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Review

Affordable Enteric Virus Detection Techniques Are Needed to Support Changing Paradigms in Water Quality Management

In light of water quality monitoring paradigms shifting to a more holistic approach, it is essential that environmental microbiologists embrace new methodological developments in clinical virology to create rapid, laboratory-free methods for the identification of wastewater pollution. It is widely accepted that routine monitoring of fecal indicator bacteria (FIB) does not adequately reflect human health risks associated with fecal pollution, especially risks posed by viruses. Enteric viruses are typically more resistant to wastewater treatment and persist longer in the environment than FIB. Furthermore, enteric viruses often have extremely low infectious doses. Currently, the incorporation of sanitary surveys, short-term monitoring of reference pathogens, exploratory quantitative microbial risk assessments, and predictive ecological models is being championed as the preferred approach to water management. In addition to improved virus concentration methods, simple, point-of-use tests for enteric viruses and/or improved viral indicators are needed to complement this emerging paradigm and ensure microbial safety worldwide.

Keywords: Fecal indicator bacteria; Fecal pollution; Immunostrip tests; Quantitative microbial risk assessment; Waterborne pathogens

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1 Introduction

1.1 Microbial diversity of wastewater

Domestic wastewater, raw or with treatment, is a complex, patchy matrix of organic material containing a diverse microbial community of bacteria, archaea, viruses, fungi, and parasites [1]. These microbes, which result from pathogen shedding due to active infection, host excretion of native gut flora, and passive transport of dietary microbes, vary in composition and concentrations based on geographic location, pathogen epidemiology, and animal host diversity [1–4]. Metagenomic surveys of viral communities exemplify the high diversity and varying origins of microbes in wastewater, with sequences from over 50 different viral families recovered, as well as numerous sequences too divergent to classify [2, 5]. DNA metagenomes from wastewater are dominated by unknown sequences and bacteriophage sequences that likely represent viruses infecting gut bacteria, while RNA metagenomes contain eukaryotic viruses infecting plants (*Virgaviridae* family; likely of dietary origin), followed by viruses infecting humans, insects, and rats. While

wastewater is dominated by microbes that are not pathogenic to humans, there are many types of viruses, bacteria, protozoan parasites, and helminth worms that can be transmitted via the fecal-oral route, are waterborne, and cause human infections of varying severity and symptoms (e.g. respiratory, encephalitis, gastroenteritis, hepatitis, dermatitis) (Fig. 1A; [1, 3, 4, 6]).

1.2 Human pathogens in wastewater

Presently, norovirus and rotavirus (viruses), *Salmonella* and *Campylobacter* (bacteria), *Ascaris* (helminth), and *Cryptosporidium* (unicellular parasite) are among the most prevalent and widespread known human/zoonotic enteric pathogens [1, 3, 4, 6]. However, the emergence of additional waterborne pathogens (e.g. microsporidia, mycobacteria, parvoviruses, coronaviruses, picornaviruses) is of growing concern [7, 8]. The frequent lack of an identifiable etiological agent of gastrointestinal (GI) illness (reviewed in [9]) and the prevalence of novel viruses dominating sewage [10, 11] further highlights the threat of additional pathogens present in wastewater to public health and economic productivity. Given the high incidence and concentration of pathogens in feces and wastewater, it is not surprising that the World Health Organization (WHO) recognizes acute gastroenteritis as one of the leading causes of human morbidity and mortality worldwide [12]. Furthermore, it is estimated that enteric viruses, particularly norovirus and rotavirus, are largely responsible for these infections and deaths due to their resistance to typical methods of disinfection, low infectious dose, and long persistence in the environment and on

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Abbreviations: FIB, fecal indicator bacteria; GI, gastrointestinal; NASBA, nucleic acid sequence-based amplification; PCR, polymerase chain reaction; QMRA, quantitative microbial risk assessment; qPCR, quantitative PCR; WHO, World Health Organization

Figure 1. (A) The main sources of fecal pollution into surface waters include feces and shedding by wild and domestic animals, domestic wastewater, and direct shedding during human recreation. While the pathogens associated with wastewater and fecal pollution decay over time, FIB can survive and replicate in the environment. (B) The traditional monitoring approach for recreational water management depends upon routine monitoring of FIB using culture-based methods and subsequent beach closures when FIB concentrations exceed allowable limits. (C) The emerging holistic approach to recreational water management employs a combination of tools to make risk-based management decisions.

fomites [3, 4, 7, 13]. Since as few as ~18 viral particles can cause infection, an individual infected with norovirus can excrete up to five billion infectious doses (e.g. 10^5 – 10^{11} viral copies/g feces) into wastewater [13]. Given the high impact of enteric viruses on public health, this commentary will focus on the relation of enteric viruses to current and emerging paradigms in water quality monitoring, current methods used for enteric virus detection, and the growing need for methodological improvements (Fig. 1).

2 Water quality monitoring

2.1 Traditional monitoring approach with FIB

The main sources of fecal pollution into surface waters include feces and shedding by wild and domestic animals, domestic wastewater, and direct shedding during human recreation (Fig. 1A). According to the WHO, improvements in water management could alleviate 10% of the worldwide disease burden, resulting in net savings of approximately US\$ 72.7 billion each year until Millennium Development Goals for improved sanitation and drinking water are met [10]. Although developing countries suffer disproportionately from enteric pathogen-associated mortality and morbidity, illness due to enteric infections still has significant impacts on health and socioeconomics in developed countries with advanced wastewater and drinking water treatment [12, 14]. Therefore, a primary, longstanding goal for protecting human health worldwide is accurate identification of the presence of wastewater pollution and prediction of risks to the public associated with contaminated drinking, shellfish harvesting, and recreational waters. Since the early 1900s, non-pathogenic enteric bacteria (FIB; e.g. *Enterococcus*, fecal coliforms, and *Escherichia coli*) have been used throughout the world as indicators for the presence of enteric pathogens and have been utilized for subsequent prediction of human health risk due to

microbial pathogens (Fig. 1B; [14–17]). The traditional monitoring approach for recreational water management depends upon routine monitoring of FIB using culture-based methods and subsequent beach closures when FIB concentrations exceed allowable limits. FIB concentrations are also used to determine microbial quality of both treated and untreated wastewater and to calculate the associated health risks associated with reuse in agriculture [1]. For example, the 2006 WHO guidelines suggest using quantitative microbial risk assessments (QMRA), with an assumed ratio of 0.1–1 human norovirus or rotavirus per 10^5 *E. coli*, to calculate human health risk of viral infection from wastewater reuse in agriculture. Although this method is known to be flawed (see below), FIB are still widely used as an indicator of enteric pathogens and human health risks due to their consistent presence in wastewater and the readily available, low cost, culture-based methods for detection that require minimal laboratory training [14–17].

2.2 Shortcomings of the current FIB paradigm

Despite their convenience and widespread use, it has been recognized since 1979 that FIB do not consistently correlate with the presence of enteric pathogens, particularly those that are not bacteria, or with human health risks in any water matrix (Table 1; [14–20]). Even with the introduction of quantitative PCR (qPCR) to measure FIB, the lack of a consistent correlation between FIB and human health risks continues to hold true in the majority of studies. One exception is a recent study that demonstrated a correlation between qPCR-derived *Enterococcus* concentrations and the incidence of GI illness due to recreational activities at a beach exposed to point-source wastewater pollution [18]. The general lack of correlation between FIB and enteric viruses is not surprising due to differences in stability and persistence of these microbes. In comparison to enteric viruses, FIB are more susceptible to wastewater and drinking

Table 1. The advantages and disadvantages of the traditional monitoring and emerging holistic recreational water management approaches

	Advantage	Disadvantage
Traditional routine monitoring of FIB	<ul style="list-style-type: none"> • Relatively simple lab tests • Able to identify contamination due to infrastructure failure 	<ul style="list-style-type: none"> • FIB do not consistently correlate with the presence of all pathogens types and health risks • FIB are excreted by other animals; thus, they do not always represent human contamination • FIB replicate in the environment post-contamination causing high incidence of false-positive beach closures • Culture-based FIB quantification does not provide same day results
Emerging holistic water management approach	<ul style="list-style-type: none"> • Alleviates need for routine monitoring • Water management and policy are based upon minimizing health risks • More cost effective than traditional monitoring 	<ul style="list-style-type: none"> • Relies solely on predictive modeling and QMRA to mitigate health risks; thus, could fail to identify contamination due to unexpected infrastructure failures • Creation of effective QMRA and modeling is dependent upon sufficient data derived from expensive and sophisticated molecular methods

water treatment, are excreted by all warm-blooded animals, and have higher die-off rates in the environment, even though they can replicate and persist in the sediment after a contamination event (Fig. 1A). Drinking water, shellfish harvesting areas, recreational water, and wastewater designated for reuse that are considered safe based upon FIB concentrations can actually contain high concentrations of human enteric viruses [10, 15–17, 20, 21]. Furthermore, FIB-based QMRAs for wastewater reuse have been shown to greatly underestimate the risk of norovirus illness; in one study, actual norovirus concentrations were 1000-fold greater than those predicted by the FIB-norovirus ratio [21]. The lack of an indicator to encompass all sources of fecal pollution and the heterogeneous distribution of FIB as well as their highly variable ratio with non-bacterial pathogens in wastewater and environmental waters has significantly hindered improvements in microbial safety related to drinking water, wastewater reuse, shellfish consumption, and recreational waters [14, 15, 22].

2.3 Emerging holistic management approach

As a result of the inadequacy of FIB monitoring to determine human health risks associated with enteric pathogens, particularly viruses, alternative approaches to traditional microbial quality monitoring have been recommended (Fig. 1C). These holistic approaches incorporate sanitary surveys that inform water quality studies, which directly quantify reference pathogens (e.g. norovirus, rotavirus) and drive exploratory QMRA and subsequent management decisions [6, 15, 19, 21, 23, 24]. Three main lines of evidence suggest that this approach will improve water quality monitoring efforts. First, the power of incorporating actual enteric virus measurements into exploratory QMRAs with multiple scenarios for driving site-specific microbial safety guidelines for recreational waters was recently demonstrated [15]. Similarly, the need to quantify enteric virus concentrations and resulting health risks was also highlighted to improve QMRAs for wastewater reuse and consequent public health guidelines [21]. Finally, the utility of incorporating specific enteric viruses and/or a viral indicator to identify wastewater pollution/poor microbial quality and to better predict human health risks related to wastewater exposure has been demonstrated throughout the world [14–16, 22, 24, 25]. Furthermore, improvements in microbial safety depend on improved treatment processes, enteric pathogen modeling, and QMRAs that directly measure reference enteric viruses and/or an improved viral

indicator instead of relying upon FIB-to-model pathogen ratios [3, 15, 19–21, 23, 24].

3 Viral indicators of fecal pollution

3.1 Proposed viral indicators

Due to the hypervariability of environmental persistence and epidemiology of individual enteric viruses, it is unlikely that a single viral indicator will be sufficient to represent all enteric viruses [7, 14, 22, 25]. Despite this challenge, viruses from the families *Adenoviridae*, *Caliciviridae*, *Picornaviridae*, and *Reoviridae* as well as the genera *Anellovirus*, *Picobirnavirus*, and *Polyomavirus* have been incorporated into water quality studies throughout the world [14, 16, 26]. Additionally, several different bacteriophages (viruses that infect bacteria), particularly F-specific RNA coliphages, have been proposed as indicators for enteric viruses (reviewed in [22]); however, none reliably correlate with the presence of enteric viruses in the environment or throughout disinfection processes [16, 17, 20, 25]. The fairly consistent, relatively high concentrations of adenoviruses and polyomaviruses in wastewater make them possible indicator viruses (Table 1); however, their low concentrations in contaminated environments still present methodological difficulties for detection [3, 14, 16]. Metagenomic studies have shown the dominance of plant viruses (family *Virgaviridae*) in human feces and wastewater [2, 5, 11, 27]. Consequently, the use of a pepper plant pathogen, pepper mild mottle virus, has also been proposed as an alternative enteric virus indicator due to its high concentration in wastewater (average $\geq 10^6$ particles/mL) and dietary (i.e., human infection-independent) origin in feces [14, 25, 26, 28]. Further research is needed to understand its correlation to infectious enteric viruses throughout different geographic regions, disinfection processes, and contamination scenarios.

3.2 Emerging molecular methods for virus detection

Conventional methods for the detection and/or quantification of enteric viruses in environmental matrices are culture- or molecular-based and involve two key steps, virus concentration and target detection [3, 9, 26]. A variety of virus concentration techniques are available, optimized for different environmental matrices, which take advantage of the physiochemical properties of viruses (e.g.

adsorption/elution) and/or utilize particle size separation (e.g. filtration). The efficiency of virus isolation and concentration ranges widely, with values from 5 to 92% reported [26]. While culture-based methods can determine the concentration of infectious enteric viruses, they are expensive, laboratory intensive, yield delayed results, and fail to measure many waterborne viruses of concern (e.g. human norovirus, which has not yet been obtained in cell culture) [3, 26]. For these reasons, molecular methods, particularly those that are quantitative, have become increasingly popular (Fig. 1C). The most common methods are amplification based and include: (reverse transcription) (RT-) qPCR and nucleic acid sequence-based amplification (NASBA) [29].

3.3 Current methodological limitations

Since exposure to just a few virus particles (e.g. norovirus) can cause illness, virus detection methods must be sensitive enough to detect low concentrations in the environment [3, 15, 16, 24, 29]. Given that an appropriate enteric virus indicator has not been identified to date, investigators rely largely on pathogen-specific molecular assays [14, 15, 24]. However, poor method sensitivity is one the major limitations associated with using a human pathogen as an indicator for enteric viruses. Improvements in virus concentration methods or the use of an indicator that is found in higher concentrations (e.g. pepper mild mottle virus) could alleviate these problems. Other limitations of qPCR and NASBA include laboratory time, expense of equipment and reagents, co-concentration of inhibitors (e.g. humics, organics), target virus selection, primer specificity, proper standards and controls. Standards exist for the correct interpretation of qPCR results; however, these guidelines are not widely used among water quality studies [30]. Despite attempts to remove non-infectious viruses prior to amplification-based molecular methods, no effective molecular method exists to differentiate infectious and non-infectious enteric viruses [3, 14, 29].

3.4 Need for affordable, lab-free methods

To date, the field of environmental microbiology has focused on increasingly high-tech, molecular methods while overlooking the need for affordable, rapid, and practical approaches to detect enteric viruses [15, 26]. Despite the increasing availability and affordability of molecular methods for the detection of enteric viruses in research settings [3], these methods remain far out of reach for routine monitoring in high and low income countries alike [29]. This is particularly true for developing countries that have poor sanitation coverage, lack safe drinking water, and rely on wastewater reuse in agriculture [1, 12]. Despite the inadequacy of FIB to predict human health outcomes, lab-free, user friendly, relatively inexpensive FIB culture-based methods exist for different water matrices (IDEXX Laboratories, USA; AguaGenX, LLC, USA). Over the last decade, advancements in nanotechnology have facilitated the development of immunostrip tests (dipstick tests similar in format to a pregnancy test) for the detection of a variety of targets, including human viruses (reviewed in [31]). Furthermore, other immuno-based rapid detection tests for norovirus (evaluated by [32]) and even simpler, more affordable tests for the detection of *Salmonella* Typhi via a flow-through membrane immunoassay platform have been developed [33]. The application of these technologies to various matrices, likely requiring viral concentration prior to detection of enteric viruses, is essential for the advancement of microbial safety worldwide.

4 Conclusions

Waterborne viruses associated with wastewater are and will continue to be a major source of morbidity and mortality worldwide. Current microbial quality monitoring of FIB as well as FIB-based QMRAs frequently underestimate viral presence and associated health risks. In order to advance disinfection processes and improve microbial safety with respect to recreational waters, shellfish harvesting areas, and wastewater reuse, it is necessary to incorporate measures of enteric viruses as a means to guide public health policies seeking to minimize risk. As water management practices move away from routine FIB monitoring in favor of a holistic, risk-based approach, it is necessary to improve methods for the concentration and detection of viral pathogens. In conjunction with water management advances, the development of simple, affordable, lab-free tests for the rapid detection of enteric viruses and/or viral indicators is essential for ensuring worldwide improvements in microbial safety. These tests will be particularly valuable to identify unpredictable wastewater contamination events (e.g. infrastructure failure, natural disasters) or in situations where implementing reference pathogen measurements are not economically feasible. Additionally, the availability of user-friendly, point-of-use tests will enable individuals to ensure their own microbial safety.

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