MICROBIAL INDICATORS OF FECAL CONTAMINATION: APPLICATION TO MICROBIAL SOURCE TRACKING

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INTRODUCTION

The direct detection of pathogenic bacteria and viruses, and cysts of protozoan parasites requires costly and time-consuming procedures, and well-trained labor. The task would be enormous if one contemplates the monitoring of hundreds of pathogens and parasites on a routine basis in water and wastewater treatment plants, receiving waters, soils, and other environmental samples. Therefore, indicators of fecal pollution were much needed. As early as 1914, the U.S. Public Health Service (U.S.P.H.S.) adopted the coliform group as an indicator of fecal contamination of drinking water. Later on, other microorganisms were added to the list of indicators. Research in the last few decades has shed some light on the fate of microbial indicators in the environment and their suitability as representatives of the hardier viruses and protozoan cysts.

The criteria for an ideal indicator organism are the following (Bitton, 2005):

1. It should be a member of the intestinal microflora of warm-blooded animals.
2. It should be present when pathogens are present, and absent in uncontaminated samples.
3. It should be present in greater numbers than the pathogen.
4. It should be at least equally resistant as the pathogen to environmental factors and to disinfection in water and wastewater treatment plants.

5. It should not multiply in the environment.

6. It should be detectable by means of easy, rapid, and inexpensive methods.

7. The indicator organism should be non pathogenic

In this report, we will review the major microorganisms which have been proposed as fecal indicators, the methodology for their detection in environmental samples, and their contributions in reducing the risks to public health. We will also review the major methods proposed to track the source(s) of fecal contamination in environmental samples.

REVIEW OF INDICATOR MICROORGANISMS AND METHODOLOGY FOR THEIR DETECTION

Proposed or commonly used microbial indicators are discussed below (APHA, 1998; Bitton, 2005; Ericksen and Dufour, 1986; Leclerc et al., 2000) **(Figure 1):**

1. Coliform Bacteria

a. Characteristics of the coliform group

The **total coliform group** belongs to the family enterobacteriaceae and includes the aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas production within 48 hours at 35^{o}C (APHA, 1998). Total coliforms include *Escherichia coli, Enterobacter, Klebsiella*, and *Citrobacter*. These coliforms are discharged in relatively high numbers (2 x 10^{9} coliforms/day/capita) in

human and animal feces, but not all of them are of fecal origin. These indicators are useful for determining the quality of potable water, shellfish-harvesting waters, and recreational waters. They are less sensitive, however, than viruses or protozoan cysts to environmental stresses and to disinfection. Some members (e.g., *Klebsiella*) of this group may sometimes grow under environmental conditions in industrial and agricultural wastes. In water treatment plants, total coliforms are one of the best indicators of treatment efficiency of the plant.

Fecal coliforms are thermotolerant bacteria that include all coliforms that can ferment lactose at 44.5^{o}C. The fecal coliform group comprises bacteria such as *Escherichia coli* or *Klebsiella pneumonae*. The presence of fecal coliforms indicates the presence of fecal material from warm-blooded animals. Some investigators have suggested the sole use of *E. coli* as an indicator of fecal pollution as it can be easily distinguished from the other members of the fecal coliform group (e.g., absence of urease and presence of β-glucuronidase). Fecal coliforms display a survival pattern similar to that of bacterial pathogens but their usefulness as indicators of protozoan or viral contamination is limited. Coliform standards are thus unreliable with regard to contamination of aquatic environments with viruses and protozoan cysts. Coliforms may also regrow in the environment. Detection of *E. coli* growth in pristine sites in a tropical rain forest, suggest that it may not be a reliable

indicator of fecal pollution in tropical environments (Bermudez and Hazen, 1988; Hazen, 1988).

b. Standard Methods for the Detection of Total and Fecal Coliforms

Total coliforms have the ability ferment lactose with gas production within 48 hours at 35°C. They are detected via most probable numbers (MPN) technique or via the membrane filtration method. These procedures are described in detail in *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998). Fecal coliforms produce gas when grown in EC broth at 44.5°C (MPN method) or they form blue colonies when grown in m-FC agar at 44.5°C (membrane filtration method).

Several factors influence the recovery of coliforms, among them the type of growth medium, the diluting solution, membrane filter used, the presence of non-coliforms, and the sample turbidity. Another crucial factor affecting the detection of coliforms is the occurrence of injured coliform bacteria in environmental samples. These debilitated bacteria do not grow well in the selective detection media used (presence of selective ingredients such as bile salts and deoxycholate) under temperatures much higher than those encountered in the environment (Domek et al., 1984; McFeters et al., 1982). The low recovery of injured coliforms in environmental samples may underestimate their numbers. We now know that injured pathogens may retain their pathogenicity following injury (Singh and McFeters, 1987). A

growth medium, m-T7 agar, was proposed for the recovery of injured microorganisms (LeChevallier et al., 1983; Reasoner et al., 1979).

c. Some Rapid Methods for Coliform Detection

Enzymatic assays provide an alternative approach for rapid and sensitive detection of total coliforms and *E. coli* in environmental samples.

In most tests, the detection of total coliforms is based on the β-galactosidase activity. The enzyme substrates used are chromogenic substrates such as ONPG (*o*-nitrophenyl-β-D-galactopyranoside), CPRG (chlorophenol red-β-D galactopyranoside), X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or cyclohexenoesculetin-β-D-galactoside. Fluorogenic substrates are also used and include 4-methylumbelliferone-β-D-galactoside (MUGA) or fluorescein-di—β-galactopyranoside (FDG) (Bitton et al., 1995; James et al., 1996).

Rapid assays for detection of *E. coli* are based on the hydrolysis of a fluorogenic substrates, 4-methylumbelliferone glucuronide (MUG) by β-glucuronidase, an enzyme found in *E. coli*. The end product is fluorescent and can be easily detected with a long-wave ultraviolet lamp. β-glucuronidase is an intracellular enzyme found in *E. coli* as well as some *Shigella* species (Feng and Hartman, 1982). These tests have been used for the detection of *E. coli* in clinical and environmental samples (Trepeta and Edberg, 1984). The assay consists of incubating the sample in

lauryl-tryptose broth amended with 100 mg/L MUG, and observing the development of fluorescence within 24 hr incubation at 35°C. This assay can be adapted to membrane filters since β-glucuronidase-positive colonies are fluorescent or have a fluorescent halo when examined under a long-wave UV light.

A commercial test, Colilert, was developed to enumerate simultaneously in 24 hr both total coliforms and *E. coli* in environmental samples (Edberg *et al.,* 1990). The test is performed by adding the sample to tubes that contain powdered ingredients consisting mainly of salts and specific enzyme substrates, which also serve as the only carbon source for the target microorganisms. The enzyme substrates are *o*-nitrophenyl-β-D-galactopyranoside (ONPG) for detecting total coliforms, and 4-methylumbelliferyl-β-D-glucuronide (MUG) for specifically detecting *E. coli*. After 24-hr incubation, samples positive for total coliforms turn yellow whereas *E. coli*-positive samples fluoresce under a long wave UV illumination in the dark. Several surveys concerning coliform detection in drinking water have shown that Colilert had a similar sensitivity as the standard multiple tube fermentation method, or the membrane filtration method for drinking water (Edberg et al., 1988; Katamay, 1990).

ColiPAD™ is another detection test for total coliforms and *E.coli* in environmental samples. It is based on the hydrolysis of chlorophenol

red-β-D-galactopyranoside (CPRG) and 4-methylumbelliferone glucuronide (MUG) for the rapid detection on an assay pad of total coliforms (purple spots) and *E. coli* (fluorescent spots), respectively. Monitoring of wastewater effluents and lake water showed a good correlation between results obtained by ColiPAD and the standard multiple tube fermentation method (Bitton et al., 1995)

The modified mTEC method proposed by EPA uses a medium that contains the chromogen 5-bromo-6-chloro-3-indolyl-β-D glucuronide. Following sample filtration, the filters are placed on modified mTEC medium and incubated for 2 h at 35°C and then for 20-22 h at 44.5°C. Magenta colonies are counted as *E. coli* (Francy and Darner, 2000).

E. coli can also be detected, using monoclonal antibodies directed against outer membrane proteins (e.g., *OmpF* protein) or alkaline phosphatase, an enzyme localized in the cell periplasmic space (Joret et al., 1989). Polymerase chain reaction (PCR) is used to detect *E. coli* by targeting genes such as *LacZ*, *lamB*, or *uidA* genes (Bej et al., 1990).

2. Fecal Streptococci

This group comprises *Streptococcus faecalis, S. bovis, S. equinus* and *S. avium*. Since they commonly inhabit the intestinal tract of humans and warm-blooded animals, they are used to detect fecal contamination in water. Members of this group survive longer than other bacterial indicators but do

not reproduce in the environment. A subgroup of the fecal streptococci group, the enterococci (*E. faecalis* and E*. faecium*, *E. durans*, *E. gallinarum* and *E. avium*) have the ability to grow at 6.5% NaCl, high pH (pH=9.6) and high temperature (45C). This group has been suggested as useful for indicating the presence of viruses, particularly in biosolids and marine environment.

Fecal streptococci/enterococci can be detected, using selective growth media in most probable numbers or membrane filtration formats. Enzyme-based methods are available for the detection of fecal streptococci. These indicators can be detected by incorporating fluorogenic (MUD = 4-methylumbelliferone β-D-glucoside) or chromogenic (indoxyl-β-D-glucoside) substrates into selective media. The enterococci group can be rapidly detected via fluorogenic or chromogenic enzymatic assays. These tests are based on the detection of the activity of two specific enzymes, pyroglutamyl aminopeptidase and β-D-glucosidase (Manafi and Sommer, 1993). Miniaturized tests, using microplates and MUD, were successful in the selective detection of this group in environmental samples (Hernandez et al., 1990; 1993; Pourcher et al., 1991). Enterolert is marketed as a 24-hr MPN test for the detection of enterococci, and is based, as shown for Colilert, on the use of a methylumbelliferyl substrate (Budnick et al., 1996).

Molecular methods for the detection of enterococci will be covered in the section on microbial source tracking.

3. Anaerobic Bacteria

Some of the anaerobic bacteria are important part of fecal flora. We will cover three important anaerobic bacteria that have been proposed as indicators of fecal contamination.

a. *Clostridium perfringens*

Clostridia are mostly opportunistic pathogens but are also implicated in human diseases such as gas gangrene (*C. perfringens*), tetanus (*C. tetani*), botulism (*C. botulinum*) or acute colitis (*C. difficile*) (Payment et al., 2002). *C. perfringens* is an anaerobic gram-positive, endospore-forming, rod-shaped, sulfite-reducing bacterium found in the colon and represents approximately 0.5% of the fecal microflora. It is a member of the Sulfite Reducing Clostridia (SRC) group and is commonly found in human and animal feces and in wastewater. In Europe, SRC have been traditionally used as indicators of water quality but new European Union (EU) regulations consider more specifically *C. perfringens* as the indicator of choice. The EU standard was set at 0/100ml of drinking water supply (European Union, 1998). Some argue that the hardy spores make this bacterium too resistant to be useful as an indicator organism. Thus, it could be useful as an indicator of past pollution and as a tracer to follow the fate of pathogens. Payment

and Franco (1993) recommended its use as an indicator of the presence of viruses and parasitic protozoa in water treatment plants, and as an indicator of the quality of recreational waters (Fujioka, 1997). This bacterium is generally much more resistant to oxidizing agents and to UV than bacterial and phage indicators. However, it's use in microbial source tracking is doubtful.

b. Bacteroides spp.

These non-spore forming obligate anaerobic bacteria occur in the intestinal tract at concentrations in the order of 10^{10} cells per gram of feces. They represent about one-third of the human fecal bacteria, outnumbering the fecal coliform bacteria (Holdeman et al., 1976). Strict anaerobic bacteria are desirable because they are restricted to warm-blooded animals, and do not survive long once deposited in waters (Meays et al., 2004). However they are more difficult to grow in the laboratory than coliforms or enterococci..

c. Bifidobacteria

Bifidobacteria are anaerobic, non-spore-forming, gram-positive bacteria that live in human and animal guts. They have long been suggested as fecal indicators. *Bifidobacterium* is the third most common genus found in the human gut. Since some of the bifidobacteria (e.g., *B. bifidum*, *B. adolescentis, B. infantis, B. dentium)* are primarily associated

with humans, they may serve as another tool to differentiate human from animal fecal contamination sources (Bitton, 2005). The human isolates of bifidobacteria have the ability to ferment sorbitol and can be selectively detected in sorbitol agar (Mara and Oragui, 1983; Rhodes and Kator, 1999. They can also be detected with rRNA probes (Bonjoch et al., 2004; Langendijk et al., 1995). However, if bifidobacteria are considered as indicators of fecal pollution, their sensitivity to environmental factors is a problem.

4. Bacteriophages

Bacteriophages have a basic structure similar to that of animal viruses. They infect a wide range of bacteria. They initiate a lytic cycle, which results in the production of phage progeny and the destruction of the bacterial host cells.

Phage detection in environmental samples consists of concentrating the sample using one of several published procedures, decontaminating the concentrate, and carrying out the phage assay by the double or single-layer methods. A wide range of bacterial host cells have been used as some are more efficient than others in hosting phages. Somatic coliphages can be assayed on an *E. coli* C host, while the assay of male-specific phage requires the use of specific host cells such as *Salmonella typhimurum* strain WG49 or *Escherichia coli* strain *HS[pFamp]R* , but may be complicated by the growth

of somatic phages. The U.S. EPA has proposed two methods (methods # 1601 and 1602) to detect somatic coliphages (host is *E. coli* CN-13) and F-specific coliphages (host is *E. coli* F-amp) in aquatic environments. Method 1601 include an overnight enrichment step (water is supplemented with the host, $MgCl_2$, and tryptic soy broth) followed by "spotting" onto a host bacterial lawn. In Method 1602, a 100-mL water sample is supplemented with $MgCl_2$, host bacteria, and double-strength molten agar. The mixture is poured onto Petri dishes and the plaques are counted after overnight incubation (U.S. EPA, 2001a; 2001b). Bacterial phages can also be detected by reverse transcriptase-polymerase chain reaction (RT-PCR) technique as shown for F^+-specific coliphage in fecally contaminated marine waters (Rose et al., 1997).

Three groups of bacteriophages have been considered as indicators: somatic coliphages, male-specific RNA coliphages (FRNA phages) and phages infecting *Bacteroides fragilis* (Berger and Oshiro, 2002; Leclerc et al., 2000):

a. <u>Somatic coliphages</u>

They infect mostly *E. coli* but some can infect other enterobacteriaceae. They have been used as water quality indicators in estuaries, seawater, freshwater,potable water,wastewater and biosolids (Mocé-Llivina et al., 2003). Phages can also serve as biotracers to identify

pollution sources in surface waters and aquifers (Harvey 1997; McKay et al. 1993; Paul et al. 1995). They may also serve as indicators for assessing the removal efficiency of water and wastewater treatment plants (Bitton, 1987). Genetically modified phages have been proposed to avoid interference with indigenous phages present in environmental samples.

A unique DNA sequence was inserted into the phage genome which then can be detected, using polymerase chain reaction (PCR) or plaque hybridization (Daniell et al., 2000).

b. F+ coliphages

F+ coliphages include the families *Inoviridae* (FDNA) and *leviviridae* (FRNA). They have single-stranded DNA or RNA and they infect *E. coli* cells that contain the F plasmid which codes for the F or sex pilus to which the phage attach. Their presence in high numbers in wastewaters and their relatively high resistance to chlorination contribute to their consideration as indicators of wastewater contamination (Havelaar et al., 1990; Nasser et al., 1993; Yahya and Yanko, 1992). As regards shellfish contamination and depuration, FRNA (or male-specific phages) provide a suitable model for studying the fate of animal viruses in shellfish (Doré and Lees, 1995). They also appear to be suitable indicators for viral contamination in the marine environment.

c. Phages infecting Bacteroides fragilis

Phages active against *Bacteroides fragilis* HSP 40 were detected in feces (found in 10% of human fecal samples but not in animal feces), sewage, and other polluted aquatic environments (river water, seawater, groundwater, sediments) and were absent in non polluted sites (Cornax et al., 1990; Tartera and Jofre, 1987). Unlike coliform bacteria, these phages do not multiply in the environment. These indicators were found to be more resistant to chlorination than bacterial indicators and even viruses (Abad et al., 1994). The higher resistance to chlorine of bacterial phages as compared to bacterial indicators was confirmed for sewage effluents (Durán et al., 2003). They are more resistant to water treatment processes than bacterial indicators, other phages (somatic and male-specific phages), and enteroviruses (Jofre et al., 1995). They are also more resistant to natural inactivation in freshwater environments than fecal coliforms and other phages (Duran et al., 2002).

5. Bacterial Spores

Aerobic spores are non pathogenic, ubiquitous in aquatic environments, occur at much higher concentrations than the parasitic protozoan cysts, do not grow in environmental waters, and their assay is simple, inexpensive and relatively quick. *Bacillus* spores may serve as good surrogates to assess the removal efficiency of *Cryptosporidium* oocysts or *Giardia* cysts and disinfection efficiency in water and wastewater treatment

plants (Chauret et al., 2001; Nieminski, 2002; Nieminski et al., 2000; Radziminski et al., 2002).

The detection of bacterial spores is relatively simple and consists of pasteurizing the sample (60° C for 20 min), passing it through a membrane filter which is incubated on nutrient agar supplemented with 0.005% bromothymol blue (Francis et al., 2001).

6. Heterotrophic Plate Count (HPC)

The heterotrophic plate count (HPC) represents the aerobic and facultative anaerobic bacteria that derive their carbon and energy from organic compounds. This group includes gram-negative bacteria belonging to the following: *Pseudomonas, Aeromonas, Klebsiella, Flavobacterium, Enterobacter, Citrobacter, Serratia,Acinetobacter, Proteus, Alcaligenes, Enterobacter, Moraxella* and nontubercular mycobacteria. Some members of this group are opportunistic pathogens (e.g., *Aeromonas, Flavobacterium* but little is known about the effects of high numbers of HPC bacteria on human health. Segments of the population particularly at risk of infection with opportunistic pathogens are newborn babies, elderly and sick people. HPC level in drinking water should not exceed 500 organisms/ml. Numbers above this limit generally signal a deterioration of water quality in distribution systems (Bitton, 2005).

HPC is useful to water treatment plant operators with regard to the following (Reasoner, 1990):

● Assessing the efficiency of various treatment processes, including disinfection, in a water treatment plant.

● Monitoring the bacteriological quality of the finished water during storage and distribution.

● Determining the potential for regrowth or aftergrowth in treated water in distribution systems.

7. Concluding Remarks

We have reviewed the characteristics and detection methodology for the traditional and less traditional microbial indicators used for assessing contamination of aquatic and other environments by pathogenic microorganisms. There is still an ongoing debate among the public health community on which microorganism should be used as an indicator, as there is a weak relationship between some indicators and the pathogens or parasites they are supposed to represent. There is probably no universal ideal indicator microorganism that fulfills all the criteria outlined earlier, and that works for all pathogens under all circumstances. Short of direct detection of pathogens and parasitic cysts and oocysts, we may have to use of battery of indicator microorganisms.

A useful development is the advent of molecular techniques for rapid detection of small numbers of bacterial or viral pathogens and protozoan parasites in environmental samples. Furthermore, multiplex PCR can be used to detect a wide range of pathogenic microorganisms and parasites in the same sample. The road is open to direct, rapid and possibly inexpensive methods for detecting pathogens and parasites in the environment.

We might also consider the use of an enterovirus indicator (e.g., poliovirus) for enteric viruses, a protozoan indicator (e.g., *Giardia* or *Cryptosporidium*) for cysts of parasitic protozoa, and possibly coliform bacteria or enterococci for bacterial pathogens.

MICROBIAL SOURCE TRACKING

B. Total Maximum Daily Load (TMDL)

Despite the passage of the Clean Water Act in 1972, many of the Nation's surface waters do not meet water quality standards and are classified as impaired as a result of pollution by pathogens, metals, organics, nutrients, solids, to name a few. Section 303 (c) of the Act addresses the total maximum daily loads (TMDL) that must be established for impaired waters. TMDL must address each pollutant and consider both point sources and non point sources for a given pollutant (USPA, 2001c). A TMDL is defined as the maximum pollutant load that a water body can receive and still meet water quality standards. TMDL is the driving force behind the development of methodology to distinguish human from animal sources. It requires the setting of source load allocations to determine allowable pollution loads.

Identification of pollution sources could aid in the restoration of water quality, reduce the amount of nutrients in watersheds or reduce the risk of infectious diseases. Management of water quality is made easier if the source of the pollutant can be identified. Best management practices are considered once the TMDL is developed for a given pollutant. As regards the risk of infectious diseases, source identification could help in the improvement of water quality. In Virginia, it was estimated that over 60% of

cases of impairment of water quality were dues to violations of the coliform standards (Hagedorn et al., 2003). Source tracking is particularly important for waters used for water supply, swimming or shellfishing and impacted by pathogenic microorganisms.

B. Microbial Source Tracking (MST)

Fecal contamination of surface waters is caused by surface runoffs from agricultural lands and cattle feedlots, domestic animals, inadequate septic tanks, wastewater effluents, and fecal discharges by wildlife. The entry of fecal coliform bacteria in streams and rivers is a widespread problem often contributed by agricultural activities. Microbiological TMDL addresses the entry of health-related microorganisms into an aquatic environment.

In MST, the clonal population structure of bacteria is used to classify microorganisms on the basis on their phenotypic or genotypic fingerprints. However, the successful outcome of MST depends on several assumptions (Gordon, 2001; Samadpour, 2002):

1) within a given species of bacteria, some members have adapted to living under specific environmental conditions or specific host.

2) the clonal composition of the populations changes with the locality.

3) bacterial strains display host specificity.

4) the clonal composition of populations is stable through time.

MST is crucial to water quality management and is helpful in partitioning loads among sources of fecal contamination. As an example, the use of MST in a small watershed in Virginia showed that the fecal contamination source was from cattle, leading to recommendations of best management practice (BMP) for fecal discharges from cattle (Hagedorn et al., 1999).

TMDL is carried out by (Simpson et al., 2002):

1) determining the numbers of traditional microbial indicators (total and fecal coliforms, *E. coli*, fecal streptococci/enterococci. This topic has been covered in a previous section of this report.
2) Choosing an MST methodology: Traditional indicator microorganisms are not limited to humans but are also found in the gastrointestinal tract of other warm-blooded animals. Thus, methodology for tracking the source of the fecal pollution (Human vs animal sources) is essential. MST is based on the premise that different microbial strains are related to specific animal hosts. MST tracks the genotypic and phenotypic differences in traits acquired as a result of exposure to a given host or environment. When bacterial indicators are specifically tracked, the operation is called bacterial source tracking (BST). Sometimes, the tracking is able to distinguish between different animal sources. The target microorganisms have included bacteria (e.g.,

Escherichia, *Enterococcus*, *Bacteroides*, and *Bifidobacterium*), protozoa (e.g., *Cryptosporium* oocysts), phage, or enteric viruses.

C. Approaches used in MST

There are several approaches used in MST (Field et al., 2003; Simpson et al., 2002; Scott et al., 2002). The source of fecal contamination can also be identified, based on the antibiotic resistance pattern of coliforms (Kaspar et al., 1990) or fecal streptococci (Knudtson and Hartman, 1993; Wiggins, 1996; Hagedorn et al., 1999), multiple antibiotic resistance (MAR) profiles (Parveen et al . 1997), ribotyping, pulsed-field gel electrophoresis, biochemical fingerprinting (Manero et al., 2002), phenotypic fingerprinting with carbon source utilization profiles, using the Biolog system (Hagedorn et al., 2003) or amplified fragment length polymorphism (AFLP) which can distinguish between non-pathogenic and pathogenic strains of *E. coli* (Leung et al., 2004). MST can also be carried out by directly tracking human or animal pathogens. All of the above methods necessitate the construction of a reference library. Library-independent methods include the direct tracking of human and animal viruses, FRNA coliphage, *Bacteroides* and bifidobacteria genotyping, enterotoxin biomarkers and immunological tests. **Table 1** shows the two-way classification of MST methods (Bernstein et al., 2002).

1. Phenotypic Approach

Bacterial pathogens have been traditionally identified by using methods such as biochemical tests, outer membrane protein profiles, phage susceptibility, serology, fatty acid methyl esters (FAME) or fimbriation, to cite a few (Parveen and Tamplin, 2002).

a. Antibiotic Resistance Analysis (ARA)

The most commonly used phenotypic method is antibiotic resistance analysis (ARA) for MST in small watersheds. Microorganisms develop resistance to antibiotics to which they are regularly exposed following therapeutic administration of the drugs to human or animal hosts. Thus, ARA uses the antibiotic resistance patterns of the microbial isolates as fingerprints for distinguishing human from animal sources. ARA requires culturing a large number of isolates, screening them for resistance against an array of antibiotics at various concentrations, and analyzing the fingerprints by discriminant analysis. The fingerprints are compared to a reference database composed of bacterial isolates from known sources. This analysis generates an average rate of correct classification (ARCC). ARA has been mostly used for *E. coli* and enterococci.

ARA and discriminant analysis were used to differentiate between human and animal fecal isolates from surface waters in Florida. The average rate of correct classification for fecal streptococci and fecal coliforms were 62.3% and 63.9%, respectively (Harwood et al., 2000). Others reported

ARCCs for streptococci isolates from one of four possible groups (human, cattle, poultry, and wild animals) ranged from 64 to 78% (Wiggins et al., 1999). Discriminant analysis of antibiotic resistance profiles in fecal streptococci in natural waters showed that human vs. animal isolates were correctly classified at an ARCC of 95% while human vs. wildlife isolates had an ARCC of 98% (Wiggins, 1996). Hagedorn et al. (1999) used a larger database consisting of ARA patterns from 7,058 fecal streptococci isolates from known human, livestock, and wildlife sources. ARCC was 87% for the entire database and was as high as 93% for human isolates. This database was field-tested at a watershed in Viriginia and showed an ARCC of 88% and the predominance of cattle fecal pollution as the source of the fecal streptococci.

ARA of enterococci in samples taken in a rural community in Virginia yielded correct classification rates of 94.6% for 203 human isolates, 93.7% for 734 livestock isolates, and 87.8% for 237 wildlife isolates (Graves et al., 2002). Field application of ARA in an urban watershed in Florida revealed that the majority of fecal coliforms isolated from failing septic tank systems were of human origin and the majority of the isolates from Stevenson Creek were from wild animals, humans, and, to a lesser extent, dogs (Whitlock et al., 2002). Choi et al. (2003) considered ARA (7 antibiotics at four concentrations) to identify the source of fecal enterococci in Huntington

Beach, CA. The sources of seawater isolates was from sewage (39%), birds (30%), marsh sediments (24%) and urban runoff (6%). However, the investigators observed a temporal variation of the dominant sources. ARA (use of 6 antibioitics at 4 concentrations each) was conducted to test the source of fecal contamination in the Big Creek watershed in Georgia (CDM, 2000). A total of 800 FC isolates were subjected to ARA and the results were compared to a database of 1125 FC isolates. Discriminant analysis of antibiotic resistance patterns of FC from human and nonhuman sources generated an ARCC of 94%. The distinction between the animal sources was somewhat less accurate. Parveen et al. (1997) studied the multiple antibiotic resistance (MAR) patterns, using 10 antibiotics, of 765 *E. coli* isolates collected in Apalachicola National Estuarine Research Reserve in Florida. *E. coli* strains from human sources generally displayed higher resistance to antibiotics and higher MAR index than strains from non-human sources. ARA was also applied to the tracking of fecal streptococci in a shellfish growing area in Australia, using 4 concentrations of 4 different antibiotics. The library was composed of isolates from beef and dairy cattle, chickens and humans. No single significant source of fecal contamination was identified (Geary and Davies, 2003).

Simpson et al. (2002) concluded that the results from various studies showed that the average rate of correct classification (ARCC) ranged from 62 to 94% when individual species were compared.

Some potential problems associated with ARA are:

* antibiotic resistance genes are carried by plasmids which may be lost upon cultivation. The gain or loss of a plasmid may change the antibiotic resistance pattern of bacteria. However, Wiggins et al. (2003) showed that the antibiotic resistance patterns were stable for at least 1 year.

* ARA necessitates the construction of a library of phenotypic fingerprints obtained from bacteria isolated from the feces of known human and animal sources. The database should be designed to include sufficient representatives from the most likely sources of contamination in a given watershed (Choi et al., 2003]. The appropriate size of a representative library is still not well known. A library should be large enough to represent a large geographical area. Wiggins et al., (2003) reported that libraries from six watersheds in Virginia could be merged to produce a representative library, although the ARCC of the library was only 57%.

* We do not know if antibiotic resistance patterns in a given geographic area can predict the source of fecal contamination in a different area (Harwood et al., 2000).

* Changes in antibiotic use may change the antibiotic resistance pattern of fecal bacteria.

* Some suggested that ARA might not be suitable for wildlife isolates because wildlife might consume feed destined for livestock as the results of their close proximity (Meays et al., 2004).

b. Carbon utilization profiles (CUP)

Using the CUP approach, bacterial isolate identification is based on differences in the utilization pattern of various carbon and nitrogen sources. The BIOLOG system is based on the use of 96-well microplates which contain 95 different carbon substrates. The utilization pattern of these substrates is scored for each isolate. The utilization profile is compared to the Biolog database that comprises more than 2,000 microorganisms. This approach was used to differentiate microbial communities in wastewater treatment systems (Liberty et al., 1996) and to identify microorganisms of clinical importance (Holmes et al., 1994). Carbon utilization profiles were obtained for 365 *Enterococcus* isolates collected from four different geographical areas. Discriminant analysis showed that the derived ARCC by source was 92.7% for a human vs non-human two-way classification (Hagedorn et al., 2003). ARCC was however lower for a three-way classification (Hagedorn, 2002).

An advantage of CUP is its simplicity, necessitating only a microplate reader to determine carbon source utilization. This method requires less skills than ARA or genotypic methods. Biolog now offers microplates with different sources of N, P and S, that could potentially be used for a more accurate classification of *E. coli* and *Enterococcus* strains (Hagedorn, 2002).

While this method has been extensively used in clinical microbiology, and soil and aquatic microbiology, its wide application to microbial source tracking needs further research (Simpson et al., 2002).

2. Genotypic Approach

Genetic techniques have helped alleviate the dependence on microbial phenotypic characteristics which may be unstable and less specific. In this section, we will cover the most popular genotypic methods used in microbial source tracking.

a. Ribotyping

Ribosomal ribonucleic acids (rRNA) are integral part of all living cells, and the genes coding for rRNA tend to be very highly conserved (Farber, 1996; Samadpour, 2002). Bacteria harbor numerous copies of ribosomal RNA.

Ribotyping consists of using oligonucleotide probes to detect rRNA sequences, thus generating fingerprints for microbial isolates. The fecal bacterial isolate is cultured, its DNA is extracted and digested with one or

more restriction enzymes. It is then subjected to gel electrophoresis, transferred to a membrane (e.g., nylon membrane) and hybridized with a labeled rRNA probe. This generates several bands, forming ribotype patterns that can be used to identify bacterial strains. The fingerprints are analyzed by discriminant analysis and compared to a reference database. The unique riboprints help to determine the host of the environmental isolate. Ribotyping using two restriction enzymes, *Eco*RI and *Pvu*II, showed a higher resolution than using one of the enzymes (Aarnisalo et al., 1999; Carson et al., 2003; Farber, 1996; Meays et al., 2004; Samadpour, 2002). Ribotyping main steps are summarized in **Figure 2**. (Aarnisalo et al., 1999).

Using ribotyping, Samadpour and Chechowitz (1995) were able to identify 71% of isolates from human and non-human sources in Seattle, WA. Parveen et al.(1999) isolated 238 *E. coli* from samples collected in Florida from the Apalachicola National Estuarine Research Reserve, sewage treatment plants, and directly from human and animals feces. Discriminant analysis and ribotyping showed that the average rate of correct classification (ARCC) was 82% for isolates from both human and non human sources. ARCC was slightly higher (84%) for isolates from human and animal feces. Ribotyping of 287 *E.coli* isolates from human and non human (cattle, pigs, horses, turkeys, chicken, migratory geese, dogs) sources showed that the

rates of correct classification (RCC) were 95% and 99.2% for human and non human sources, respectively (Carson et al., 2001). A total of 482 fecal *E. coli* isolates from humans (136 isolates) and animal (cattle, swine, horses, dogs, chickens, turkeys, and migratory geese) (346 isolates) was analyzed by ribotyping and rep-PCR (see below for more details on this technique). The RCCs for human and nonhuman (pooled) ribotyping patterns were 87.5% for human and 86.4% for nonhuman sources. The ARCC was 86.9%. The RCCs for human and nonhuman (pooled) rep-PCR patterns were 97.0% for human sources and 96.2% for nonhuman sources. The ARCC was 96.6% (Carson et al., 2003). This method was used for *E. coli* to distinguish human and animal sources but was not capable of distinguishing *E. coli* isolates from the different animal species (Scott et al., 2003).

Some drawbacks of this method are (Carson et al., 2003; Field et al., 2003; Hartel et al., 2002):

● Ribotyping can distinguish human from animal sources but does not differentiate between animal sources.

● Need to grow a large number of isolates and the method may take a long time to complete. Manual ribotyping requires a total of 10 to 12 days for total processing. However, ribotyping can be automated.

● Ribotyping require large databases for comparison. There are regional differences in ribotypes in fecal bacteria from humans and animals.

Thus, there is a need to establish a database for each watershed under study.

● Ribotyping req uires more skilled technician time and the cost is higher than rep-PCR.

● Diet was found to influence *E. coli* ribotype diversity in deer (comparison of wild deer to captive deer). This finding led the investigators to conclude that database should include isolates from wild deer rather than from captive deer (Hartel et al., 2003).

Some advantages of ribotyping are excellent reproducibility, good discriminatory power, and ease of interpretation. An added advantage is that the procedure can be automated (use of a riboprinter) and the results can be obtained in less than a day (Farber, 1996).

b. Pulsed field gel electrophoresis (PFGE)

The PFGE technique involves the digestion of chromosomal DNA, using low-frequency restriction endonucleases (these are enzymes that recognize 6 to 8-base sequences instead of 4-base sequences), resulting in a small number of large well resolved fragments. The high-molecular weight fragments are separated by gel electrophoresis by alternating pulsed-electric fields (Parveen and Tamplin, 2002). The main steps of PFGE are illustrated in **Figure 3** (Swaminathan and Matar, 1993). PFGE is similar to ribotyping, but

instead of analyzing rRNA, it uses the whole DNA genome (Meays et al., 2004).

Tynkkynen et al. (1999) typed two strains of *Lactobacillus* (*L. rhamnosus* and *L. casei*), using ribotyping, PFGE, and RAPD (randomly amplified polymorphic DNA). PFGE was the most discriminatory of the three methods. PFGE revealed 17 genotypes for 24 strains studied, as compared to 15 and 12 genotypes for ribotyping and RAPD, respectively.

Working with 32 *E. coli* isolates, Parveen et al. (2001) were not able to distinguish human from non-human sources, showing no association between the isolate pattern and the contamination source. Using PFGE to classify 439 *E. coli* isolates, Simmons et al. (2000) showed that the predominant sources were wildlife and dogs.

Some advantages of PFGE are excellent reproducibility, discriminatory power and ease of interpretation (Farber, 1996). The use of this technique in MST deserves further study.

c. Repetitive Extragenic Palindromic Element- PCR (rep-PCR)

Repetitive DNA elements are scattered throughout the bacterial genome and are separated by distances which vary according to the bacterial species or strain (Farber, 1996). In rep-PCR, DNA is amplified by PCR between adjacent repetitive extragenic elements and this leads to strain-specific DNA fingerprints. The PCR products are size-fractionated by

agarose gel electrophoresis, leading to DNA fingerprint patterns. A computer software is used for pattern recognition (Dombek et al., 2000; Nakatsu et al., 2002).

A total of 482 fecal *E. coli* isolates from humans (136 isolates) and animal (cattle, swine, horses, dogs, chickens, turkeys, and migratory geese+) (346 isolates) was analyzed by ribotyping and rep-PCR. The RCCs (i.e., rate of correct classification) for human and nonhuman (pooled) rep-PCR patterns were 97% for isolates from human sources and 96.2% for isolates from nonhuman sources. The ARCC was 96.6% . The RCCs for REP-PCR were higher that those obtained using ribotyping (Carson et al., 2003) The use of rep-PCR technique for 154 *E. coli* isolates from different sources showed that 100% of the chicken and cow isolates and between 78 and 90% of the human, goose, duck, pig, and sheep isolates were assigned to the correct source groups (Dombek et al., 2000). Genthner et al. (2005) used rep-PCR in combination with ARA to detect the source of *E. faecalis* isolates from samples in Pensacola Beach, FL. This combined approach added more confidence into isolate identification. It was found that the main source of contamination was from seagulls. The authors caution that *E. faecalis* represents only 32% of the enterococci isolated from the beach. However, Holloway (2001) found no significant clustering of *E. coli* or *E. faecalis* strains by animal type, due possibly to too few strains tested .

Rep-PCR is reproducible, relatively simples and has moderate discriminatory power (Carson et al., 2003; Farber, 1996).

d. Mitochondrial DNA

Humans and animals excrete a large amount of cells (e.g., blood cells, epithelial cells) in their stools (Iyengar et al., 1991). Martellini et al. (2005) developed a PCR protocol that target the nucleic acids of the host instead of the microorganisms they excrete into the environment. Thus, the eukaryotic genetic markers serve as the tracking agents of the fecal source. Mitochondrial DNA sequences were used to design PCR primers specific for human, bovine, ovine and porcine DNA using PCR protocols. Many of the approaches discussed in this review are able to differentiate human from animal sources. This method is however able to distinguish between bovine, ovine and porcine sources.

e. Sequence-based source tracking of Escherichia coli

The enzyme, β-glucuronidase, is found in approximately 95% of *E. coli* in the environment (Martins et al., 1993). Several enzymatic detection methods (e.g., Colilert, , mColiBlue, ColiPAD) for *E. coli* are based on detection of β-glucuronidase in environmental samples (Bitton, 2005; Bitton et al., 1995; USEPA, 2001b). A PCR-based denaturing-gradient gel electrophoresis (PCR-DGGE) method was used to partiallly sequence the β-glucuronidase gene (*uidA*) for specific detection and differentiation of

Escherichia coli populations in freshwater samples according to variations in *uidA* sequence (Farnleitner et al., 2000).

An MST method was proposed recently and is based on detecting the genetic variability (i.e., specific sequence differences between *E. coli* strains) of the *E. coli* gene, *uidA*, which codes for the production of β-glucuronidase (Ram et al., 2004). A library was constructed and comprised 182 *E. coli* isolates from various fecal samples (humans, ducks, geese, gulls, dogs, cows, horses). 81 alleles (genetic variants of *uidA*) were identified in the isolates. When matching the environmental data set (from the Lake St. Clair and Clinton River watershed in Michigan) with the library data set, the average rate of correct classification (ARCC) was 60% to 75%. It is worth noting that while certain alleles were found in all fecal samples (e.g. *uidA*1), others were specific for humans (e.g., *uidA*9, *uidA*13 and *uidA*15) or birds (*uidA*5 and *uidA*11) (Ram et al., 2004).

3. Library-Independent Methods

The construction of a library is a main drawback of library-dependent MST methods. The library must be large enough to be representative of large geographical areas (Wiggins et al., 2003). This is why some investigators are proposing library-independent methods such as direct tracking of

human or animal pathogens, bacterial and coliphage indicators that are associated with human or animal pollution or virulence factors of pathogenic strains of *E. coli*.

a. Fecal coliform to fecal streptococci ratio (FC/FS ratio)

This ratio has served for many years as an indicator of the origin (human vs. animal sources) of fecal pollution of surface waters. In general, a ratio of ≥ 4 indicates a contamination of human origin whereas a ratio of < 0.7 is indicative of animal pollution (Geldreich and Kenner, 1969). This ratio is only valid, however, for recent (24 hours) fecal pollution and is unreliable for chlorinated effluents, and some investigators have questioned its usefulness (Pourcher et al., 1991). As a result of these findings, the American Public Health Association (APHA) no longer recommends the use of the FC/FS ratio as a means of differentiating human from animal sources of pollution (APHA, 1998).

b. Bacteroides spp.

We have mentioned that anaerobic bacteria are generally difficult to cultivate under laboratory conditions. Molecular methods circumvents the problems of growing anaerobic bacteria. Genetic marker sequences in *Bacteroides* were used to design specific PCR primers that distinguish human from ruminant fecal contamination. The specific 16S rDNA were amplified by

PCR, cut with restriction enzymes and screened by length-heterogeneity polymerase chain reaction (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP) (Bernhard and Field, 2000a; 2000b; Field et al., 2003). These markers can trace fecal pollution in natural environments. Thus, amplification of genetic markers from *Bacteroides* provides a sensitive and accurate method of fecal source identification without the need to grow the bacteria. Using rDNA libraries, *Bacteroides* and *Prevotella* sp. sequences were detected both in horse manure and downstream samples, using group-specific primers. The analysis of these sequences produced an equine-specific phylogenetic cluster. However, the use of these bacterial groups in MST needs further research (Simpson et al., 2004).

c. Bifidobacteria

We have seen that some of the bifidobacterial species can serve as a tool to track the source of fecal contamination. The development of molecular techniques has made this task easier. A combination of genus-specific PCR and denaturing gradient gel electrophoresis (DGGE) was used to monitor fecal bifidobacteria in a human feeding trial. DNA was extracted from human feces and bifidobacterial 16S rDNA sequences were amplified by PCR. The PCR fragments were separated by DGGE to obtain a profile (Satokari et al., 2001). Enrichment for bifidobacteria followed by detection of *Bifidobacterium adolescentis* via colony hybridization have shown that this bacterium can serve as a specific indicator of human fecal contamination (Lynch et al., 2002). A multiplex PCR approach showed that *B. adolescentis*

and *B. dentium* were found exclusively in human sewage (Bonjoch et al., 2004).

d. Phages infecting Bacteroides fragilis

These microorganisms display a positive correlation with enteroviruses and rotaviruses (Jofre et al., 1989) and their persistence is similar to that of enteric viruses in seawater and shellfish (Chung and Sobsey, 1993). They may be suitable indicators of human fecal pollution and their use enables the distinction between human and animal fecal pollution.

Drawbacks are the difficulty in performing the *B. fragilis* phage assay (i.e., use of anaerobic *Bacteroides* as a host) and the occurrence of these phages at low numbers in environmental samples (Sinton et al., 1998).

e. F+ phages

It was mentioned earlier that this phage category comprises FRNA and FDNA phages. As regards MST, most of attention has been focused on the FRNA phages.

There are four subgroups of FRNA phages based on serological and phylogenetic analyses. With a few exceptions, genotypes II and III are generally associated with human feces while subgroups I and IV are associated with animal wastes (Furuse, 1987; Hsu et al., 1995). It was suggested that FRNA phages could be used as broad indicators of the source

(human vs animal sources) of fecal contamination (Schaper et al., 2002). However, they cannot distinguish between animals sources (Sobsey, 2002). Genotyping of FRNA phage is generally more successful than serotyping for distinguishing the four subgroups of FRNA phages (Scott et al., 2002).

The FDNA phages are filamentous and contain single-stranded DNA. They could possible be used as tools for MST. Recently, Vinje et al., (2004), developed and validated a reverse line blot hybridization (RLB) assay which allows for the simultaneous detection and genotyping of both FRNA as well as FDNA phages. According to the authors, the RLB method is rapid, reproducible, low-cost, and easy to perform with a high throughput of samples, and could be used in MST.

f. Direct Monitoring of human or animal pathogens

The ultimate indicators of fecal contamination are the pathogens of concern. We now have sophisticated techniques to detect bacterial and viral pathogens, and cysts and oocysts of protozoan parasites. We also have relatively efficient concentration procedures for detecting small numbers of pathogens and parasites in environmental samples (Bitton, 2005). The giant advances in molecular technology have greatly facilitated this enormous task. One has to remember that many of the molecular techniques do not provide information about the viability of pathogens and parasites found in

the environment. However, this information is not necessary when addressing source tracking of fecal contamination.

One can use conventional PCR, RT-PCR (Reverse transcription PCR) and quantitative PCR (Q-PCR) to detect human viruses in the environment, thus differentiating between human and animal pollution. It was proposed that library-independent source tracking can be based on the detection of human (adenoviruses, enteroviruses) viruses (Noble et al., 2003). PCR assays targeting human enteroviruses (HEV), bovine enteroviruses (BEV), and human adenoviruses (HAdV) were used to track the major source**s** of fecal contamination in the lower Altamaha River, Georgia (Fong et al., 2005). Ovine and porcine viruses have also been considered as tools to track animal fecal contamination (Maluquer de Motes et al., 2004). Adenoviruses are double-stranded DNA viruses belonging to the family adenoviridae and causing respiratory infections in humans. Over 40 adenoviruses have been described in the literature. Some investigators have proposed that adenoviruses can serve as indicators of fecal pollution from human sources (Jiang, 2002; Jiang et al., 2001; Pina et al., 1998). Similarly, bovine enteroviruses (BEV), found in cattle could be used as indicators of fecal pollution originating from animals (Ley et al., 2002).

Some suggested that the PCR-based detection of virulence factors would be a better indicator of the health significance of coliforms and a tool

for source tracking (Gordon, 2001). Biomarkers, based on enterotoxin genes in *E. coli* have also been proposed (Olson et al., 2002). A biomarker was developed to specifically identify swine fecal pollution in water, using a portion of the STII (heat-stable toxin II) toxin gene from enterotoxigenic *E. coli*. Cross-reactivity data showed that STII occurrence outside of swine was rare. This method is a presence-absence one (Khatib et al., 2003). Scott et al. (2005) proposed a PCR method for detecting a virulence factor, the enterococcal surface protein (*esp*) in *Enterococcus faecium,* as an index of human fecal pollution. The *esp* marker was detected in 97% of sewage and septic tank samples but was absent livestock waste lagoons or in bird or animal fecal samples.

Another example is the fecal indicator bacterium *Enterococcus faecalis* which has a limited host range. Some investigators reported that the use of selective media in combination with ribotyping made it possible to differentiate among isolates from human and chicken sources.

4. Chemical Targets

Fecal sterols

Biochemical tracers such as fecal sterols offer the potential of distinguishing between human and animal sources. Fecal materials contain sterols and the breakkdown products, stanols. In the GI tract cholesterol is degraded to coprostanol in humans and epicoprostanol in animals. Plant-

derived 24-ethylcholesterol is reduced in the gut of herbivores to 24-ethylcoprostanol and 24-ethylepicoprostanol, and is reduced in the environment to 24-ethylcholestanol (Leeming *et al*., 1996). Coprostanol appears to be the main stanol in human sewage where its concentration is much higher than in animals. A good relationship was found between levels of *E. coli* and coprostanol concentrations in tropical regions (R^2 varying from 0.81 and 0.92) but was affected by temperature (Isobe et al., 2004). These compounds persist in the environment, especially in the sediments, making it difficult to gain information about recent fecal contamination (Isobe et al., 2002; Pitt, 2001). More information is needed about the persistence of these chemicals in aquatic environments.

The measurement of microbial and chemical indicators in three rivers from New Zealand showed that the ratios of fecal sterols to stanols (coprostanol:24 ethylcoprostanol and coprostanol:epicoprostanol) increased downstream from the fecal pollution sources, thus indicating human fecal pollution (Gilpin et al., 2002). The use of fecal sterols to trace the fecal contamination of the surf zone at Huntington Beach, CA. showed that the contamination source was due to birds fecal input and not to sewage input (Noblet et al., 2004).

Bile Acids

Bile acids (e.g., deoxycholic and lithocholic acids) are associated with human fecal contamination. They are more resistant to degradation than coprostanol and can help in distinguishing between human and animal contamination sources (Elhmmali et al., 1997; 2000).

Caffeine

Caffeine is found in several beverages such as coffee, tea and soft drinks, and was proposed as a suitable indicator of human sewage pollution. A significant correlation was found between watershed scale land-use and the presence of caffeine and consumer product fragrance materials in wastewater treatment plant effluents (Standley et al., 2000).

Fluorescent whitening agents and other chemicals

Wastewater from human sources often contain fluorescent whitening agents (FWA) which are included in detergents and washing powders. They have been used to indicate contamination from septic tanks or gray water discharge (Close et al., 1989). Sodium tripolyphosphate, linear alkylbenzene sulphonates (LAS) have also been under consideration for source tracking (Sinton et al., 1998). FWA are detected by HPLC with fluorescence detection (Kramer et al., 1996; Poinger et al., 1996). These compounds adsorb to

particles and are not readily biodegradable in aquatic environments (Poinger et al., 1998).

Boron, a major ingredient of laundry detergents was proposed to be used as a tracer of sewage pollution. However, boron may not be suitable due to changes in formulations in modern laundry detergents (Pitt, 2001).

The ratio of ammonia to potassium can be used to indicate whether or not the source is sanitary wastewater. Ammonia/potassium ratios greater than 0.60 would indicate likely sanitary wastewater contamination (Pitt, 2001).

Pharmaceuticals (e.g., aspirin, ibuprofen, clofibric acid from cholesterol lowering drugs) can also help identify sewage contamination.

Finally, UV absorbance at 228 nm could be useful for source tracking (Pitt, 2001).

5. Methods comparison

Three methods [ARA, amplified fragment length polymorphism (AFLP) analysis, and analysis of 16S rRNA sequences were compared to differentiate 319 *E. coli* isolates from human sewage and clinical samples as well as from the feces of cattle, poultry, swine, deer, goose, and moose. AFLP method perform the best, correctly classifying 94% of the livestock isolates, 97% of the wildlife isolates, and 97% of the human isolates (Guan et al., 2002). A battery of methods was used to identify the source of fecal

pollution in New Zealand rivers. The methods used were fluorescent whitening agents, fecal sterols and stanols, *E. coli* , and *Bifidobacterium adolescentis*. It was found that the predominant fecal pollution was of human origin (Gilpin et al., 2002). Myoda et al. (2003) compared PFGE, rep-PCR and ribotyping for identifying the source of contamination in water samples spiked with feces from wastewater, humans, dogs, cows and seagulls. The source of host of fecal contamination was correctly identified by all methods. However, the methods showed false positive rates as high as 57%. Stoeckel et al.(2004) compared seven methods to identify sources of fecal contamination in Berkeley County, WV. The seven protocols included two phenotypic methods (ARA, CUP) and five genotypic methods (ribotyping using the restriction enzyme *Hin*dIII, ribotyping using *Eco*RI, PFGE, rep-PCR and BOX-PCR). The study indicated that the methods should display better accuracy (the accuracy was less that 30%) in order to be considered for field application.

In a comparison of ARA and ribotyping, Samadpour's group reported that ribotyping had higher sensitivity, reproducibility, and host specificity than ARA (Samadpour, 2002).

Following an EPA workshop in 2002 on microbial source tracking (USEPA, 2002), it was recommended that methods comparison should be conducted in 4 phases: Phase 1 deals with repeatability, phase 2 addresses

method accuracy for laboratory samples, phase 3 addresses method accuracy for environmental samples, and phase 4 compares the methods in complex watersheds. The methods evaluation criteria agreed upon by the the EPA workshop's participants are shown in **Table 2** (Bernstein et al., 2002).

CONCLUDING REMARKS

- For the reference library-dependent MST methods, the databases are often too limited. We need to know more about the library size necessary for representing a watershed. To which degree a library developed for a given watershed is valid for another watershed? Many investigators agree that there is a need for large libraries for phenotypic and genotypic profiles (Simpson et al., 2002). Some estimate that the size of the library should be 1000 to 2000 isolates per source (Stoeckel et al., 2004). However, there are some cost considerations when building a large database.

- We need to know more about the stability of phenotypic and genotypic traits in the environment. Bacterial genotypes and phenotypes vary with time and location, within and between animal species and can be influenced by the animal diet (Johnson et al., 2004). Samadpour et al.

(2005) concluded that the phenotypic traits were less stable than the genopypic traits.

Another confounding factor which could affect the MST results is the presence of feces of dogs and other pets in domestic wastewater (Geary and Davies, 2003). Therefore, isolates from feces from these animals must be added to a given library.

- None of the methods discussed in this review is completely reliable. Some of the methods give false positives and false negatives which would hinder the interpretation of MST results. Many of the methods are time consuming, labor-intensive, and may require costly laboratory equipment. When the bacterial isolates are cultured in the laboratory, there is the problem of culture bias. The ideal method should be quick and reliable, easily performed, robust, should have good discriminatory power, should not require culturing of isolates, should be flexible with regard to sample handling and should require a minimum of costly specialized equipment and technical skill (Carson et al., 2003; Field et al.,2003; Stoeckel et al., 2004).

- So far, no MST method has been proposed as a standard method. There is a need for standardization of MST methods, and more rigorous comparison studies (Martellini et al., 2005; Meays et al., 2004; USEPA, 2002). .

- A methods-battery approach (use of a mix of library-dependent, library-independent methods, chemical tracers) would be helpful in MST. Any choice of mix of methods should include cost consideration and level of expertise required..

- Fecal bacteria (*E. coli*, enterococci) have been generally used in MST studies. However, Gordon (2001) criticized source tracking based on the use of *E. coli* and suggested other enteric bacteria, such as *Citrobacter freundii*, for source tracking.

- Finally, the library-independent methods are worth exploring further. There is a need to further validate the use of source-specific indicators (e.g., bifidobacteria, F+ phages, phages infecting *Bacteroides fragilis*) for MST studies in the future. Furthermore, future progress in molecular methods will allow the direct detection of certain pathogens which will give information about both public health significance and microbial source tracking.

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WEB RESOURCES

http://www.epa.gov/microbes/
(EPA methods on pathogens, parasites and indicator organisms)

http://bcn.boulder.co.us/basin/data/FECAL/info/FColi.html
(info about fecal coliforms)

http://oh.water.usgs.gov/micro/qcmanual/manual.pdf
(methodology for indicators and pathogens from USGS)

TMDL

http://www.epa.gov/owow/tmdl/
(Introduction to TMDL, U.S EPA)

MST WEB SITES

http://lakes.chebucto.org/H-2/bst.html#ribotyping
(Soil & Water Conservation Society of Metro Halifax: BST Methods)

http://pubs.caes.uga.edu/caespubs/pubs/PDF/B1242-7.pdf#search='microbial%20source%20tracking'
(introduction to MST; University of Georgia)

http://www.ocwatersheds.com/watersheds/pdfs/sanjuan_bb_cbi_Baby_Beach_Bact_Studies_Work_Plan.pdf#search='microbial%20source%20tracking'
(Use of MST in Baby Beach, CA, Orange County Public Health Laboratory, 2002)

http://www.chbr.noaa.gov/Newsletter/OctoberNews/sourcetracking.htm
(Microbial Source Tracking in South Carolina Surface Waters)

http://www.cropsoil.uga.edu/mst/
(From University of Georgia; contains powerpoint presentations)

http://water.usgs.gov/owq/MST_bibliography.html
(USGS bibliography of microbial source tracking))

http://soils1.cses.vt.edu/ch/biol_4684/bst/BSTprojects.htm
(Dr. Charles Hagedorn lab, Virginia Tech, VA)

http://www.bacterialsourcetracking.com/
(Dr. Mansour Samadpour, Institute of Environmental Health, Lake Forest Park, WA)

http://www.sccwrp.org/tools/workshops/source_tracking_agenda.html
(U.S EPA Workshop on Microbial Source Tracking, 2002)

http://www.usm.edu/bst/
(Microbial source tracking: University of Southern Mississippi)

http://www.cas.usf.edu/biology/Faculty/harwood.html
(Dr. Valerie Harwood, University of South Florida)

http://www.wef.org/pdffiles/TMDL/McClellan.pdf#search='microbial%20source%20tracking'
(Maptech, Inc., Slide Presentation on MST)

http://sun.science.wayne.edu/~jram/MGLPF-MSTProject.htm
(Dr. Jeffrey Ram, Wayne State University)

http://soils1.cses.vt.edu/ch/biol_4684/bst/BST.html
(MST website, Virginia Tech)

http://www.vetmed.wsu.edu/research_vmp/MicroArrayLab/Webpages/MST.asp
(MST, Washington State University)

http://www.forester.net/sw_0105_detecting.html
(Detecting bacteria in coastal waters; Stormwater journal)

http://dmsylvia.ifas.ufl.edu/msp/Ribotyping.pdf

(Ribotyping protocol)

Table 1. Two-way classification of some of the more widely used source tracking methods[1]

	Library-dependent Methods	Library-independent Methods
Genotypic	Ribotyping	F+ coliphages (FRNA & FDNA phage)
	Bacterial community fingerprinting	Direct pathogen detection (PCR, RT-PCR)
	Rep-PCR[2]	*Bacteroides* genotyping
	PFGE[2]	Enterotoxin biomarkers
	Mitochondrial DNA	
Phenotypic	Antibiotic resistance analysis (ARA)	*Bifidobacterium*
		Phage infecting B. fragilis
	Carbon source profiling (CUP)	F+ coliphage serotyping

[1]adapted from Bernstein, B.B., J.F. Griffith, and S.B. Weisberg. 2002. Summary of proceedings. In: *Microbial Source Tracking Workshop*. See www.sccwrp.org/tools/workshops/source_tracking_workshop.html

[2]rep-PCR = Repetitive Extragenic Palindromic Element- PCR; PFGE =Pulsed field gel electrophoresis

Table 2: Method Evaluation Criteria Adopted by Participants in EPA Workshop[1]

Category of Criteria	Specific Evaluation Criteria
Tier 1: Measurement reliability	Reproducibility of results within and across laboratories
	Classification accuracy of isolates (for library-dependent methods)
	Confidence that an identified indicator is from the presumed source (for library-independent methos)
	Discrimination power (i.e., level of resolution)
	Matrix stability
	Geographical stability
	Temporal stability
	Confirmation by peer review
Tier 2: Management relevance	Relationship to actual source of contamination
	Relationship to public health outcomes
	Relationship to commonly used water quality indicators
	Ease of communication to the public
	Ease of communication to management audiences
Tier 3: Costs and logistics	Equipment and laboratory facilities required
	Training required
	Library size required
	Library development efforts
	Implementation time
	Cost of ensuring results are legally legally defensible
	Cost per sample
	Sample turnaround time

[1]from Bernstein, B.B., J.F. Griffith, and S.B. Weisberg. 2002. Summary of proceedings. In: *Microbial Source Tracking Workshop*. See www.sccwrp.org/tools/workshops/source_tracking_workshop.html

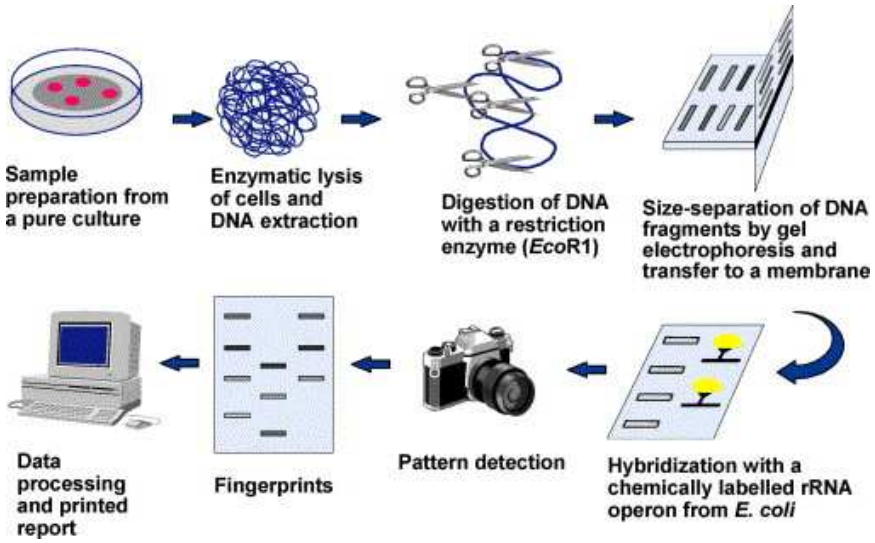

Figure 1: Ribotyping Procedure

From: Aarnisalo, T.J. Autio, J.M. Lunden, M.H. Saarela, H.J. Korkeala and M.L. Suihko, Subtyping of *Listeria monocytogenes* isolates from food industry with an automated riboprinter microbial characterization system and pulse field gel electrophoresis (PFGE), *VTT Biotechnology*, VTT Technical Research Centre of Finland (1999).

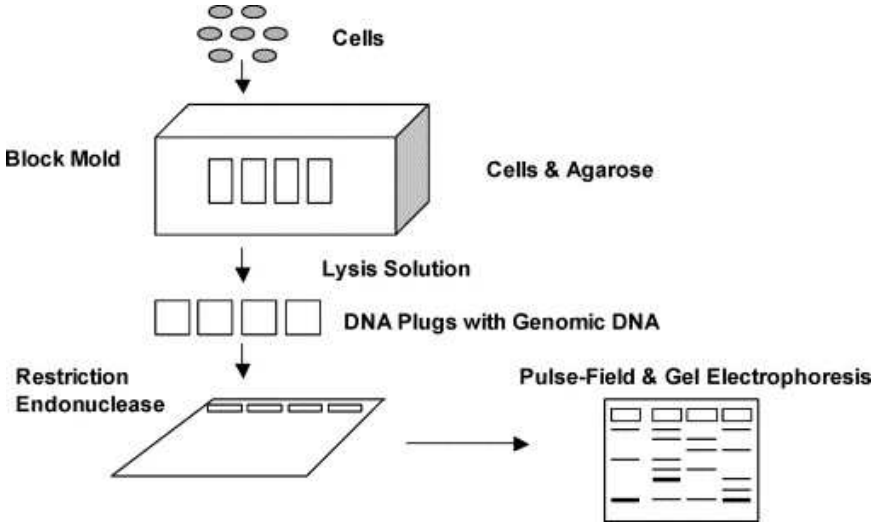

Figure 2: Pulse-Field Gel Electrophoresis (PFGE) Procedure

From: J.M. Farber, An Introduction to the hows and whys of molecular typing, *Journal of Food Protection* **59** (1996) (10), pp. 1091–1101.