Bacterial Markers for Fecal Pollution Detection

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The goals of fecal pollution detection include fecal waste source tracking and identifying the presence of pathogens, therefore assessing potential health risks.

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1. Bacterial Markers

One major purpose of fecal pollution detection is to identify the presence of pathogens to identify the fecal waste source and potential health risks. With the realization that waterborne transmission of bacterial diseases was a public health concern in the 18th century, governments established sanitary systems to monitor drinking water safety and build water treatment facilities [1]. A question arises—should one monitor waterborne pathogens directly or employ proxy indicators? There are many bacterial, protozoan, and viral enteric pathogens that can cause diseases. Their methods of detection are often laborious, time-consuming, and require specialized expertise and biosafe laboratory settings. Pathogens in environmental water are sporadic and sparse, making it difficult or costly to measure these pathogens [2]. In addition, waterborne pathogens in the environment are less dangerous at low concentrations compared to those in GI tracts, but their laboratory enumeration often involves concentration from large volumes of water samples; therefore, these concentrated pathogens may cause severe human diseases during their measurement [3][4].

Water quality monitoring to detect fecal pollution can employ biological fecal indicators to proxy many potential pathogens. A typical fecal indicator should fulfill the following criteria [3][2][5]:

(1) It should be detected only in feces-polluted environments and should not be detected in non-fecal-polluted environments; i.e., it should have a high association with fecal pollution with little or no background noise;
(2) It should be detectable in the same host as the pathogens, and its abundance should reflect pathogen abundance both inside and outside the host; i.e., it should have tight correlations with pathogens/diseases;
(3) It should be detectable in the host intestine, feces, and also fecal-polluted environmental waters so that it can be assessed using inexpensive and simple methods; i.e., it should have good detection sensitivity;
(4) It should not multiply outside the host intestine but should be viable or in a detectable form for some time comparable to fecal pathogens;
(5) It should be safe to measure for field and lab workers, not causing severe human diseases.The value of common fecal indicator bacteria (FIB) is that they occur widely in the feces of birds, mammals, and humans, posing a robust way to detect fecal pollution. However, lack of host specificity is also a problem when determining the specific source of fecal pollution, i.e., fecal pollution source tracking. Advances in the field of microbial source tracking have now provided new fecal indicators that can be used to identify specific fecal pollution sources (e.g., human sewage). If a proxy is used to detect human fecal pollution, ideally it should be exclusively of human sources and not be found in animals. Here researchers introduce well-validated bacterial, viral and molecular indicators, emphasizing their association with pathogens, their presence in fecal wastes, their host specificity, their persistence in the environment, and their pathogenicity.

Host specificity is one of the most critical criteria for any fecal indicator because it determines whether a given fecal indicator can be used to identify the specific sources of fecal pollution, i.e., fecal source tracking [6]. Accurate source tracking helps water quality regulators to locate pollution sources, such as fecal pollution from a nearby WWTP or an animal farm. Host specificity can be readily assessed by the proportion of the false-positive results detected in nontarget fecal pollution sources [7]. Fecal indicators with absolute human specificity must be detected only in humans and must not be detectable in any animals, whereas those of poor human specificity may also be detected in animals, plants, or environments without association with fecal pollution. Livestock in a farm/slaughterhouse [8][9] and wild animals in forests/natural reserves [10] can also be sources of fecal pollution and pathogens. Thus, fecal indicators specific to one animal host are worth noticing because they help identify the species of animal hosts and the possible source locations.

Sensitivity is another critical criterion which is evaluated by the positive proportions in target fecal pollution sources. Fecal indicators of perfect sensitivity should be detectable in all target fecal pollution sources, whereas those of poor sensitivity may be detectable in a minor proportion of target fecal pollution sources in situations where pollution is high. Sensitivity measurement is, of course, affected by fecal indicator concentrations. Even fecal indicators of high sensitivity may be undetectable in target samples if they are diluted or have undergone significant losses through sample processing steps, causing false negatives.

2. Coliforms

Coliform bacteria, or total coliforms or simply coliforms, are a group of Gram-negative, facultatively anaerobic, non-spore-forming and rod-shaped bacteria that can ferment lactose to acid and gas at ~37 °C within 48 h, constituting ~10% of gut microorganisms [11]. Traditionally, total coliforms included four closely related genera within the family Enterobacteriaceae: *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter* [12]. But today, some species of over 19 bacterial genera (e.g., *Hafnia*, *Serratia* and *Raoultella*) can be classified as coliforms based on the phenotypic characteristics above [13]. Total coliforms are used to evaluate water treatment efficacy in fecal wastes removal and assess the integrity of water distribution systems [14] because they mainly originate from the GI tracts of humans and warm-blooded animals (i.e., mammals and birds), and thus, their presence in water can indicate possible fecal pollution. In 1975, the U.S. EPA issued the National Primary Drinking Water Regulations, which allowed a maximum of 5% drinking water samples positive for total coliforms tested in a month [15]. Then, in 1989, the U.S. EPA published the first Total Coliform Rule, which supplemented a Maximum Contaminant Level Goal of zero total coliforms in nationwide drinking waters [16]. To date, total coliforms are still routinely monitored in drinking water.

The recognition of free-living coliforms in non-enteric environments, such as freshwater, soil and vegetation, that do not have a history associated with fecal pollution [17][18] has cast doubt on the exclusive association between total coliforms and fecal pollution. For example, *Klebsiella variicola* is a member of total coliforms but is typically a plant endosymbiont [18]. Thus, not all members of total coliforms are suitable indicators of fecal pollution, and presence of total coliforms in drinking water does not necessarily indicate fecal pollution [14]. To overcome this issue, fecal coliforms, which are defined as coliforms originating from the intestines of warm-blooded animals capable of fermenting lactose at 44.5–45.5 °C, are proposed as a monitoring target [19]. As a subgroup of total coliforms, fecal coliforms have better associations with fecal pollution because the non-fecal-origin part is excluded [20]. Fecal coliforms detected in aquafarm harvesting areas such as an oyster bed are indicative of fecal pollution, causing ecological issues and economic losses [21]. Note that neither total coliform nor fecal coliform is a natural taxon but a method-based bacteria category for convenience. Researchers now understand that even within the category of fecal coliforms, some are not exclusively of fecal origin, hence the transition to *Escherichia coli* as a better fecal indicator. Among fecal coliforms, *E. coli* is the dominant species isolated from water (>95%) and is the most frequently used indicator [22].

Since coliforms can be cultured with ease, there are culture-based and culture-independent methods to detect and enumerate coliforms in water. Frequently used culture-based enumeration methods include the (i) classical plate count on violet-red-bile-lactose agar medium [23] or chromogenic media based on the activities of β-D-galactosidase (of coliforms) and β-glucuronidase (of *E. coli*) [24], (ii) most probable number procedure in lauryl tryptose broth [25] and IDEXX systems, (iii) membrane filtration in selective M-Endo medium [26] and (iv) lateral-flow immune-chromatographic assay using antibodies recognizing coliform genera [27]. Culture-independent methods include (i) real-time PCR targeting the *lacZ* gene of coliforms [28][29], (ii) fluorescence-based DNA microarray probing *E. coli* [30], (iii) flow cytometry [31] and (iv) next-generation sequencing or metagenomics that profile all microorganisms, including total/fecal coliforms [32].

Except for a few strains such as *E. coli* O157:H7, fecal coliforms are generally not considered to be pathogenic. However, drinking water free of coliforms or not exceeding coliform regulatory thresholds is not necessarily safe to drink. Seven waterborne disease outbreaks, affecting more than 3000 people across 5 American states between 1991 and 1992, occurred in drinking water not exceeding the 1989 Maximum Contaminant Levels [33]. Protozoal parasites *Giardia* and *Cryptosporidium* were identified as the etiological agents. Therefore, coliforms should not be the sole indicator for successful drinking water quality assessment.

3. *Escherichia coli*

Escherichia coli is used as a fecal indicator as part of drinking water regulations [1]. The U.S. EPA added *E. coli* to the water monitoring and assessment list in 1985 [34]. According to an early investigation by the U.S. EPA, *E. coli* has the highest correlation to swimming-associated diseases at freshwater beaches on the east coast of the U.S. [35]. First, it shows a strong association with fecal pollution, as it is found in all feces of warm-blooded animals and a few reptiles at

high concentrations [1][36], and remains at detectable levels in waters subject to animal fecal pollution, such as sewage, rivers, lakes, groundwater and coastal areas [37][38]. In addition, *E. coli* is a facultative anaerobe and is easy to culture, so it can be readily measured using both culture-based and culture-independent methods. Second, *E. coli* is highly associated with a fecal origin and is less likely to be present in natural environments free of animal feces. It was believed to be of exclusively fecal origin when selected as a fecal indicator, which, however, is no longer accurate with the recognition of “naturalized” *E. coli*, which persists and may replicate in non-enteric/non-fecal environments such as soil, sediment, beaches and aquatic plants [39][40]. “Naturalized” *E. coli* may not come from a recent fecal input, though it is still believed to have diverged from *E. coli* of fecal origins.

It is important to distinguish between fecal pollution caused by human wastes and that caused by animals, because human fecal pollution is generally associated with high health risks from human-specific pathogens and high loads of antibiotics and ARBs. One major limitation of *E. coli* as a fecal indicator lies in its poor specificity to human feces. Despite the substantial genetic diversity of *E. coli* fecal isolates, epidemiological studies have identified weak associations between a particular *E. coli* clone and a given host [41][42]. For example, a B1 subgroup clone that exhibits the *hly* virulence gene is unique to animals [41], and a B2 subgroup VIII, O81 serotype clone is specific to humans [43]. *E. coli* strains showing stringent host specificity may be specialized candidates for fecal source tracking indicators. Still, most *E. coli* strains discovered in human fecal samples are not specific to humans, causing difficulties in fecal pollution source tracking.

E. coli was initially believed to have poor survival and to be unlikely to multiply after being released into the environment through deposition of fecal wastes because of the environment being so different from animal gastrointestinal tracts and because of environmental stresses such as insufficient organic matter [44], sunlight inactivation and high salinity [45]. However, prolonged *E. coli* survival can be found at low temperatures [46], and enhanced *E. coli* replication is seen at higher temperatures, where relatively sufficient nutrients are likely to support its regrowth [47]. Lake sediments rich in organic contents also favor the survival of *E. coli* at lower temperatures [48]. When grown as biofilms, *E. coli* has a significantly higher tolerance against chlorine than planktonic *E. coli* [49]. In conclusion, the persistence of *E. coli* is greatly affected by environmental conditions.

Changes in climate, such as an increase in temperature and rainfall, may cause fluctuations of *E. coli* counts in waters [50][51][52]. A rainfall event can result in up to a three-fold increase of *E. coli* counts in recreational waters compared to before rainfall, and this increase can persist for up to 12 h [52][53]. After a rainfall event, increases in *E. coli* counts may occur due to the “first flush” from stormwater drainage or agricultural waste runoff coming along with the flow [53][54]. Furthermore, bacteria including *E. coli* tend to form flocs, adhere to rocks and other particles, then settle down to the sediment of freshwater ecosystems. A heavy rain or storm event may resuspend these bacterial cells from sediments, leading to a 30–37 times higher bacterial load in water [55][56]. Thus, climate and meteorological parameters should be monitored before and during water sample collection for *E. coli* testing to help interpret data.

Most strains of *E. coli* are commensal or symbiotic to their animal hosts in the gastrointestinal tracks [57]. For example, indigenous gut *E. coli* strains can be highly competitive for proline, and their depletion of proline helps inhibit the colonization of pathogenic *E. coli* strain O157:H7 [58]. These non-pathogenic strains are considered safe fecal indicators (categorized as biosafety Level I organisms in the U.S.) and can be enumerated by similar methods, as described for coliforms in environmental waters. In addition, there are specialized methods that only detect certain *E. coli* strains, such as ATCC 47,076, in drinking water [59]. Pathogenic *E. coli* strains, including the aforementioned O157, a Shiga toxin-producing *E. coli* (STEC) serotype categorized as a biosafety Level II organism, caused 5441 infections across the United States in 1996 [60]. The previous study [61] identified a nine base-pair conserved DNA sequence insertion located in the *ybiX* gene that is unique to O157 strains and can be used for O157 quantification by qPCR. Pathogenic *E. coli* strains may have better correlations with diseases, and therefore, their enumeration can be a means to assess potential health risks to the public.

4. Enterococcus

Enterococci share traits with *E. coli* that are useful for environmental monitoring. First, they both are facultative anaerobic bacteria detected abundantly in the GI tracts of humans and many animals and are shed in feces at high concentrations. Furthermore, both are easy to culture, both have generally low pathogenicity (at Biosafety Level 1, except for a few species), both have abundances highly associated with GI diseases, and both are subsequently common indicators for water quality monitoring, fecal pollution detection and health risk assessment by culture-based and culture-independent methods [1]. Apart from in humans, enterococci have been detected in insects (e.g., drosophila), domestic animals (e.g.,

cattle and cats), plants (e.g., forage crops) [62][63][64], as well as non-enteric environments (e.g., soil and beach sand) [1]. These discoveries indicated a limited host specificity of enterococci and possible origins other than feces.

Both *E. coli* and enterococci have long been used as FIB because of their association with waterborne diseases [65]. Two epidemiological studies recognized enterococci as good indicators of GI diseases among swimmers at both freshwater and marine beaches [66][67]. Enterococci have been the sole recommended FIB by U.S. EPA for marine waters since 1986 [68] because they have higher survival in salt water than coliforms (including *E. coli*) and are thought to be more tightly associated with human pathogens [69][70]. However, using enterococci as fecal indicators has been criticized as well because enterococci are not exclusively of fecal origin and have limited host specificity, as aforementioned. These characteristics may hinder accurate fecal pollution source tracking because non-fecal-origin enterococci may not be associated with health risks, and animal-specific enterococci-associated pathogens may have variable survival strategies in the environment and health risks from human-specific pathogens [71].

The persistence of enterococci in the non-enteric environment is robust, as enterococci can survive across a wide range of temperatures (from 10 to 45 °C) [1], are intrinsically resistant to multiple antibiotics (e.g., ampicillin, cephalosporin and kanamycin) [72], and can survive high salinity (6.5% *w/w* NaCl) and alkaline (up to pH 10) [73]. *Enterococcus faecium* and *Enterococcus faecalis* are generally the two most abundant species of *Enterococcus* found in human fecal microbiota [74]. They generally have a longer persistence in marine water than *E. coli* [75][76]. They tend to adhere to biotic or abiotic surfaces to form biofilms, protecting them from environmental stresses such as temperature fluctuations, UV radiation, predation, and wave action [77]. Heavy rain or a storm can disrupt these biofilms and release the enterococci into the water column [78]. In environmental waters, adverse conditions, such as solar radiation, starvation, and low temperatures, may decrease enterococci culturability and induce them to enter the VBNC state [79][80]. VBNC cells are unlikely to be detected via culture-based methods.

Epidemiological studies support an association between enterococci from human fecal sources (e.g., urban sewage) and waterborne diseases in recreational waters [66][81][82]. Higher enterococci densities are associated with an increasing occurrence of GI illnesses among beachgoers at beaches impacted by treated sewage effluent [66]. In addition, human pathogens such as *Giardia* and *Salmonella* are found in higher densities and positively correlated with enterococci concentrations in recreational waters impacted by sewage effluent [81][82]. Along with human fecal pollution, exposure to animal feces poses health threats. Globally, one-third of childrens' deaths due to GI illnesses in 2015 were caused by pathogens that are found in animal feces [83]. It is predicted that enterococci densities as low as 35 CFUs/100 mL from cattle manure pose health risks [71]. Thus, enterococci levels are indicative of general health risks from both human and animal fecal pollution. However, without associated source tracking information, it is difficult to unambiguously identify the fecal hosts/sources of enterococci.

5. *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, spore-forming, biofilm-forming, anaerobic and pathogenic species (at biosafety Level II) associated with feces [84]. It is among the first fecal indicators in aquatic environments [85]. *C. perfringens* can be found in the gut of fish and warm-blooded animals, such as pigs and ducks [86][87][88], as well as in some non-enteric environments, such as soil, streams and marine sediments [89][90]. Despite having limited host specificity, *C. perfringens* can be present in higher abundances in human feces than animal feces. As a pathogenic species, its presence itself indicates possible health risks to humans and animals.

Species of the genus *Clostridium* are obligate anaerobes and cannot multiply in environmental waters. Spores of *C. perfringens* are coated by a thick peptidoglycan cortex structure that provides resistance against environmental stresses such as chlorination, heat and UV radiation and resistance against toxins in industrial wastes [91]; thus, *C. perfringens* spores can survive longer in polluted waters than other FIB, especially in freshwaters [91][92]. The inability to multiply in non-enteric environments and the prolonged environmental viability of its spores make *C. perfringens* a better indicator of long-term or accumulative fecal pollution. Criticisms against the usage of *C. perfringens* also focus on its prolonged viability and wide distribution in aquatic environments. Its spores can be detected in places far from the pollution input site, indicative of either remote or old fecal pollution [93]. Besides, its abundance does not always correlate with abundances of other FIB [89]. Hence, combined usage of *C. perfringens* and other FIB may better predict pathogens.

Detection and quantification of *C. perfringens* can be conducted by culture-based and culture-independent methods. It can be isolated from water samples by the membrane filtration and Rapid Fung double tube methods [94], then cultivated in anaerobic chambers at 44–46 °C [95]. Available media include mCP medium, tryptose-sulfite-cycloserine agar, and tryptone-sulfite-neomycin agar [96][97]. Rapid and culture-independent enumeration methods include qPCR targeting the

cpn60 gene [98], DNA sequencing targeting 16S rDNA genes or the whole genome, and the newly developed Saltatory Rolling Circle Amplification targeting the *cpa* gene, which is conducted at a constant temperature and does not require a thermocycler or gel electrophoresis [99].

6. Bacteroides

Species of genus *Bacteroides* are frequently used fecal indicators for source tracking purposes. They are low-pathogenic indicators (biosafety Level I) with promising host specificity compared to facultative anaerobic FIB [100]. *Bacteroides* are obligate anaerobes of gut origins, unlikely to multiply in a non-enteric environment, and are present at significantly higher densities in the GI tracts and feces of humans than coliforms or enterococci [7]. *Bacteroides* can be found in warm-blooded animal hosts such as humans, dogs, pigs, and also fish, which are cold-blooded [101][102][103]. Unlike coliforms or enterococci, *Bacteroides* spp. isolated from humans and some animal hosts are unique to one or a group of hosts [104].

Unlike facultative anaerobic FIB, the isolation and cultivation of *Bacteroides* cells require anoxic chambers, which is costly, time-consuming and labor-intensive [105]. *Bacteroides* can grow at 37 °C in many selective media, such as Brain Heart Infusion medium, Bacteroides Bile Esculin agar, and Blood Hemin Vitamin K medium containing antibiotics [105][106][107]. In most applications, *Bacteroides* grows into visible colonies on plates within 24 h, and colonies will be large enough to enumerate in 36 and 48 h. Some *Bacteroides* species can be identified to the species level within four hours by a few biochemical tests, such as the API ZYM system that detects activities of *Bacteroides*-specific enzymes [108].

The use of biological molecular markers bypasses the difficulties of anaerobic cultivation and enables the routine detection of *Bacteroides* spp. The aforementioned host-specific 16S rDNA markers can be used to detect and quantify *Bacteroides* in environmental samples and thus enable source tracking of the fecal pollution. Among human-specific *Bacteroides* 16S rDNA sequences, the region to be amplified by the primer set HF183F (Human Fecal 183 forward primer) and Bac708R (reverse primer) ranks among the most recognized markers to assess human fecal pollution and to discriminate it from animal fecal pollution because of its high sensitivity and specificity to humans [109]. Although HF183 fluorescent probe-based assays may sometimes cross-react with animal feces, its concentration in human feces can be 1000-fold more, or higher, than in animal feces [110]. In 2012, the U.S. EPA recommended an illness-based recreational water quality threshold of 32 illnesses per 1000 primary contact recreators using enterococci or *E. coli* [68]. In 2020, as *Bacteroides* HF183 becomes widely used, a threshold of 525 HF183 copies/100 mL recreational water was proposed by Boehm and Soller [111]. Apart from 16S rDNA sequences, there are other *Bacteroides* human molecular markers, such as genes encoding a hypothetical protein of *Bacteroidales*-like cell surfaces [112], which showed a >97% human specificity.

Obligate anaerobes such as *Bacteroides* cannot grow or multiply in environmental waters and have shorter survival periods than aerobes, facultative anaerobes, or viruses [7]. This property can provide a time progression of fecal pollution in environmental waters after *Bacteroides* spp. are released [113]. Molecular markers of both viable and non-viable cells can be detected using qPCR and DNA sequencing, so that they are less affected by cell states [114]. For example, the portion of culturable enterococci and *E. coli* in unfiltered freshwater can be reduced by 90% within 1.2 h and 1.4 h in sunlight, whereas it takes more than 6 h for the same reduction for HF183 and human polyomavirus [110].

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