

MOLECULAR MARKERS AND SENTINEL ORGANISMS FOR ENVIRONMENTAL MONITORING

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Summary:

Molecular methods are useful for both to monitor anthropogenic viral, bacterial, and protozoan enteropathogens, and to track pathogen specific markers in a complex environment in order to reveal sources of these pathogens. Molecular genetic markers for fecal viruses, bacteria, and protozoans hold promise for monitoring environmental pollution and water quality. The demand for microbiologically safe waters grows exponentially due to the global demographic rise of the human population. Economically important shellfish, such as oysters, which are harvested commercially and preferentially consumed raw can be of public health importance if contaminated with human waterborne pathogens. However, feral molluscan shellfish which do not have an apparent economic value serve as indicators in monitoring aquatic environments for pollution with human waterborne pathogens and for sanitary assessment of water quality. Current technology allows for multiplexed species-specific identification, genotyping, enumeration, viability assessment, and source-tracking of human enteropathogens which considerably enhances the pathogen source-tracking efforts.

KEY WORDS : microbiological monitoring, fecal contamination, fecal source discrimination, molecular markers, sentinel organisms.

Fecal contamination of aquatic environments afflicts many regions of the world with associated human health risks and environmental damage. Fecal pollution spreads a variety of dangerous viral, bacterial, and protozoan pathogens such as hepatitis, Norovirus, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Escherichia coli* (*E. coli*) O157:H7, *Cryptosporidium* spp., *Giardia lamblia*, *Cyclospora cayetanensis*, and human-virulent microsporidia (*i.e.*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *E. bellem*, and *E. cuniculi*)

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(Field *et al.*, 2003; Graczyk *et al.*, 2007a). *Cryptosporidium*, *Giardia*, and microsporidia inflict considerable morbidity on healthy people and can cause mortality (*e.g.*, *Cryptosporidium*) in immunosuppressed individuals (Graczyk *et al.*, 2007a). *Cryptosporidium* and *Giardia* are very frequently transmitted via water, and numerous reports indicate involvement of water in the epidemiology of microsporidian spores (Graczyk *et al.*, 2007a). They transmissive stages, *i.e.*, oocysts, cysts, and spores, are environmentally-robust and therefore ubiquitous in aquatic habitats (Graczyk *et al.*, 2007a). These protozoan enteropathogens are category B biodefense agents on the National Institute of Health list, and microsporidian spores are on Contaminant Candidate List of the U.S. EPA because spore identification, removal, or inactivation in drinking water is technologically challenging. Drinking water source or recreational waters are not routinely monitored for these pathogens, and considerable evidence demonstrates their direct zoonotic association with animals. Very often the source of fecal contamination cannot be determined because failing septic systems, wastewater discharges, and surface runoff from point and non-point sources may equally contribute to environmental contamination) (Field *et al.*, 2003). Sewage wastewater discharges are worldwide risk factors for the introduction of human enteropathogens into surface waters. In addition, although water sediments are known to be a source of human enteropathogens, the contribution of pathogen pollution deposited in sediments and re-suspended during storm events relative to other sources is unknown) (Field *et al.*, 2003). In order to adequately assess human health risks and develop management plans for watersheds that providing drinking water and supporting recreation and fisheries, it is necessary to know the sources of fecal contamination (Graczyk *et al.*, 2007a).

BACTERIAL PATHOGENS AND METHODS FOR DETECTING THEIR SOURCES

For bacterial pathogens, the standard method of measuring fecal pollution does not distinguish between human and animal sources (Field *et al.*,

2003). The method requires growing fecal coliforms or *E. coli* from water samples to estimate their concentrations. Because these bacteria are found in a variety of warm-blooded animals, this method does not distinguish between human and animal sources (Field *et al.*, 2003). In addition, coliform-based methods can be confounded by growth of coliforms in receiving water and sediments (Field *et al.*, 2003). Coliforms adhere to sediments and are transported in and out of the water column, affecting the measure of fecal pollution in unknown ways (Field *et al.*, 2003). It is remarkable that although coliforms are the gold standard for measuring fecal pollution, the influences of these important factors are still so poorly understood (Field *et al.*, 2003). Several methods for identification of the source of fecal pollution have been used, but each has drawbacks. The ratio of fecal streptococci to fecal coliforms has been used to differentiate human *vs* animal sources (Sinton *et al.*, 1993). However strains of coliforms and streptococci have different environmental survival rates, and because the ratio changes over time – it is unreliable. Antibiotic resistance patterns of fecal streptococci or coliforms can distinguish among sources of fecal pollution (Wiggins *et al.*, 1999). However this method requires culturing a large number of fecal streptococcal isolates, and therefore, it is labor-intensive and time-consuming. *Escherichia coli* ribotyping (Carson *et al.*, 2001) and rep-PCR DNA fingerprinting (Dombek *et al.*, 2001) are genotyping methods that depend on growing a large number of isolates. Ribotyping can discriminate between human and non-human isolates, but is less efficient at identifying animal sources. Both these methods require large local collections of strains for comparison purposes. Phage can be used as indicators of fecal pollution (Havelaar *et al.*, 1993). Although coliphage abundance is well correlated with sewage effluent (Paul *et al.*, 1997); coliphage are also associated with non-point discharge (Paul *et al.*, 1997). Coliphage can indicate the presence of human enteric viruses in water (Havelaar *et al.*, 1993); however, their use is limited because only a small portion of fecal bacteria may contain phages, and virus concentrations in sewage are highly variable (Havelaar *et al.*, 1993). All of the aforementioned methods are time consuming, labor intensive, require specialized equipment or extensive strain collections, are subject to culture bias, and may have unacceptably low rates of accuracy.

Source-tracking is commonly used to relate to bacterial overload and waterborne transport. However, fecal coliform source-tracking technology is not easily adoptable by wastewater facilities because of its sophistication. In addition, multiple studies have already shown the inadequacy of standard fecal coliforms (*i.e.*, *E. coli*, enterococci, fecal and total coliforms) as indicators of contamination of drinking, recreational, and wastewaters with human viral and protozoan enteropathogens.

RECOVERY AND IDENTIFICATION OF *CRYPTOSPORIDIUM* FROM ENVIRONMENTAL MATRICES

Recovery of *Cryptosporidium* oocysts from environmental-matrices remains a technologically complex process, but even more challenging is subsequent species-specific identification and viability assessment of the oocysts (Graczyk *et al.*, 2007b). Microscopy has a low sensitivity and requires a skilled microscopist. Immunofluorescent antibody (IFA) usually overestimates the *Cryptosporidium* load because IFA cross-reacts with other species of *Cryptosporidium* not virulent for humans, small unicellular algae, and non-viable oocysts (Graczyk *et al.*, 2007b). PCR-based methods, although very sensitive and specific, do not allow for viability assessment, and are highly sensitive for massive amounts of PCR inhibitors present in environmental samples. Vital dyes, *e.g.*, DAPI/PI stains, can be used for viability assessment; however, these stains are not *Cryptosporidium*-specific and produces positive reactions with any organism that have nucleic acid (Graczyk *et al.*, 2007b).

There is currently no consensus on the best method for determining species/genotype of *Cryptosporidium*. Since *Cryptosporidium* species cannot be differentiated based on oocyst morphology, a number of molecular techniques have been developed for species identification and analysis of genetic diversity. There is very little data on how results generated using different genotyping methods correlate. All of these techniques are based on PCR amplification of a DNA region with reasonable genetic variability between and within species. In some cases, a single round of PCR amplification is sufficient, though more often, a nested PCR is performed to improve sensitivity. Following PCR amplification, species determination is most often carried out by restricted fragment length polymorphism (RFLP), in which the PCR product is digested with restriction enzymes and the resulting DNA fragments are visualized and compared to standard fragment profiles for each known species. *Cryptosporidium* genes that are frequently used for this purpose include the small subunit rRNA gene (SSU rRNA), the *Cryptosporidium* outer wall protein (COWP) gene, the heat shock protein 70 (HSP70) gene, the glycoprotein 60 (GP60) gene and the thrombospondin-related adhesive protein (TRAP) gene. Once the species has been determined, further genotyping can be carried out by DNA sequence analysis of the amplified gene. Some alternative methods that don't involve DNA sequencing are multi-locus genotyping by microsatellite analysis and single strand conformation polymorphism (SSCP) analysis of the internal transcribed spacer (ITS-2) region of the nuclear

ribosomal DNA gene. Both of these alternative methods can be used to determine species without RFLP analysis. A recent study compared multiple genotyping methods. Though all methods tested provided valuable results, SSCP analysis of the ITS-2 rDNA gene was superior in identifying genetic variants and mixed infections. This method was also considered to be most cost-effective for analyzing large numbers of samples.

ENUMERATION AND ASSESSMENT OF *CRYPTOSPORIDIUM* INFECTIVITY/VIABILITY

Most of the PCR-based methods used for screening samples and genotyping provide no information on numbers of *Cryptosporidium* oocysts present or the viability/infectivity of those oocysts. Several assays have been developed to provide this type of epidemiologically-important data. The gold standard is an animal bioassay using neonatal mice. This method is frequently used to assess oocyst infectivity after treatment with disinfectants. Treated oocysts are administered to neonatal mice orally, and after a sufficient incubation period, oocyst infectivity can be assessed by quantifying oocysts shed in fecal matter or identifying developmental stages of the parasite in fixed and stained sections of the intestine. Alternatively, sections of intestine can be homogenized and parasites present in the homogenate can be detected by flow cytometry or PCR. However, there are several disadvantages to using animal bioassays. First, neonatal mice cannot be infected with all species of *Cryptosporidium*, including the human-specific species, *C. hominis*. Though a gnotobiotic pig model has been developed to overcome this limitation, bioassays are significantly more expensive than *in vitro* assays for infectivity and bring up issues of animal bioethics. Several cell culture-based methods have been shown to be just as effective as animal bioassays for assessing oocyst infectivity and inactivation by disinfectants. After exposing cell cultures to treated oocysts, infectivity can be assessed by staining infected cells using a *Cryptosporidium*-specific IFA. Alternatively, *Cryptosporidium* DNA can be extracted from the infected cultures and detected by PCR amplification. The use of a quantitative real-time PCR on DNA extracted from infected cell cultures can allow quantification of oocyst infectivity. The fluorescent *in situ* hybridization (FISH) provides both, species-specific quantitative identification with simultaneous viability assessments of *Cryptosporidium* oocysts (Graczyk *et al.*, 2006). The FISH method utilizes fluorescently-labeled oligonucleotide probes, *i.e.*, CRY-1, designed to hybridize with specific sequences of 18S rRNA of *C. parvum* and *C. hominis*. Because rRNA is only present in large copy numbers in viable

organisms, FISH allows species-specific identification by providing visualization of viable oocysts and cysts, and facilitates their enumeration (Graczyk *et al.*, 2006). Furthermore, multiplexed FISH has been combined with a direct immunofluorescent antibody (IFA) against the wall antigens of *Cryptosporidium*, and this approach has been successful for simultaneous detection of *C. parvum*, and *C. hominis* in environmental and clinical samples (Graczyk *et al.*, 2006).

HUMAN WATERBORNE PATHOGENS IN MOLLUSCAN SHELLFISH

Molluscan shellfish are suspension- or sediment-feeding organisms, which filter unicellular algae, bacteria, other microorganisms, and detrital particles of approximately 1-30 μ m size range. Bivalves have an important role in aquatic habitats; by filtering suspended particles they clarify the water and generally improve water quality. The diameter of transmissive stages of *Cryptosporidium*, *Cyclospora*, and *Toxoplasma* does not exceed 6 and 10 μ m, respectively, and *Giardia* cysts are oval and no longer than 15 μ m. Microsporidian spores range from 1.5 to 4 μ m. Thus, cystic stages of these parasites fall within the range of particles filtered by bivalve mollusks. Multiple *in vitro* and *in vivo* experimental studies demonstrated that aforementioned parasites can be efficiently recovered from water, then retained and concentrated in shellfish. Historically, *C. parvum* oocysts of waterborne origin were first identified in the tissue of blue mussels in Ireland (Chalmers *et al.*, 1997), initiating worldwide investigation of this pathogen in molluscan shellfish (Graczyk, 2003). Since then, multiple studies demonstrated that these filter-feeding organisms can harbor environmentally derived protozoan parasites as a result of concentrating the recovered particles (Graczyk, 2003). A recent and interesting epidemiological discovery is the identification, for the first time, of human-infectious microsporidia spores, *i.e.*, *E. intestinalis* and *E. bienersi* in molluscan shellfish, zebra mussels (*Dreissena polymorpha*) (Graczyk *et al.*, 2004). Microsporidia infects a variety of vertebrate and invertebrate hosts, and approximately 14 species have been reported to infect people. Of these *E. intestinalis* and *E. bienersi* have been reported to be zoonotic and to infect domestic animals and livestock (Słodkiewicz-Kowalska *et al.*, 2006). Although the actual transmission route of this specific spore species is not known, it is quite possible that infectious spores of human or animal origin passed to the aquatic environments via feces or urine (Graczyk *et al.*, 2004).

Cryptosporidium oocysts have also been identified in feral bivalves, supporting the concept that estuarine

shellfish can be used in the sanitary assessment of water quality as biological indicators for contamination of water and sediment (Graczyk *et al.*, 2004). Zebra mussels and *Corbicula* clams very efficiently concentrate *C. parvum* and *G. lamblia* in relation to low ambient concentrations (Graczyk *et al.*, 2004). Bivalves such as zebra mussels or *Corbicula* clams are convenient for such purposes because they form dense populations and clusters that facilitate the collection of large samples, do not have economic value, have a relatively small size, and are easily collected throughout the year (Graczyk *et al.*, 2004).

QUANTITATIVE ESTIMATION OF REMOVAL OF WATERBORNE PATHOGENS BY MOLLUSCAN SHELLFISH

Zebra mussels collected from the St.-Lawrence River, Canada, near a wastewater discharge site contained on average approximately 440 *C. parvum* oocysts/mussel (Graczyk *et al.*, 2001). Knowing the *C. parvum* retention rate as 4.9×10^2 oocysts/mussel/24 h and *D. polymorpha* densities of approximately 30000 specimens/m² for adult (> 1-year-old) mussels, it has been calculated that during 24 h approximately 1.3×10^7 waterborne *C. parvum* oocysts can be removed by each square meter of mussel bed in the St.-Lawrence River (Graczyk *et al.*, 2001).

The concentration of *C. parvum* observed in zebra mussels from the Shannon River, Ireland (Graczyk *et al.*, 2004) was much lower than that reported from the St.-Lawrence River (Graczyk *et al.*, 2001). However, in the St Lawrence River, mussels originated from sites impacted by wastewater discharge, and in the Shannon River, no apparent sources of water contamination have been identified near any of the sites. Considering the natural densities of zebra mussels and the fact that on average approximately eight parasites/mussel have been identified in the Shannon River study (Graczyk *et al.*, 2004), at least 2.4×10^5 pathogens/24 h can be potentially removed per each square meter of zebra mussel bed in the Shannon River.

A PUBLIC HEALTH THREAT FROM SHELLFISH CONTAMINATED WITH WATERBORNE PATHOGENS

Prior to 1992, the association between contamination derived from animal fecal wastes and the occurrence of shellfish-vectored illnesses was inconclusive (Graczyk, 2003). In 1994, enterohemorrhagic

E. coli 0157 became a major concern. This bacterium has not been associated with shellfish; however, its frequent occurrence in cattle indicated potential public health problems with shellfish harvested from waters affected by runoff from cattle farms. Beginning in 1998, multiple studies worldwide indicated that molluscan shellfish intended for human consumption can be contaminated with *Cryptosporidium*. So far there has been no reported outbreak (or case) of foodborne cryptosporidiosis linked to consumption of raw oysters in the US. However, (A) over 40 % of all foodborne infections linked to oyster consumption are in the category of an unknown etiologic agent; (B) 20 % of the general US population are vulnerable to *C. parvum* infection; (C) epidemiology of enteric infections, *i.e.*, cryptosporidiosis, indicates an association with consumption of raw shellfish; and (D) it is believed that in the United States and Canada the true incidence of shellfish-vectored gastroenteritis is underestimated as much as 20-fold. Since there is no mandatory federal requirement for reporting of gastroenteritis of an unspecified nature, physicians and state health departments are not forwarding case reports to federal authorities. In intensive seafood production regions such as northwest Galicia, Spain, where molluscan shellfish production is the most important industry, cases of self-limiting diarrhea associated with consumption of raw oysters and clams are often reported (Graczyk, 2003).

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