molecular signal for *Bacteroides distasonis* persisted for up to 14, 5, and 2 days at 4°C, 14°C, and 24°C, respectively, in unfiltered bulk river water. Furthermore, Seurinck and colleagues (2005) found that the human-specific marker (HF183) persisted for up to 24 days at 4°C and 12°C and for up to 8 days at 28°C in fresh river water (Seurinck et al., 2005).

The impact of temperature variation on the survival of the *Bacteroidales* group is one of the most studied environmental persistence determiners, with a positive relationship between temperature and decay rates being found (Bell et al., 2009; Okabe and Shimazu, 2007). This is thought to be largely caused by increased eukaryotic activity and thus predation at higher temperatures. Moreover, it is known that DNA degrades more rapidly at higher temperatures. Because temperature

plays a significant role in the persistence of *Bacteroidales* and its DNA in the environment, variability in DNA detection between summer and winter must be considered in studies utilizing molecular techniques such as PCR and qPCR. Significant predation combined with high DNA degradation rates under high-temperature conditions may lead to misdetection of the marker when fecal pathogens are still viable. Or conversely, the persistence of the DNA at lower temperatures could present falsely high risk scenarios.

The impact of salinity on decay rates was also studied by Okabe and Shimazu at salinities ranging from 0% to 30% at 10°C. The salinity effect was negligible compared to the effects of temperature, but decay rates were consistently lower in the higher-salinity microcosms (Okabe and Shimazu, 2007).

Several studies have also examined the impact of light on the persistence of *Bacteroidales*, and have showed mixed results. (Bae and Wuertz, 2009; Walters and Field, 2009; Walters et al., 2009). The results of Bae and Wuertz did suggest small if not negligible differences in decay rates due to light at a salinity of 33‰ (Bae and Wuertz, 2009), which is similar to the findings of Walters and Field (2009) in freshwater microcosms. However, the reported decay rates were significantly different. The small impact sunlight does have on the persistence of

*Bacteroidales* is thought to be caused by the increased reactive oxygen species in the environment in the presence of sunlight.

In summary, short survival periods, on the scale of a few hours, have been described for culturable *Bacteroides* species (Saunders et al., 2009; Savichtcheva et al., 2005). In contrast, the DNA of fecal *Bacteroides* species remains detectable from day to weeks and possibly even months, depending on the conditions (Okabe and Shimazu, 2007; Seurinck et al., 2005). Moreover, *Bacteroidales* is thought to persist longer under natural conditions as sewage influent often contains flocculent material that bacteria can colonize small, thus creating anaerobic or anoxic microniches that may provide a refuge for *Bacteroidales* within an overall aerobic environment for a period of time (Tay et al., 2002). However, *Bacteroides* spp. have also shown higher die-off rates than fecal coliforms and enterococci, regardless of temporal variability (Ballesté et al., 2010). Hence, *Bacteroides* spp. are indicative of recent fecal pollution. Moreover, higher correlations between detection and persistence of enteric pathogens exist for *Bacteroides* than for other fecal indicator bacterial groups (Savichtcheva et al., 2005; Walters et al., 2006; Walters et al., 2009), supporting the validity of *Bacteroides* as a fecal indicator.

### **BACTEROIDES IN BIOFILMS**

Because *Bacteroidales* bacteria are believed to originate from the intestinal tracts of warm blooded animals, there have been no studies

investigating the potential for *Bacteroides* to form biofilms within the natural environment. It is well documented, though, that *Bacteroides* are able to form biofilms within the human body. *Bacteroides* have been found to be one of the predominant anaerobes associated with biofilms formation in layer of mucosal tissue (Probert and Gibson, 2002) that extends from the stomach to the colon in the gastrointestinal tract (Atuma et al., 2001).

Moreover, *Bacteroides* has shown the ability to form biofilms within wastewater treatment plant (WWTP) influent pipes. Savichtcheva and colleagues (2007) showed that while total coliforms (TC) and fecal coliforms (FC) outnumbered *Bacteroides* 16s rRNA markers in wastewater treatment plant (WWTP) bulk influent and in fecally contaminated surface waters, *Bacteroides* genetic markers outnumbered TC and FC in biofilms in WWTP influent pipes (Savichtcheva et al., 2007). And while 16S rRNA gene copies cannot be directly related to cell counts because the 16S rRNA operon copy numbers of the genera *Bacteroides* and *Prevotella* are not presently known, these results do indicate that *Bacteroides* preferentially integrate into pipe biofilms over FC's and TC's. This fact could reflect the difference in survival and persistence of FCs and anaerobic *Bacteroides* spp. in biofilm environments. Fecal anaerobes, especially *Bacteroides* spp., may survive better inside biofilms because the deeper parts of the biofilms are most likely anoxic (Okabe et al., 1999).



Furthermore, Van der Wielen and colleagues have posited that *Bacteroidales* growth within non-chlorinated water distribution systems is possible (van der Wielen et al., 2010). Their particular study revealed higher levels of *Bacteroides* 16s rRNA gene markers in tap water than in source water for over ten utilities in the Netherlands. The possible growth of *Bacteroides* in water distribution systems (WDS) seems more likely to occur in WDS biofilms where anoxic conditions may exist than in the generally aerobic bulk water.

It is well known that bacteria growing in the surface associated communities that are known as biofilms are different physiologically from bacteria growing in a bulk liquid (Hall-Stoodley et al., 2004). As the bacterial cells adapt to growth in these surface-associated communities, they express phenotypic traits that are often distinct from those that are expressed during growth in the bulk fluid (Stewart and Franklin, 2008). The scientific literature has demonstrated that significant physiological adaptations can occur to bacteria following attachment and growth in biofilms, such as the production of extracellular organelles, such as pili and flagella (Belas, et al., 1986). Figure 4 shows electron micrographs of *B. fragilis*.

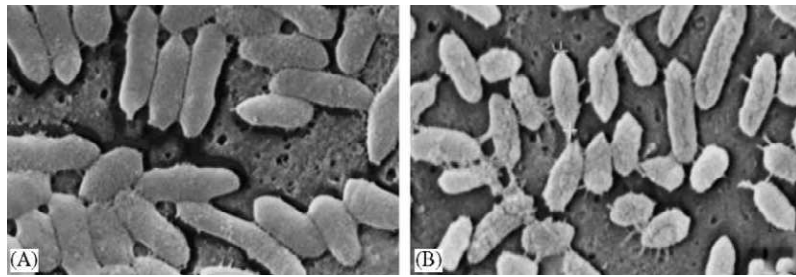


Figure 4-Scanning Electron Micrograph of *B. fragilis* NCTC 9343 (Pumbwe et al., 2006)<sup>3</sup>

It remains possible that although *Bacteroides* does not generally persist in the environment, there may exist localized regions within WDS biofilms where the adaptation of *Bacteroides* to a biofilms environment may cause the persistence and survival of *Bacteroides* to be anomalous to general trends or theory.

#### **BACTEROIDES ISOLATION AND IDENTIFICATION METHODS**

This section will go over different techniques for *Bacteroides* isolation and identification including culture-based as well as molecular-based methods such as polymerase chain reaction (PCR), real-time PCR (qPCR), and isothermal amplification.

**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

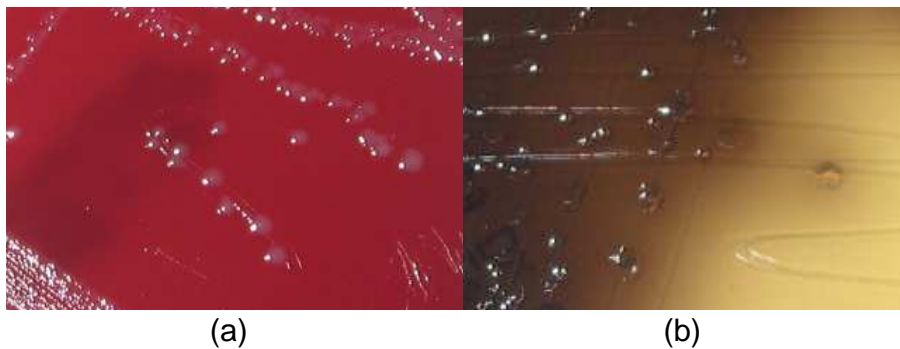
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<sup>3</sup> Figure shows: (A) uncultured cells and (B) cells treated with 0.15% bile salts which resulted in production of pili-like appendages

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). The KVLB agar is designed in such a way to inhibit growth of most facultative anaerobic bacteria while allowing selective isolation mainly of *Prevotella* species and *Bacteroides* species. BBE is selective for isolation of most of the *Bacteroides* species that include the *Bacteroides fragilis* group.

Utilizing selective media allows 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 bigger than one millimeter in diameter and are 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. Thus, the tolerance to the 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 non-*B. fragilis* group. Some examples of such microorganisms are *Bacteroides splanchnicus*,

*Fusobacterium mortiferum*, *Klebsiella pneumoniae*, *Enterococcus* species, and yeasts. 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 or PCR must be performed to ensure the cultured microorganisms are *Bacteroides* (Finegold et al., 1986; Lennette et al., 1985). Figure 5 shows cultured *Bacteroides* in both blood agar and BBE agar.



*Figure 5-Bacteroides fragilis* Pure Culture (ATCC #23745) Cultured on Blood Agar (a) and on *Bacteroides* Bile Esculin Agar (b) (Photos: ASU Environmental Microbiology lab)

**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. Moreover, isothermal methods have been developed that could have application to the detection of fecal indicators.

**PCR and other Conventional Techniques.** 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 has been reported (Grove and Clark, 1987).

In addition, the 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, first an appropriate restriction endonuclease is utilized to digest total genomic DNA, and then, the resulting digested DNA is electrophoresed on an agarose gel. Obtained patterns are species related to Restriction Fragment Length Polymorphism (RFLP). To simplify RFLP patterns, 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 field gel electrophoresis (PFGE). This technique separates the large DNA fragments that are generated with rare-cutting restriction endonuclease (Shaheduzzaman et al., 1997).

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* specific 16S rRNA gene molecular markers. The species specific molecular markers for *Bacteroides 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 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 *Bacteroides fragilis*, *B. distasonis*, *B. ovatus*, *B. eggerthii*, *B. thetaiotaomicron*, *B. uniformis* and *B. volgatus*.

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 relies on the cultivation-independent, host-specific targets, 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.

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 *Bacteroides* 16S rRNA gene of different hosts such as pig, horse, dog, elk, bovine and cow has been developed. However, it must be noted that cross-amplification of published human specific primers has been reported. One example is the findings of McClain and colleagues, who reported cross-amplification of fish feces with human specific primers (McClain et al., 2009).

**Isothermal Amplification Methods.** Since the introduction of PCR in 1987, molecular amplification has become a ubiquitous presence in all fields relating to bioscience. The success and widespread use of PCR has encouraged further studies into alternative means of molecular amplification that could be used for clinical research, with much emphasis put on isothermal methods. After NASBA was first introduced in 1991, there have been other isothermal amplification methods that have been proposed. These include Ligase Chain Reaction (LCR) (Barany, 1991), Strand Displacement Amplification (SDA) (Little et al., 1999), Branched DNA Amplification (bDNA) (Smolina et al, 2004), DNA Cleavage Based Amplification also known as Invader (Hall et al., 2000), Thermophilic Helicase Dependent Amplification (Vincent et al., 2004) and Rolling Cycle Amplification (RCA) (Wiltshire et al., 2000). All have shown good sensitivity and are compatible with many detection techniques, such as fluorescence, chemiluminescence, or gel electrophoresis. These methods have shown success in some capacity, but the complicated nature of the some of these assays and their associated costs have the potential to limit their popularity. For these reasons we chose to investigate two of the more straightforward methods, a simplified NASBA procedure and tHDA.

**Nucleic Acid Sequence Based Amplification (NASBA).** The first isothermal molecular amplification method that was investigated was based on the concept of Nucleic Acid Sequence Based Amplification



(NASBA). NASBA is a transcription-based amplification system (TAS) for the specific replication of nucleic acids *in vitro*. The amplification reaction is performed at the isothermal temperature of 41°C. Three enzymes are involved in this homogeneous reaction: avian myeloblastosis virus (AMV) reverse transcriptase (RT), RNase H and T7 DNA dependent RNA polymerase (DdRp). Because the reaction incorporates RT into the amplification process, the method is especially suited for RNA analytes like mRNA, rRNA or genomic RNA (Deiman et al., 2002). Table 3 shows some of the basic characteristics of NASBA.

Table 3

*Characteristics of NASBA* (modified from Deiman et al., 2002)

<b>Characteristics of NASBA</b>
<ul style="list-style-type: none"><li>• The reaction is carried out in a single isothermal temperature profile.</li><li>• Especially suited for RNA analytes because of the integration of RT into the amplification process.</li><li>• The use of a single temperature eliminates the need for special thermocycling equipment.</li><li>• Efficient ongoing process resulting in exponential kinetics caused by production of multiple RNA copies by transcription from a given cDNA product.</li><li>• Unlike amplification processes, such as PCR in which the initial primer level limits the maximum yield of product, the amount of RNA product obtained in NASBA exceeds the level of primers by at least one order of magnitude.</li><li>• The intermediate cDNA product can be made double-stranded, ligated into plasmids, and cloned.</li><li>• Three enzymes are required to be active in the same reaction conditions.</li><li>• Low temperature can increase the nonspecific interactions of the primers. However, these interactions are minimized by the inclusion of DMSO.</li><li>• The primers are not incorporated in the amplicon and thus labeled primers cannot be used for detection.</li><li>• The length of the target sequence to be amplified efficiently is limited to approx 100 to 250 nucleotides.</li></ul>

For visualization purposes, a schematic of NASBA for the detection of RNA is presented in Figure 6.

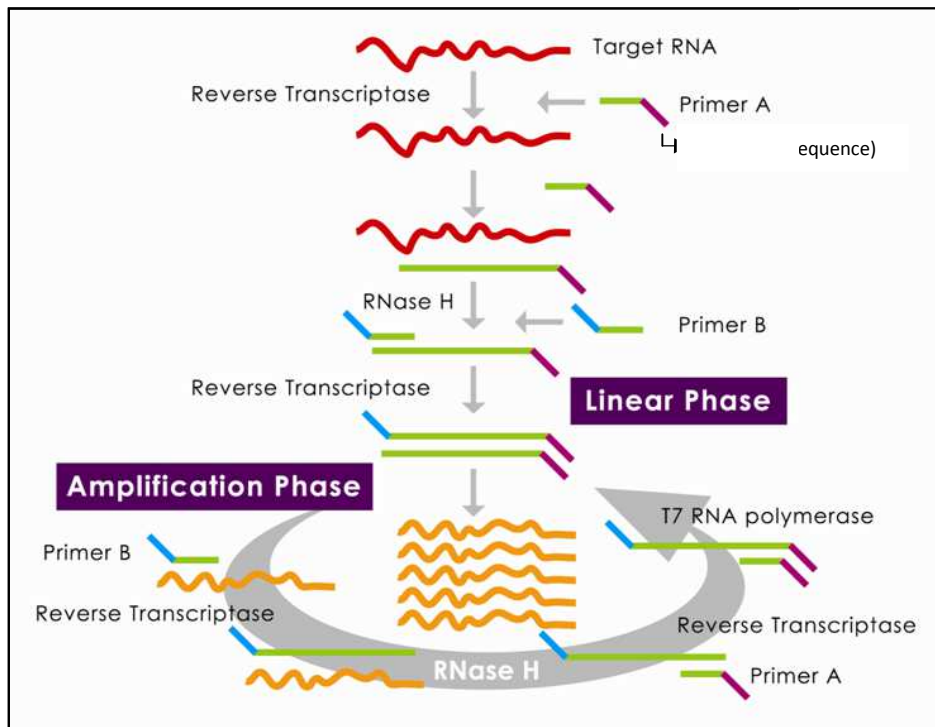


Figure 6-Schematic of NASBA for Detection of RNA analyte<sup>4</sup>

Figure 6 shows that NASBA relies on the ability of T7 RNA polymerase to recognize a known promoter sequence and synthesize an RNA amplicon at an isothermal temperature profile. However, because there is a need for a more straightforward protocol for water quality testing, specifically a protocol that does not work with RNA, we attempted to simplify NASBA. As explained above, NASBA relies on T7 RNA Polymerase to produce an RNA amplicon that can be detected. However, it has also been shown that T7 RNA Polymerase is able produce a DNA amplicon when in the presence of dioxynucleotides (dNTP's).

<sup>4</sup> Source: <http://www.foodsafetylabs.com/images/tech/nasba2l.png>

Moreover, RNase H has shown the ability to denature, or unzip, double stranded DNA, though the mechanism by which RNase H does this is unknown. Therefore, utilizing these two enzymes, we attempted to develop a simplistic, novel technique to amplify a genomic DNA target sequence in a one-step isothermal reaction that results in a DNA amplicon, effectively eliminating RNA from the reaction sequence.

#### **Thermophilic Helicase-Dependent Amplification (tHDA).**

Helicase-dependent amplification (HDA) is an isothermal *in vitro* DNA amplification method based upon the enzymatic activities of helicases to separate double stranded DNA and the ability of DNA polymerases to synthesize DNA. First presented in 2004 (Vincent et al., 2004), the helicase *E. coli* UvrD was used with two necessary accessory proteins, MutL and single-stranded DNA-binding protein (SSB) for separation of double stranded DNA. In an effort to improve the specificity and performance of HDA, a thermostable UvrD helicase (Tte-UvrD) was purified from *Thermoanaerobacter tengcongensis* by (An et al., 2005). The Tte-UvrD helicase unwinds blunt-ended DNA duplexes as well as substrates possessing 3' or 5' ssDNA tails. Though the Tte-UvrD helicase has been known to require single-stranded 3' tails several nucleotides in length to begin separation of dsDNA, An's group utilized the fact that at temperatures between 60-65°C dsDNA exhibits thermal breathing where several base pairs at blunt ends of dsDNA separate to use the Tte-UvrD

helicase without the need for accessory proteins. The so called thermophilic HDA (tHDA) system is more efficient, displaying heightened amplification sensitivity without the need for the MutL and SSB accessory proteins (An et al., 2005). The simplistic nature of the tHDA platform makes the technology ideal for use where rapid identification of microorganisms is needed at the point-of-need. A schematic of the tHDA system is provided in Figure 7.

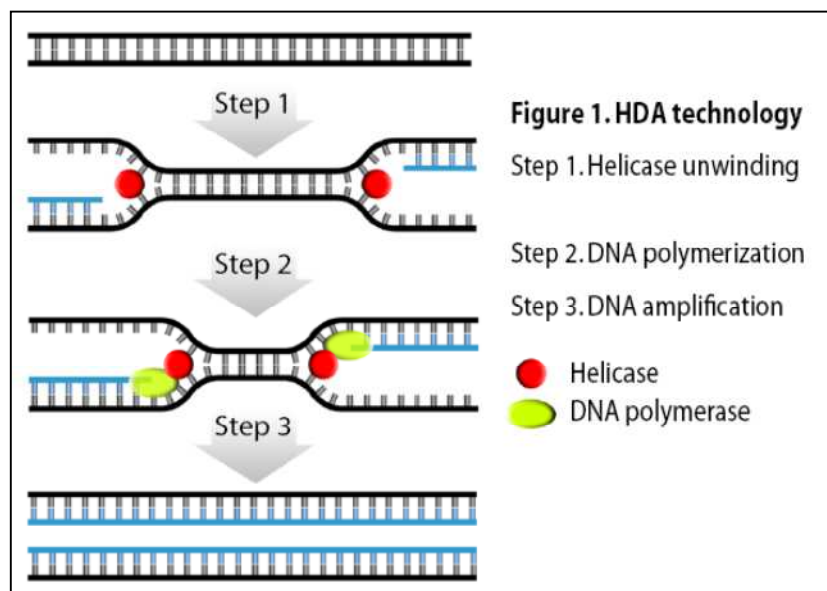


Figure 7-Schematic of tHDA (Biohelix Corp.)

## Chapter 3

### *BACTEROIDES* PERSISTENCE IN WATER DISTRIBUTION SYSTEMS

#### **ABSTRACT**

*Bacteroides* have been suggested as alternative indicators of fecal pollution due to their high abundance in feces, low potential for growth in the environment, and high degree of host specificity. The success of *Bacteroides* species in identifying fecal pollution in recreational waters has led to their suggested use as fecal indicators in drinking water. However, recent literature suggests that *Bacteroides* can potentially survive within nonchlorinated water distribution systems. The objective of this study was therefore to investigate the ability of *Bacteroides* to persist in water distribution systems. A nonchlorinated pilot scale PVC water distribution system with a total volume of 50 L that had been continuously running for more than 10 years was inoculated with a culture containing  $10^9$  *Bacteroides fragilis* cells. Although *Bacteroides* could not be detected in bulk water or biofilm samples by culture-based techniques after the first hour since inoculation, the *Bacteroides* DNA was detected in the model distribution system biofilms via PCR for up to two months after inoculation. Moreover, field studies were performed on two large chlorinated water distribution systems in central Arizona to investigate the ability of *Bacteroides* to persist in these systems. Biofilms collected from the inlet of domestic water meters were analyzed by PCR. The *Bacteroides* 16S

rRNA genetic marker was detected in 30 of 65 samples, and sequencing results confirm 27 of these samples were *B. fragilis*. These results suggest the interactions between *Bacteroides* and biofilms could play an important role in understanding *Bacteroides* persistence in environments where biofilms are significant, such as water distribution systems.

## **INTRODUCTION**

Microbial quality of drinking water is measured in many parts of the world by testing for the presence of two bacteria groups, coliforms and fecal streptococci, as indicators of fecal contamination (Madema et al., 2003). However, despite the wide-spread use of *Escherichia coli* and intestinal enterococci as measures of microbial water quality, these fecal indicator organisms do possess limitations. The concentration of *E. coli* and enterococcus bacteria in mammalian feces is low compared to other organisms within the intestinal tract (Harmsen et al., 2002) and they have shown potential for growth in the environment (Rhodes and Kator, 1988; Desmarais et al., 2002).

In the search for alternative fecal indicators, bacterial species that belong to the order *Bacteroidales*, an order that includes the genus *Bacteroides*, have been proposed (Post et al., 1967; Fiksdal 1985). *Bacteroides* is the predominant bacterial group in the complex intestinal flora of almost all mammals, with *Bacteroides coprocola*, *B. uniformis*, *B. fragilis*, *B. dorei*, and *B. volgatus* being the prevailing species in human

feces (Hong et al., 2008; Bernhard and Field, 2000). Species within *Bacteroidales* are thought to be restricted to reservoirs within body cavities of animals, with no known species adapted for growth within aquatic environments (Walter and Fields 2006).

Recent studies have proposed using members of the *Bacteroides* genus as alternative indicators of fecal pollution due to their abundance in feces (Madigan et al., 2003), low potential for growth in the environment (Salyers, 1984; Sghir et al., 2000), and high proportions in fecal matter compared to other fecal bacteria such as fecal coliforms and enterococci (Sghir et al., 2000). Moreover, *Bacteroides* possess a high degree of host specificity that likely reflects differences in source animal digestive systems (Bernhard & Field, 2000; Simpson et al., 2004; Dick et al., 2005).

Historically, the use of *Bacteroides* species as fecal indicator organisms has been hindered by the difficulty of cultivating these microorganisms. However, in recent decades the advancement of molecular methods has made it possible to detect *Bacteroides* without the difficulties associated with culture-based techniques. These advances have opened the door for use of *Bacteroides* as a molecular screening tool for fecal contamination with the potential for source identification. Numerous microbial source tracking studies in the past ten years have demonstrated that *Bacteroidales* species are able to effectively indicate fecal contamination in recreational waters and provide some level of fecal



source identification (Betancourt and Fujioka, 2006; Dick and Field, 2004; Savichtcheva et al., 2007).

The success of *Bacteroidales* species as fecal indicators in recreational waters has led to their suggested use as fecal indicators in drinking water (Saunders et al., 2009). However, van der Wielen and Medema in a recent study showed that *Bacteroides* DNA could be found in tap water from nonchlorinated water distribution systems in the Netherlands (van der Wielen and Medema, 2010). It was hypothesized that the high number of *Bacteroidales* genetic markers detected in tap water in relation to source water could suggest environmental growth of *Bacteroidales* species in nonchlorinated water distribution systems. If true, this would cast doubt on the suitability of *Bacteroidales* species as fecal indicators for drinking water and raise further questions about the persistence of *Bacteroides* in the environment.

Moreover, few data exists on the persistence of fecal bacteria genetic markers in water distribution systems, even for traditional indicators such as *E. coli*. It remains possible that the genetic markers of fecal indicator bacteria could be detected past the time when the bacteria can be detected via culture-based techniques. Furthermore, since *Bacteroides* is detected in environmental samples by molecular methods, the persistence of its genetic marker in water distribution systems is an important factor when considering *Bacteroides* as an indicator for drinking

water. The objectives of this study were, therefore, to examine the ability of *Bacteroides* to persist in a non-chlorinated model water distribution system and to investigate the occurrence of *Bacteroides* in chlorinated water distribution systems in central Arizona, USA.

## **MATERIALS AND METHODS**

This study consisted of two parts: experiments with a pilot distribution system under controlled laboratory conditions and a field study.

**Pilot Distribution System.** Experiments were performed using a laboratory scale model water distribution system consisting of an independent pipe loop constructed of PVC (Figure 8) that had been continuously running for more than 10 years. The pipe loop system had an eighteen foot long (two-inch diameter) distribution main (connected to a reservoir) and a four foot long dead end. Distribution mains and dead end lines consisted of six and two removable pipe sections (2 feet in length), respectively. The total volume of water contained in the model distribution system (MDS) was fifty liters. The water was continuously recirculated in the main distribution line and reservoir using a self-priming, thermally protected magnetic-drive electric pump (Little Giant Pump Company, Oklahoma City, OK). A rotameter and an inline ball valve were installed for controlling the flow at 1 foot per second. City of Tempe (Arizona) tap water was received into an open tank to allow chlorine evaporation before the water was transferred by gravity to the reservoir. A residence time of 48-

72 hours and a temperature of 22°C–27°C (mean 25°C) were maintained in the MDS. At the time of this study, the MDS was in operation for more than ten years.



Figure 8-Pilot scale PVC water distribution system (back). Also shown is a cast iron pilot scale water distribution system (front)

### **Stock Propagation, Sampling Methodology, and Cultural**

**Analysis of *Bacteroides*.** *Bacteroides fragilis* (ATCC# 23745) was

cultured onto Chopped Meat Medium (Hardy Diagnostics, Santa Maria, CA, USA) and placed in a Bio-Bag<sup>TM</sup> Type A system (BD-Diagnostic Systems, Franklin Lakes, NJ, USA) to maintain anaerobic conditions, then incubated at 37°C for three days. The culture was then washed and enumerated via a hemocytometer.

Upon stock propagation,  $10^9$  *B. fragilis* cells were inoculated into the MDS. To collect representative water samples from the distribution main, 300 ml of water was flushed from the sampling port prior to sample collection. Water samples for microbial analyses were collected in sterile 200 ml glass bottles. Biofilm samples were collected by terminating flow through the system and isolating a pipe segment. Six in<sup>2</sup> of biofilm was scraped off the pipe segment wall using a wire brush and resuspended in PBS buffer in 10ml conical tubes (VWR).

Biofilm and water samples were immediately cultured upon collection onto *Bacteroides* Bile Esculin (BBE) agar plates (Hardy Diagnostics). Culture plates then were placed in Bio-Bag<sup>TM</sup> Type A systems (BD-Diagnostic Systems) and incubated at 37 °C. To make sure the anaerobic atmosphere was maintained, an indicator was placed within the bio-bag which turns white in the absence of oxygen. BBE plates were examined after three days for representative colonies, i.e. those with similar morphology and color to *Bacteroides*. Procedures for isolation and identification of *Bacteroides* were followed as described in Wadsworth-

KTL Anaerobic Bacteriology Manual (Jousimies-Somer et al., 2002) and the Manual of Clinical Microbiology (Jousimies-Somer et al., 2003).

During the first day after inoculating the MDS, five water and biofilm samples were collected. One water and biofilm sample was collected daily on days 2 to 7. During weeks 2 to 7, water and biofilm samples were collected once a week. PCR analyses of water and biofilm samples were performed at two months and four months after inoculating the system. The protocol used for PCR assays is described below.

**PCR Analysis of MDS Water and Biofilm Samples.** PCR analyses were performed on water and biofilm samples at two and four months after spiking the MDS with *Bacteroides*. To do this, 100 ml of each water sample was centrifuged 3000 rpm for 20 minutes, and the pellet was resuspended in 1 ml of PBS. Three in<sup>2</sup> representative biofilm samples were also concentrated to 1 ml in a similar manner. DNA extraction was then performed using the Zymo Fecal DNA Kit (Zymo Research, Orange, CA) according to manufacturer's protocol. Standard PCR was performed using universal *Bacteroides* primers Bac32F (5'-AACGCTAGCTACAGGCTT-3') and Bac708R (5'-CAATCGGAGTTCTTCGTG-3') (Bernhard and Field, 2000). All PCR reactions were performed using a Promega GoTaq® Green Master Mix (Promega Corp., Madison, WI, USA) with 200 nM of each primer in a 25 µl reaction mixture. PCR was performed using a GeneAmp PCR System

9700 (PE Applied Biosystems, Foster City, CA) with the following protocol: 5 min at 94°C followed by 35 cycles consisting of 30s at 94°C, 1 min at 53°C, and 2 min at 72°C, ending with a final extension time of 6 min at 72°C. The reaction mixtures were stored at 4°C until they were analyzed by agarose gel electrophoresis. Agarose gel electrophoresis was performed in 1.5% agarose gels containing 0.5µl of SYBR safe DNA gel stain (Invitrogen) per ml. The gels were electrophoresed for 1 h at a constant voltage of 100V and analyzed by a Kodak Gel Logic 112 Digital Imaging System (Carestream Molecular Imaging, New Haven, CT, USA). *B. fragilis* DNA (ATCC# 23745) was used as a positive control.

**Domestic Water Meter Collection and Biofilm Sampling.** The second part of this study involved investigating *Bacteroides* occurrence in central Arizona water distribution systems. A total of 65 water meters were collected within a 20 mile radius from two water distribution systems in central Arizona. Water meter ages ranged between 5-45 years old. A sample meter is shown in Figure 9. Upon removal for repair by city personnel, meters were immediately submerged in a clean bucket of tap water and transferred to the environmental microbiology laboratory at Arizona State University. Within two hours of removal from the ground, meter biofilms were collected by scraping the inlet of each meter using a bleach-sterilized nylon brush. Six in<sup>2</sup> of biofilm was collected and vortexed in 20 ml of sterile water to recover bacterial samples.

**Cultural Analyses of Water Meter Biofilm Samples.** Water meter biofilm samples were immediately cultured onto *Bacteroides* Bile Esculin agar and placed in Bio-Bag™ Anaerobic Environmental Chambers, as previously described for the MDS experiments.



*Figure 9-* Sample domestic water meter collected from large water distribution system in central Arizona

**DNA Extraction, PCR Amplification, and DNA Sequencing of Water Meter Biofilm Samples.** PCR assays were performed on every biofilm sample collected from the domestic water meters. During this process, lab cultures of *Bacteroides* were kept sealed in the freezer to prevent contamination of water meter samples. DNA extraction was

performed on the samples using the Zymo Fecal DNA Kit (Zymo Research). To ensure the extraction process was working efficiently, spiked control samples were used. One out of every 10 samples was selected randomly and spiked with *Bacteroides* in order to ensure *Bacteroides* was being detected by the PCR process. PCR assays were carried out using the universal *Bacteroides* primers Bac32F and Bac708R as previously described. The limit of detection for these primers was  $6.8 \times 10^{-7}$  g [dry weight] of sewage/liter.

To confirm that unexpected amplification did not occur and PCR products achieved belonged to *Bacteroides*, direct sequencing was performed by the DNA Laboratory lab at Arizona State University.

## **RESULTS AND DISCUSSION**

Water and biofilm samples were collected from the pilot scale PVC water distribution system (MDS) within 1 hour after inoculation.

*Bacteroides* cells were enumerated in these samples by culture-based techniques and average values were found to be  $10^3 \pm 10^1$  CFU ( $100 \text{ ml}^{-1}$ ) in bulk water and  $10^2 \pm 10^1$  CFU ( $1 \text{ in}^2$ ) in MDS biofilms. Bulk water and MDS biofilms were then sampled 3, 6, 12, and 24 hours after inoculation as seen in Table 4. *B. fragilis* could not be cultured from any of these samples. Two months after spiking the MDS a PCR assay was performed on water and biofilms samples, and results showed that the target *Bacteroides* 16S rRNA genetic marker was present in MDS biofilms but



not in the bulk water at detectable levels. Four months after spiking the MDS more water and biofilm samples were analyzed by PCR assay, and results revealed no *Bacteroides* 16S rRNA genetic marker in either the bulk water or MDS biofilms. This data can be seen in Table 5. The experiment was duplicated to confirm the results.

Table 4

*Culture-Based Analysis of Water and Biofilm Samples from MDS*

*Experiments*

Sample Time	Water	Biofilm
	CFU (100 ml <sup>-1</sup> )	CFU (1 in <sup>2</sup> )
1 hr	10 <sup>3</sup> ±10 <sup>1</sup>	10 <sup>2</sup> ±10 <sup>1</sup>
2 hr	0	0
6 hr	0	0
12 hr	0	0
24 hr	0	0
Day 2 - 7	0	0
Weeks 2 - 7	0	0

Table 5

*Molecular-Based Analysis of Water and Biofilm Samples from MDS*

*Experiments*

Sample Time	Water	Biofilm
2 months	-	+
4 months	-	-

These results are significant for several reasons. First, studies on the persistence of *Bacteroides* 16S rRNA genetic markers have focused on their persistence in bulk water or sewage without considering interactions with well-developed biofilms. For example, Kreader reported that *Bacteroides* molecular markers may only persist for up to 2 days in unfiltered river water when incubated at 24°C, a temperature very similar to the MDS mean water temperature of 25 °C, and up to a week longer in filtered river water (Kreader, 1998). Similarly, Walters and Field reported that the *Bacteroides* molecular marker will persist in aerobically incubated sewage for at least 24 hours (Walters and Field, 2006). Furthermore, Seurinck (2004), Okabe and Shimazu (2007), Bell (2009), and Ballesté (2010) have reported similar findings.

While these studies have been useful in helping us understand how *Bacteroides* molecular markers may persist in some surface waters, recreational waters, and bulk sewage, they may have limited ability to help

us understand the persistence of *Bacteroides* markers in environments where interactions with biofilms are significant, such as water distribution systems. The results show that *Bacteroides* 16S rRNA molecular markers may still be present in well-developed biofilms up to two months after the introduction of *Bacteroides*. Biofilm sloughing or *Bacterioides* diffusion out of the biofilm could result in genetic markers that are detected as false positives within water samples.

One possible explanation for the extended persistence of the *Bacteroides* DNA in the MDS experiments is diffusion of *Bacteroides* into anoxic zones or microniches within the biofilms. *Bacteroides* species have traditionally been thought of as obligate anaerobes and their survival in the extra-intestinal environment has been thought to have been negligible (Walter and Fields 2006). However, it is now known while most *Bacteroides* species can only survive in the presence of low oxygen concentrations for a period of a few hours, *B. fragilis*, which was formerly considered an obligate anaerobe, is now known to require nanomolar concentrations of oxygen for growth and possesses an O<sub>2</sub>-dependent cytochrome (Baughn et al., 2004). The high oxygen tolerance of *B. fragilis* has been demonstrated in its ability to grow at oxygen levels where other *Bacteroides* species can survive for only a few hours (Rocha et al., 2003). Thus it is possible that low oxygen zones or microniches within biofilms could aid the persistence of *B. fragilis* in the environment. These reasons

made the species *B. fragilis* an excellent selection for use in the MDS experiments.

Grazing protozoa have been reported as one of the most significant factors in the survival of enteric bacteria in the environment (Gonzalez et al., 1992, and this has been verified for *Bacteroides* (Ballesté and Blanch, 2010). Well-developed biofilms may limit the effect of eukaryotic predation and act as another mechanism by which biofilms may aid in the persistence of *Bacteroides* in the environment (Matz, 2007).

The literature reports no successful culturing of *Bacteroides* from environmental water or biofilm samples. However, the results from the MDS experiments suggest that *Bacteroides* may remain in a culturable state for a few hours after introduction into the aqueous environment. Although culturing *Bacteroides* from environmental water and biofilms exposed to fecal contamination remains difficult, these results indicate that a successful *Bacteroides* culture likely represents a very recent contamination event, probably within hours. Moreover, since it has been demonstrated that *Bacteroides* species are able to grow in aerobic sewage for up to a day (Walters and Field, 2006), multiple culture positive *Bacteroides* samples from environmental water or biofilms may indicate a sewage point source within the local vicinity.

These experiments were performed in a nonchlorinated MDS. In order to investigate whether *Bacteroides* 16S rRNA genetic markers could

be detected in a full-scale chlorinated distribution system, a field study was performed where biofilms from two large water distribution systems in central Arizona were collected and analyzed. Because it is difficult to obtain distribution system biofilms without disrupting supply to users, domestic water meters were collected and biofilms formed within the inlet were considered representative of distribution system biofilms. The two water utilities that participated in this study conduct ongoing water meter exchange programs that replace old water meters at domestic residences. A photo of an old water meter that was obtained through this program can be seen in Figure 9. The biofilms were analyzed by PCR using the AllBac primer set previously described. A total of 65 meters were collected, and 35 PCR assays were positive. These results can be seen in Table 6. The biofilm samples were also analyzed by culture method with BBE agar, though none of the samples were culture positive.

Table 6

*Results from Domestic Water Meter Assay for Bacteroides*

	Total Samples	PCR Positive	Culture Positive
Distribution System A	35	20	0
Distribution System B	30	15	0
Total	65	35	0

Positive PCR amplicons were purified and directly sequenced in order to confirm *Bacteroides* as the amplicon. The sequences were then compared to the NCBI database using the Blast tool, and the results can be seen in Table 7.

Table 7

*Sequence Homology from PCR Amplicons*

No. of Samples	Species Identified	% Homology from Blast search
27	<i>Bacteroides fragilis</i>	97 - 99%
2	<i>Bacteroides uniformis</i>	93%
2	<i>Prevotella conceptionensis</i>	90 - 93%
1	<i>Bacteroides graminisolvens</i>	94%
3	non-determinate	-

As expected, *B. fragilis* cells were the dominant species that was identified. The comparatively high oxygen tolerance that *B. fragilis* possesses over other *Bacteroides* species would provide an advantage in water distribution systems. Three other strains were identified, *B. uniformis*, *Prevotella conceptionensis*, and *B. graminisolvens*, though homology between sequences and the NCBI database ranged between 90 to 94%. Three samples were non-determinative due to the uncertainty of the nucleotide sequence achieved by direct sequencing.

These results confirm *Bacteroides* 16s rRNA gene as the target for the PCR reactions performed and verify *Bacteroides* presence in two large chlorinated water distribution systems in central Arizona. Moreover, positive samples were found to be geographically clustered in several locations, suggesting past contamination events as the source of *Bacteroides*. While there is no evidence to support the hypothesis that these *Bacteroides* grew in the environment, these results do confirm that the 16S rRNA genetic marker of *Bacteroides* species, especially of *B. fragilis*, possesses the ability to environmentally persist longer than previously thought by interacting with well-developed biofilms.

In summary, the results reported here support the hypothesis that *Bacteroides* 16S rRNA molecular markers may be found in water distribution systems despite no success in cultivating these bacterial cells. This study presents the importance of considering biofilm interactions with fecal indicator bacteria when performing molecular assays in environmental samples, as biofilms may provide protection from high oxygen concentrations and grazing protozoa in bulk water that limit the persistence *Bacteroides* in the environment. Although the significance of biofilm interactions with surface or recreational waters may be small, they are likely important when considering drinking water delivered through distribution systems. Moreover, this study has shown that *Bacteroides* likely remain environmentally culturable on the scale of a few hours after a

contamination event, and that their 16S rRNA genetic marker may remain PCR detectable on the scale of days to months, depending on factors previously described. Furthermore, it is possible that *Bacteroides* cells remain in a viable-but-not-culturable state when they cease to be culturable but are still detectable through molecular methods.



## Chapter 4

### ISOTHERMAL AMPLIFICATION TECHNIQUES FOR THE DETECTION OF *BACTEROIDES* IN WATER SAMPLES

#### **ABSTRACT**

Although molecular techniques such as Polymerase Chain Reaction (PCR) have been developed that possess numerous advantages over current fecal indicator detection methodologies, the water community has been slow to adopt these newer methods in part due to the costs involved in training personnel in laboratory intensive techniques and in purchasing the necessary equipment for these methods, such as thermocyclers. The purpose of the study was to investigate simple isothermal molecular amplification techniques that have the potential to provide the benefits of molecular indicator detection, i.e. rapid and specific identification of a fecal indicator and potential to identify its source, without the costs associated with current methods. In particular, this study focused on using a simplified protocol of Nucleic Acid Sequence Based Amplification (NASBA) and Thermophilic Helicase-Dependent Amplification (tHDA) to amplify the highly conserved 16s RNA gene in the genomic DNA of fecal indicator *Bacteroides*. The results of our study have shown that the simplified model of NASBA was not able to amplify the target, while continuous problems with tHDA exposed the methods lack of reliability, a crucial characteristic needed for the water community to adopt

a new method. However, amplification of the target region was achieved using standard PCR with the primer sets developed for tHDA. This further supports the claim that there needs to be higher reliability in the isothermal amplification methods.

## **INTRODUCTION TO MOLECULAR AMPLIFICATION AND DETECTION OF WATERBORNE PATHOGENS**

**Urgency of Improving Water Quality.** The United Nations has stated that at any one time half the world's hospital beds are occupied by people with waterborne disease and that every day 4,000 children die due to unsafe drinking water (UNESCO 2010). Furthermore, the UN has estimated that close to a billion people worldwide still do not have access to safe drinking water and 2.6 billion people lack access to basic sanitation services, such as toilets or latrines. In 2000 the United Nations declared that one of their Millennium Goals was to halve the population of the world without safe water and sanitation (UNESCO 2000).

However, here in the United States issues of water quality are often seen as something that only the developing world is concerned about. The facts, though, reveal otherwise as there were nearly 20 cases of microbial waterborne outbreaks in the United States during the 1990's, resulting in 500,000 illnesses (Craun et al., 2002). Clearly there is urgency in improving water quality both in our country and abroad.

**Need for Better Detection Methodologies.** The introduction of pathogenic microorganisms into the environment and subsequent exposure to human beings is often via the fecal-oral route. Subsequently, the United States Environmental Protection Agency (EPA) currently uses the fecal coliform *E. coli* as the standard indicator of fecal pollution in drinking water (EPA 2010). However, using the presence of *E. coli* as a water quality standard has several disadvantages. First, the time required to culture *E. coli* is more than a day, producing a lag in real time water quality data. Second, *E. coli* is present in the intestines of many animals and lacks identifiable host specific strains making it difficult to distinguish the source of fecal pollution using the standard method. Although molecular techniques such as Polymerase Chain Reaction (PCR) have been developed that are able to more rapidly detect microorganisms, the water community has been slow to adopt these newer methods in part due to the high costs involved in training personnel in laboratory intensive techniques and in purchasing the necessary equipment such as thermocyclers for these methods. For these reasons, and because of the sheer volume of assays that water utilities must perform on continuous basis, new methodologies are needed to make molecular amplification techniques cheaper and easier before the benefits of these technologies can be brought to the water community.

***Bacteroides* as a Fecal Indicator.** Fecal pollution in water is a serious environmental problem since it constitutes a health risk that is mainly owed to the potential introduction of pathogenic microorganisms that cause waterborne diseases (Crane & Moore 1986). Moreover, the ability to determine whether the source of fecal contamination is human or other animal is critical in developing effective pollution remediation strategies (McClain et al., 2005). In addition, several studies have proposed using members of the *Bacteroides* genus as alternative indicators of fecal pollution since they are highly abundant in feces (Madigan et al., 2003), have little potential to grow in environment (Salyers 1984; Sghir et al., 2000), and have a high degree of host specificity that likely reflects differences in source animal digestive systems (Bernhard & Field 2000; Simpson et al., 2004; Dick et al., 2005). For these reasons we chose to target *Bacteroides* rather than *E. coli* in our isothermal reactions.

*Bacteroides* is the predominant bacterial group in the complex intestinal flora of almost all mammals (Mitsuoka et al., 1965). Most *Bacteroides* strains belong to ten cultivated species that include *Bacteroides vulgatus*, *B. thetaiotaomicron*, *B. distasonis*, *B. caccae*, *B. eggerthii*, *B. fragilis*, *B. merdae*, *B. ovatus*, *B. stercoris*, and *B. uniformis* (Shah et al., 1989). A recent study has shown that *Bacteroides coprocola*, *B. uniformis* and *B. vulgatus*, are the prevailing *Bacteroides* species in human feces (Li et al., 2009). *Bacteroides* have also been identified in the

intestine of freshwater fish (Trust & Sparrow 1974) and freshwater sea gulls (Jeter et al., 2009), further supporting the need for human specific assays.

**Objectives of this Study.** The objective of this study is to develop a simple, rapid, and inexpensive technique for the detection of *Bacteroides* in water samples. In developing this technique, we wish to:

- Investigate the applicability of isothermal molecular amplification methods such as tHDA and a simplified NASBA procedure for water quality testing.
- Develop a method that is able to distinguish between human and non-human sources of *Bacteroides*.

## **LITERATURE REVIEW FOR TWO ISOTHERMAL AMPLIFICATION METHODS**

Since the introduction of PCR in 1987, molecular amplification has become a ubiquitous presence in all fields relating to bioscience. The success and widespread use of PCR has encouraged further studies into alternative means of molecular amplification that could be used for clinical research, with much emphasis put on isothermal methods. After NASBA was first introduced in 1991, there have been other isothermal amplification methods that have been proposed. These include Ligase Chain Reaction (LCR) (Barany 1991), Strand Displacement Amplification (SDA) (Little et al., 1999), Branched DNA Amplification (bDNA) (Smolina

et al, 2004), DNA Cleavage Based Amplification also known as Invader (Hall et al., 2000), Thermophilic Helicase Dependent Amplification (Vincent et al., 2004) and Rolling Cycle Amplification (RCA) (Wiltshire et al., 2000). All have shown good sensitivity and are compatible with many detection techniques, such as fluorescence, chemiluminescence, or gel electrophoresis. These methods have shown success in some capacity, but the complicated nature of the some of these assays and their associated costs have the potential to limit their popularity. For these reasons we chose to investigate two of the more straightforward methods, a simplified NASBA procedure and tHDA.

**Nucleic Acid Sequence Based Amplification (NASBA).** The first isothermal molecular amplification method that was attempted was based on the concept of Nucleic Acid Sequence Based Amplification (NASBA). NASBA is a transcription-based amplification system (TAS) for the specific replication of nucleic acids *in vitro*. The amplification reaction is performed at the isothermal temperature of 41°C. Three enzymes are involved in this homogeneous reaction: avian myeloblastosis virus (AMV) reverse transcriptase (RT), RNase H and T7 DNA dependent RNA polymerase (DdRp). Because the reaction incorporates RT into the amplification process, the method is especially suited for RNA analytes like mRNA, rRNA or genomic RNA (Deiman et al., 2002). Table 8 shows some of the basic characteristics of NASBA.

Table 8

*Traits of NASBA* (modified from Deiman et al., 2002)

<b>Characteristics of NASBA</b>
<ul style="list-style-type: none"><li>• The reaction is carried out in a single isothermal temperature profile.</li><li>• Especially suited for RNA analytes because of the integration of RT into the amplification process.</li><li>• The use of a single temperature eliminates the need for special thermocycling equipment.</li><li>• Efficient ongoing process resulting in exponential kinetics caused by production of multiple RNA copies by transcription from a given cDNA product.</li><li>• Unlike amplification processes, such as PCR in which the initial primer level limits the maximum yield of product, the amount of RNA product obtained in NASBA exceeds the level of primers by at least one order of magnitude.</li><li>• The intermediate cDNA product can be made double-stranded, ligated into plasmids, and cloned.</li><li>• Three enzymes are required to be active in the same reaction conditions.</li><li>• Low temperature can increase the nonspecific interactions of the primers. However, these interactions are minimized by the inclusion of DMSO.</li><li>• The primers are not incorporated in the amplicon and thus labeled primers cannot be used for detection.</li><li>• The length of the target sequence to be amplified efficiently is limited to approx 100 to 250 nucleotides.</li></ul>

For visualization purposes, a schematic of NASBA for the detection of RNA is presented in Figure 10 on the following page.

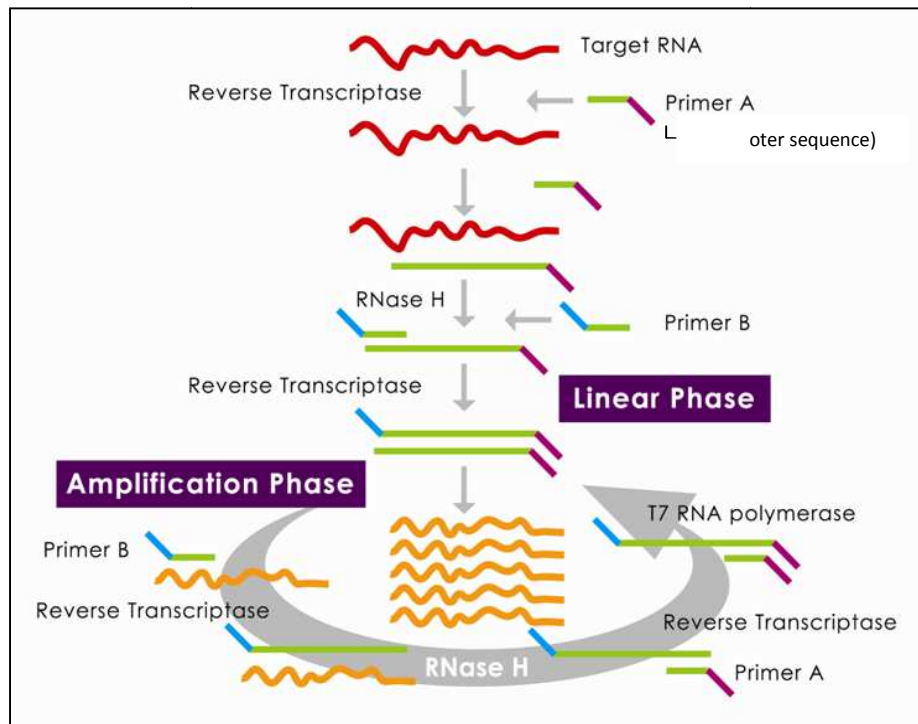


Figure 10: Diagram of NASBA for RNA analyte<sup>5</sup>

In Figure 10 target single-stranded sense RNA binds to Primer A in the linear phase. An RNA/DNA duplex is formed by the action of reverse transcriptase. RNaseH then degrades the RNA component of the hybrid and reverse transcriptase uses Primer B to make a cDNA of the target region. The cDNA is then used in the amplification phase because T7 RNA polymerase recognizes the promoter sequence in Primer A. Hence, many copies of the antisense RNA are made and these antisense RNA copies are used to make more cDNA. In this way NASBA is an exponential amplification process. However, because there is a need for

<sup>5</sup> Source: <http://www.foodsafetylabs.com/images/tech/nasba2l.png>



a more straightforward protocol for water quality testing, specifically a protocol that does not work with RNA, we attempted to simplify NASBA. As explained above, NASBA relies on T7 RNA Polymerase to produce an RNA amplicon that can be detected. However, it has also been shown that T7 RNA Polymerase is able to produce a DNA amplicon when it is in the presence of deoxyribonucleotides (dNTP's). Moreover, RNase H has shown the ability to denature, or unzip, double stranded DNA, though the mechanism by which RNase H does this is unknown. Therefore, utilizing these two enzymes, we attempted to develop a simplistic, novel technique to amplify a genomic DNA target sequence in a one-step isothermal reaction that results in a DNA amplicon, effectively eliminating RNA from the reaction sequence.

#### **Thermophilic Helicase-Dependent Amplification (tHDA).**

Helicase-dependent amplification (HDA) is an isothermal *in vitro* DNA amplification method based upon the enzymatic activities of helicases to separate double stranded DNA and the ability of DNA polymerases to synthesize DNA. First presented in 2004 (Vincent et al., 2004), the helicase *E. coli* UvrD was used with two necessary accessory proteins, MutL and single-stranded DNA-binding protein (SSB) for separation of double stranded DNA. In an effort to improve the specificity and performance of HDA, a thermostable UvrD helicase (Tte-UvrD) was purified from *Thermoanaerobacter tengcongensis* by (An et al., 2005).

The thermostable Tte-UvrD helicase unwinds blunt-ended DNA duplexes as well as substrates possessing 3' or 5' ssDNA tails. Though the Tte-UvrD helicase has been known to require single-stranded 3' tails several nucleotides in length to begin separation of dsDNA, An's group utilized the fact that at temperatures between 60-65°C dsDNA exhibits thermal breathing where several base pairs at blunt ends of dsDNA separate to use the Tte-UvrD helicase without the need for accessory proteins. The so called thermophilic HDA (tHDA) system is more efficient, displaying heightened amplification sensitivity without the need for the MutL and SSB accessory proteins (An et al., 2005). The simplistic nature of the tHDA platform makes the technology ideal for use where rapid identification of microorganisms is needed at the point-of-need. A schematic of the tHDA system is provided in Figure 11.

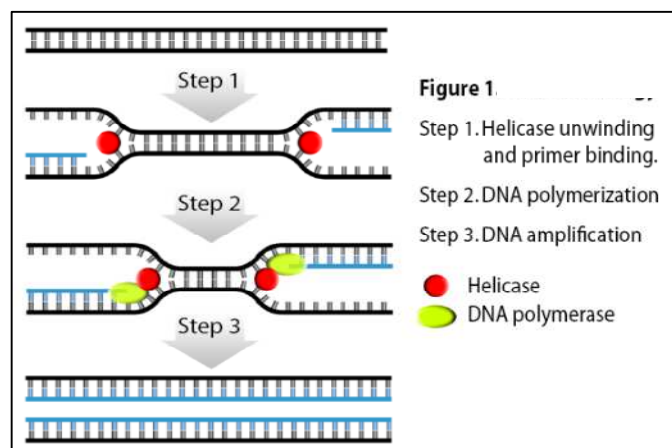


Figure 11-Diagram of tHDA (BioHelix Corp. 2010)

## MATERIALS AND METHODS

**Bacteroides Culturing and DNA Extraction.** *Bacteroides* isolates were obtained by culturing human fecal specimen under anaerobic conditions. DNA extraction was then performed to obtain pure genomic *Bacteroides* DNA, which was initially used as the template to eliminate any interference and subsequent inhibition from any impurities in the sample. Briefly, diluted human fecal samples (from 20 ml sterile PBS) were streaked onto *Bacteroides* Bile Esculin agar (BBA). The pre-made plates were purchased from (Hardy Diagnostics, Tempe, AZ, USA). Cultured plates were then placed in the Bio-Bag™ Type A system (BD-Diagnostic Systems, Franklin Lakes, NJ, USA), sealed to obtain anaerobic conditions, and then incubated at 37 °C. This system utilizes a CO<sub>2</sub> gas generator consisting of one tablet of potassium borohydride and sodium bicarbonate, an ampule of hydrochloric acid, a catalyst cup containing a palladium catalyst, and an indicator containing an ampule of resazurin. The gas generator is activated by breaking the capsule, which results in O<sub>2</sub> gas being converted to CO<sub>2</sub> gas. After incubation for three days in this anaerobic system, the BBA plates were examined and representative colonies, i.e. those bacteria of similar morphology, were randomly picked from the BBE plate and suspended in 1ml of sterile PBS buffer. DNA extraction was then performed using the Zymo Fecal DNA Kit (ZymoResearch, Orange County, CA) according to the manufacturer's

protocol to obtain pure *Bacteroides* genomic DNA to be used as a template in the isothermal reactions.

### **Nucleic Acid Sequence Based Amplification (NASBA)**

**Procedures and Reagents.** To develop a NASBA assay for identifying fecal bacteria *Bacteroides* in water, we designed a set of primers to provide as much human specificity as possible in our target region. Of the human specific primers for *Bacteroides* that have been published, those developed by Bernhard & Field, 2001 were selected. This selection was made because of the lack of human specificity reported McClain et al., 2009 for supposedly human specific primer sets that have been published. Forward primer HF134NAS and reverse primer HF183NAS were used as the basis of our primer design because of their specificity in targeting the highly conserved 16s ribosomal RNA gene in *Bacteroides* strains originating from humans. The T7 promoter sequence at the 5' end of each primer was added, which is needed for the T7 RNA Polymerase to recognize the binding site and begin extension of the target sequence. This promoter sequence is 5'- AATT CTA TACG ACTC ACTA TAGG G-3'. Furthermore, a reverse primer without a promoter sequence was tested to see the effects this would have on amplification of the target sequence. It was thought that a primer with no promoter sequence could have the potential to terminate extension T7 RNA Polymerase at the

annealing site. A summary of the discussed primers is provided in Table 9 below.

Table 9

*Primer Sets used with and without the T7 Promoter Sequence (underlined)*

<b>Primer</b>	<b>Sequence (5'-3')</b>
HF134NAS	GTCT ACT CTT GGCC
HF183NAS	ATCA TGAG TTC ACA TGT CCG
HF134NASP (w/promoter sequence)	<u>AATT CTAA TACG ACTC ACTA TAGG G</u> GTCT ACT CTT GGCC
HF183NASP (w/promoter sequence)	<u>AATT CTAA TACG ACTC ACTA TAGG G</u> ATCA TGAG TTC ACA TGT CCG

Isothermal amplification was then performed utilizing our simplified NASBA protocol. The reaction mixture volume was 30 µl for each reaction. The reaction mixture when both forward and reverse primers were used was as follows:

- 3 µl of 10x PCR buffer, 3 µl of dNTP mix (2 mM)
- 3 µl of RNase H (2.4 units/µl)
- 7.5 µl of T7 Polymerase (20 units/µl)
- 1 µl of forward primer (100 pM)
- 1 µl of reverse primer (100 pM)
- 9.5 µl of DNA pure water
- 2 µl of PCR template (from standard PCR reaction using primers HF134NAS and HF183NAS)

The reaction mixture when only a forward primer was used was identical with the exception of using 10.5 µl of DNA pure water in place of 9.5 µl in order to maintain a reaction volume of 30 µl. The amplicon was then visualized using gel electrophoresis.

### **Thermophilic Helicase-Dependent Amplification (tHDA)**

**Procedures and Reagents.** The tHDA protocol was performed on pure *Bacteroides* genomic DNA using the IsoAmpII Universal tHDA Kit<sup>®</sup> (BioHelix, Beverly, MA). Upon suggestion from BioHelix, an online program called Primer3 (<http://frodo.wi.mit.edu/primer3/>) was used to design the two primer sets. Optimal primer conditions suggested by BioHelix are provided in Table 10.

Table 10

*Optimal Primer Design Conditions for tHDA Provided by BioHelix*

#### **Optimal Primer Conditions for tHDA as Provided by BioHelix**

Product size: 80 – 120 bp  
Product T<sub>m</sub>: Min. 68; Opt. 71; Max. 75  
Primer size: Min. 24; Opt. 27; Max. 33  
Primer T<sub>m</sub>: Min. 60; Opt. 68; Max. 74  
Primer GC%: Min. 35; Opt. 44; Max. 60

These conditions were input into the Primer3 program and the output primers are shown in Table 11.

Table 11

*Primer Output from Primer3 Program for use in tHDA*

Oligo	Start	Length (bp)	Tm (°C)	GC%	3' sequence
HDA10F	10	28	66.93	39.29	TGGTGTTTTGCAGGT ACCGTATGAATAA
HDA114R	114	28	66.66	39.29	CCTTTAAACCCAAT AAATCCGGATAACG
HDAF20F	20	28	68.33	50.00	CAGGTACCGTATGA ATAAGGACCGGCTA
HDA92R	92	26	69.89	53.85	ATAACGCCCGGA CCTTCCGTATTACC

The region considered for primer design was the human specific region of the *Bacteroides* 16s ribosomal RNA gene as proposed by (Layton et al., 2006). While the entire 16s ribosomal RNA gene for *Bacteroides Fragilis* was found to be 1,533 bp in length as shown in the National Center for Biotechnology Information's BLAST tool, the human specific region of this gene proposed by (Layton et al., 2006) is 128 bp in length. This human specific region of the 16s ribosomal RNA gene in *Bacteroides* proposed by Layton provided the perfect target region length because tHDA requires amplicons between 80-120 bp, as shown in Table 9. Thus this region was entered into the Primer3 program to develop our tHDA primers. Of the output primers that were supplied by the program, two tHDA primer sets were chosen that overlapped the primers developed by (Layton et al., 2006) in the hope that the tHDA primers would exhibit some human specificity.

Using the primers listed in Table 11 the 50  $\mu$ l reaction volume was set up in a 0.2-ml micro-centrifuge tube with the following reagents:

- 29.5  $\mu$ l H<sub>2</sub>O
- 5  $\mu$ l 10X Annealing buffer II MgSO<sub>4</sub> (100 mM)\* 2  $\mu$ l
- 4  $\mu$ l NaCl (500 mM)
- 3.5  $\mu$ l IsoAmp® dNTP Solution
- 3  $\mu$ l pure *Bacteroides* DNA
- 0.75  $\mu$ l Forward Primer (5  $\mu$ M)
- 0.75  $\mu$ l Reverse Primer (5  $\mu$ M)
- 3.5  $\mu$ l IsoAmp® Enzyme Mix

The micro-centrifuge tube was then incubated at 65°C for 90 minutes using a thermocycler with a heating lid (GeneAmp PCR System 9700, PE Applied Biosystems, Foster City, CA). The tHDA product was visualized on 2% agarose gel.

The reaction was also attempted by incubating the reaction mixture at 65°C in a water bath (VWR Digital Heatblock) and an older thermocycler with no heated lid (DNA Thermal Cycler 480, Perkin Elmer, Waltham, MA) to investigate any differences in amplification efficiency.

**Use of Primers designed for tHDA in Standard PCR.** In order to evaluate the efficacy of the primers designed for tHDA, they were used in standard PCR as well. All reactions were performed using a Promega GoTaq® Green Master Mix (Promega Corp., Madison, WI, USA) with 5



$\mu\text{M}$  tHDA primers discussed above per 25  $\mu\text{l}$  reaction mixture. PCR was performed using a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA) and thermocycler conditions were as follows: 5 min at 95C followed by 35 cycles at 95C for 30 s, 60C for 45 S and 72C for 30s, ending with final extension time of 5 min. PCR products were visualized on 1.5% agarose gels using SYBR safe DNA gel stain (Invitrogen) and a Kodak Gel Logic 112 Digital Imaging System (Carestream Molecular Imaging, New Haven, CT, USA).

## RESULTS

**Culturing.** In order to culture *Bacteroides* from human samples, *Bacteroides* Bile Esculin (BBE) agar plates were used because BBE is a selective media for the growth of *Bacteroides*. This is shown in Figure 12.

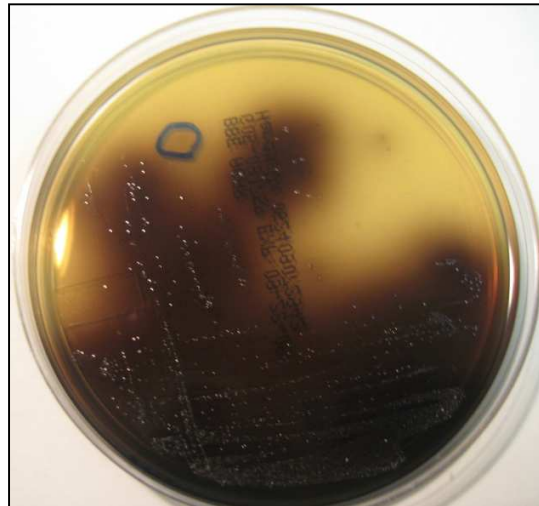
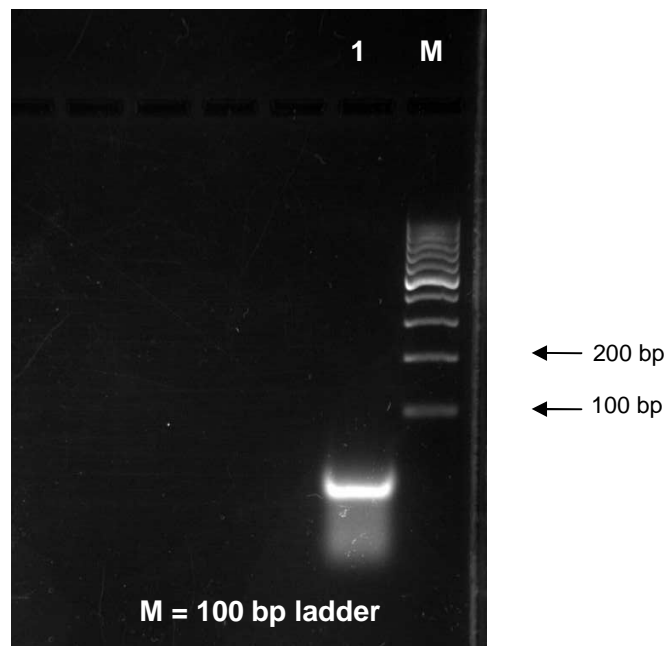
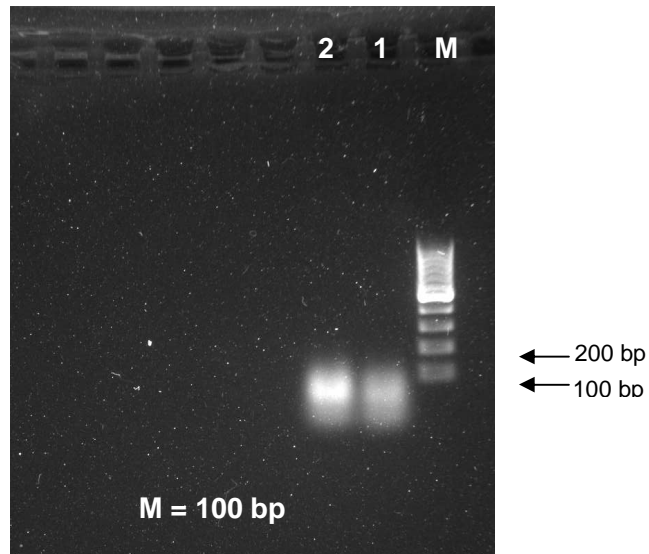


Figure 12-*Bacteroides* isolates on *Bacteroides* Bile Esculin agar (BBE)

**Nucleic Acid Sequence Based Amplification (NASBA).** Three isothermal reactions were performed. The first isothermal reaction was performed at a constant 41°C for 90 minutes using primers HF134NASP and HF183NASP. Gel electrophoresis was performed and the results are shown in Figure 13. Two more isothermal reactions were performed also at 41°C for 90 minutes using the primers HF134NASP and HF183NAS (no promoter) and primers HF134NAS (no promoter) HF183NASP. These reactions were also visualized using gel electrophoresis and can be seen in Figure 14.



*Figure 13*-Isothermal reaction using primers HF134NASP and HF183NASP. Well 1:~50 bp PCR product. M: 100 bp ladder molecular marker



*Figure 14*-Isothermal reaction using primers HF134NASP and HF183NAS in well 1 and primers HF134NAS HF183NASP in well 2. Well 1: ~50 bp PCR product. Well 2: ~50 bp product. M: 100 bp ladder molecular marker

**Thermophilic Helicase-Dependent Amplification (tHDA).** The tHDA reaction was not successful in amplifying the target. After the lack of amplification became apparent, much effort was put into identifying the reason for the lack of amplification. BioHelix provides a positive control reaction with its IsoAmpII Universal tHDA Kit that includes positive control primers and an 85 bp positive control template (all provided by BioHelix). However even this positive control showed no amplification.

### Standard PCR using Primers Developed for tHDA

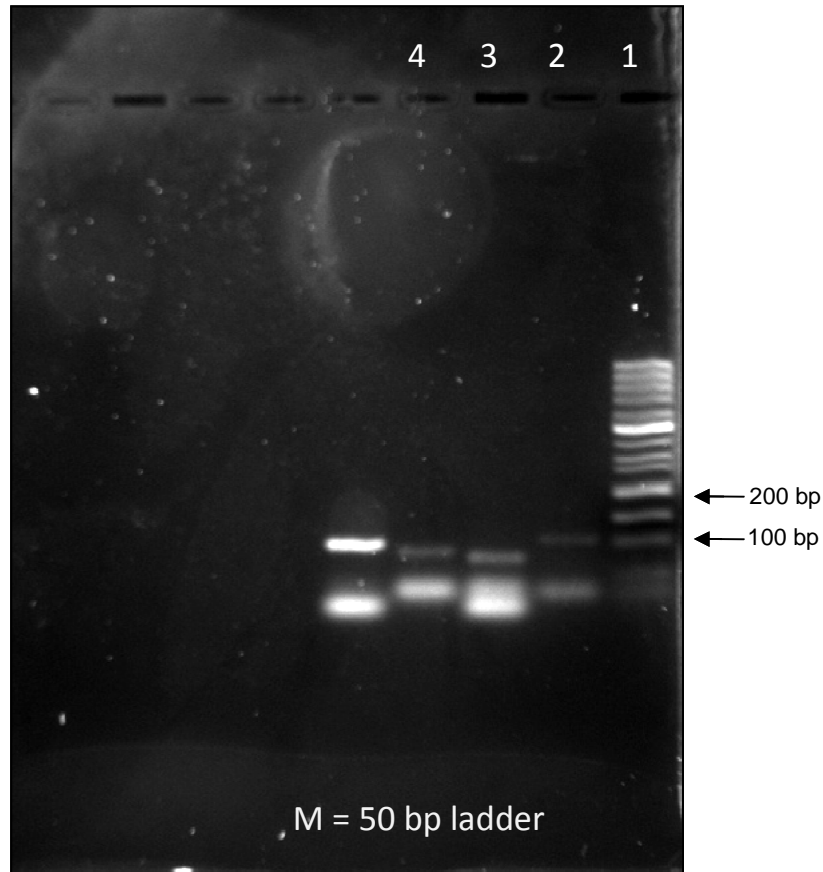


Figure 15-Standard PCR using primers developed for tHDA. Data corresponding to this photograph is present in Table 12. Lanes 1-4 show reactions with different primer sets. Negative controls using no template and *E. coli* genomic DNA were performed on a separate gel not shown. Each negative control showed no amplification

Table 12

Primer and PCR product information corresponding to Figure 15

Well No.	Forward Primer	Reverse Primer	Amplicon Length (bp)
1	HDA10F	HDA114R	104
2	HDAF20F	HDA92R	72
3	HDA10F	HDA92R	82
4	HDAF20F	HDA114R	94

Amplification of the target sequence was achieved as shown in Figure 15. Amplicon lengths with associated primers are shown in Table 12. Negative controls were run but are not shown in Figure 15.

## **DISCUSSION**

**Bacteroides Culturing and DNA Extraction.** After the human fecal sample was incubated for three days under anaerobic conditions, brown to black colonies were observed as shown Figure 12. Surrounding these colonies was a dark brown to black zone in the medium. This black coloration was formed because *Bacteroides* is able to hydrolyze esculin to products that react with ferric citrate in the medium to produce insoluble iron salts that blacken the media around the colony (Kabiri et al., 2010). DNA extraction was then performed on isolates and this purified genomic DNA was then used as the template for the rest of our reactions.

**Nucleic Acid Sequence Based Amplification (NASBA).** The simplified NASBA reaction method focuses on the ability of T7 RNA Polymerase to amplify double stranded DNA in the presence of deoxynucleotides. Figures 13 and 14 reveal that amplification is occurring although the entire target sequence is not being amplified as hoped. Unspecific annealing of the primers, due primarily to the low annealing temperature of 41°C, was thought be the reason for this short amplification by causing the primers to anneal over the entire target sequence. In order to examine this possibility, a third reaction was performed by shotgun

starting the reaction with denaturation at 95°C and specific annealing of the primers at 61°C before adding the enzymes to the continuous phase of the reaction for 90 minutes at 41°C. However, the same results were seen (gel not shown).

These results suggest several things. First, it is possible that dimerization is occurring with our primers. Perhaps the low melting temperature (~41°C) is allowing the primers to anneal to one another creating the amplicon around 80-100 bp visualized in the gels. Moreover, the lack of primer dimerization in our control reactions provide strong evidenced for this thought. Secondly, it is possible that the target sequence is too large for T7 RNA polymerase to amplify the entire region. Our target DNA sequence used from (Bernhard & Field, 2001) was 523 bp long, and while some literature has showed the ability of this type of isothermal reaction to amplify a large target sequence, most suggest that the isothermal target sequences be limited to under 250 bp (Deiman et al., 2002).

However, instead of further pursuing NASBA, thermophilic helicase dependent amplification was pursued. This was done for several reasons. First, there is nothing to guarantee that our NASBA reaction provided successful amplification of any length, as primer dimerization could have been the cause for the small amplicon visualized on our gels. Second, upon further literature review, it was found that T7 RNA Polymerase

preferentially amplifies RNA even in the presence of a heavy DNA background. This would lead us to target 16s rRNA in *Bacteroides*. However, the goal of this study was to develop simple isothermal method targeting DNA and not RNA. Moreover, the method proposed by tHDA is a simpler alternative.

#### **Thermophilic Helicase Dependent Amplification (tHDA).**

Several isothermal tHDA reactions using the newly designed tHDA primers resulted in no amplification. Moreover, BioHelix provides a positive control reaction in the IsoAmpII Universal tHDA Kit<sup>®</sup> that was run numerous times. However, this positive control reaction also showed no amplification. This suggests that the lack of amplification is due to problems with reliability of the method, possibly because of the instability of the enzymes involved in the reaction.

While tHDA is a simplistic and straightforward assay, the process needs to function without problems such as we have encountered. Microbiological water quality assays need to be not only simple and straight forward, but dependable as well. Even if we are able to amplify our target using the tHDA procedure in the future, there will need to be greater evidence of the dependability of the method before adopting such methods could be considered.

**Standard PCR with Primers designed for tHDA.** Standard PCR was performed using the primers developed for tHDA. Four reactions

were performed at the temperature profile previously discussed. Although the primers were designed for a melting temperature of ~67-70°C, an annealing temperature of 60°C was used. From Figure 15 and Table 12 it is seen that amplification of the target region was achieved with each primer set. Primer dimerization can also be seen in Figure 15. These results suggest that the primers sets were successfully developed and locates tHDA problems with the enzymes involved with the reactions.

### **SUMMARY**

- Anaerobic culturing of *Bacteroides* was successfully performed on BBE agar. DNA extraction was performed on pure culture resulting in pure *Bacteroides* genomic DNA that could be used as a template for molecular amplification.
- A simplified NASBA procedure was proposed, though no successful amplification was achieved. Moreover, tHDA also showed no amplification due to unreliability in the enzymes involved in the reaction. Despite the potential of isothermal amplification to provide a simple, rapid, and, inexpensive water quality assay, this unsuccessful amplification suggests the reliability of these methods needs to be improved before the method will be suitable for water samples.
- Standard PCR successfully amplified the target region of *Bacteroides* 16s RNA gene using primers developed for tHDA.



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## Chapter 5

### CONCLUSIONS AND CONSIDERATIONS

The main objective in this study was to investigate the potential of *Bacteroides* as a fecal indicator for drinking water. This first involved examining the persistence of *Bacteroides* in drinking water distribution systems. Laboratory experiments with a nonchlorinated pilot scale PVC water distribution system revealed that *Bacteroides* DNA may still be present in well-developed biofilms up to two months after the introduction of *Bacteroides*. Biofilm sloughing or *Bacteroides* diffusion out of the biofilm could result in detection of its genetic markers that present falsely high risk scenarios.

One possible explanation for the extended persistence of the *Bacteroides* DNA in the MDS experiments is diffusion of *Bacteroides* into anoxic zones or microniches within the MDS biofilms. *Bacteroides fragilis*, the species used to inoculate the MDS, is known to have a higher oxygen tolerance than other *Bacteroides* species. Zones of lower oxygen concentration within the biofilms could preferentially assist the persistence of *Bacteroides* cells over those in the bulk water. Moreover, well-developed biofilms could potentially protect *Bacteroides* cells from eukaryotic and act as another mechanism by which biofilms may aid in the persistence of *Bacteroides* in the environment.

Field studies were also conducted that examined the occurrence of *Bacteroides* in the biofilms of two large water distribution system biofilms in central Arizona. These results of this field study verify that *Bacteroides* DNA could be detected in the biofilms of two large chlorinated water distribution systems in central Arizona. Moreover, positive samples were found to be geographically clustered in several locations, suggesting past contamination events as the source of *Bacteroides*. While there is no evidence to support the hypothesis that these *Bacteroides* grew in the environment, these results do confirm that the DNA of *B. fragilis*, possesses the ability to environmentally persist longer than previously thought by interacting with well-developed biofilms.

Simple, rapid, and inexpensive isothermal molecular methods were also investigated for the detection of *Bacteroides* in water samples. A simplified NASBA procedure was proposed, though no successful amplification was achieved. Moreover, tHDA also showed no amplification due to unreliability in the enzymes involved in the reaction. Despite the potential of isothermal amplification to provide a simple, rapid, and, inexpensive water quality assay, this unsuccessful amplification suggests the reliability of these methods needs to be improved before the method will be suitable for water samples.

In summary, although *Bacteroides* species have been able to successfully indicate fecal pollution in recreational waters, and have

shown potential for source identification, further considerations must be made when using *Bacteroides* as a fecal indicator for drinking water. In particular, this study shows that study of biofilms is also essential to be considered when performing molecular assays for *Bacteroides*, as biofilms may aid persistence through provision of anoxic zones and protection from eukaryotic predation. This could lead to prolonged molecular detection and present falsely high risk scenarios for fecal contamination

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