Reviews in Biology and Biotechnology *Vol.1, No 2, May 2001. pp. 21-26*
By The Moroccan Society of Biology in Canada *Printed in Canada*

Advanced Detection of Viruses and Protozoan Parasites in Water

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Les virus et les parasites entériques sont excrétés dans la matière fécale des individus infectés et peuvent directement ou indirectement contaminer l'eau potable. Dans la matière fécale, ces microbes sont à une densité très élevée, 10^{5-11} par gramme, et sont communément isolés des eaux usées même après décontamination de celles-ci. Une fois libérés dans l'environnement, ces microorganismes peuvent survivre plusieurs mois si les conditions leurs sont favorables. Parmi les sources de contamination des sols et des eaux courantes, il y a les eaux usées, les déchets urbains, les épandages agricoles et les enfouissements sanitaires.
Cet article présente une description des approches biotechnologiques pour l'analyse des eaux et la détection des microorganismes pathogènes et les compare aux méthodes conventionnelles.

Introduction

More than several hundreds of enteric microbial pathogens are known to infect man. Enteric viruses and parasites are excreted in the feces of infected individuals and may directly or indirectly contaminate water intended for drinking. These microbes are excreted in high numbers, 10^5-10^{11} per gram of feces of infected individuals and are commonly isolated in domestic wastewater, even after disinfection. Once in the environment they can survive for long period of time, even months under the right conditions. Ground andsurface water may be subjected to fecal contamination from a variety of sources, including sewage treatment plant effluents, on-site septic waste treatment discharges, land runoff from urban, agricultural and natural areas, and leachates from sanitary landfills.

The purpose of this paper is to describe biotechnology approaches and compare with conventional methods for the analysis of water samples for the detection of pathogenic microorganisms.

Enteric Viruses in Water

The enteric viruses include the enteroviruses, rotaviruses, Hepatitis A & E, Norwalk and Norwalk like viruses, adenoviruses, reoviruses, and others. These enteric viruses are transmitted by the fecal-oral route, infect the gastrointestinal tract and are capable of causing a wide range ofillness including diarrhea, fever, hepatitis, paralysis, meningitis and heart disease.

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Evidence for fecal contamination of surface and ground waters is provided by the detection of enteric viruses in both surface and groundwater and the continued occurrence of outbreaks of waterborne disease. For example, in the United States between 1971 and 1985, 502 drinking waterborne outbreaks of disease involving 111,228 cases of illness were reported in the U.S., of which 49% were associated with groundwater sources and 51% were associated with surface water sources (Craun, 1988; Craun 1992). Many of the reported outbreaks were due to enteric viruses (hepatitis A virus, Norwalk virus, and rotaviruses) likely that many of the waterborne disease outbreaks for which no etiological agent was identified (half of all reported outbreaks) were caused by viruses because of the failure to look for them and the limitations of current detection methodology.

The enteroviruses (poliovirus, coxsackie A and B viruses, echovirus) can cause a variety of illnesses ranging from gastroenteritis to myocarditis and aseptic meningitis (Melnick, 1990). Numerous studies have documented the presence of enteroviruses in raw and treated drinking water (Keswick et al., 1984, wastewater (Payment 1981) and sludge (Craun, 1984). Enteroviruses in the environment pose a public health risk because these viruses can be transmitted via the fecal-oral route through contaminated water (Craun, 1984) and low numbers are able to initiate infectionin humans. The enteroviruses are approximately 27 nm in diameter and have a positive polarity, single stranded RNA genome of approximately 7400 bp. These viruses are relatively well known with regards to their replication cycle and genome organization. The full genome of several enteroviruses has been sequenced permitting comparative nucleic acid and protein alignments (Palmenberg, 1988).

Hepatitis A virus (HAV) is an important waterborne virus because of the severity of the disease it may cause in susceptible individuals. HAV is the cause of acute infectious hepatitis and was the first enteric viruses for which a waterborne outbreak was documented in the United States. This virus survives more than four months at 5 and 25°C in water, wastewater and sediments (Sobsey et al., 1988). As with the enteroviruses, the full genome of various strains of HAV has been sequenced.

Rotaviruses are a significant cause of acute diarrheal illness, especially in young children. Rotaviruses group A has been documented as causes of waterborne outbreaks in humans (Gerba and Rose, 1990). These viruses have a segmented genome consisting of 11 segments of double stranded RNA. Segments designating subgroup and serotype specificity have been sequenced for several strains and serotypes.

Protozoan Parasites in Water

The enteric protozoan *Giardia* and *Cryptosporidium* are intestinal parasites that can cause gastroenteritis in humans when they are ingested. *Giardia* is the most common cause of parasitic infections in humans in the United States (Craun, 1988; Craun 1986) and can cause a lengthy diarrhea in infected individuals (Wolfe, 1984). Numerous waterborne outbreaks of giardiasis and cryptosporidiosis have been documented (Moore et al., 1994. Low numbers of *Giardia* cysts and *Cryptosporidium* oocysts are usually found in water supplies (Craun, 1986). Cysts and oocysts are the infectious units of the microorganisms, and the development of approaches for the detection of cysts and oocysts in water samples necessitates a simple, efficient and cost effective methods. In addition, since an infection in humans can be initiated by as few as 1-10 viable cysts (Akin and Jakubowski, 1986) detection methods and viability assays need to be very sensitive.

The current methods for the detection of *Giardia* and *Cryptosporidium* in water rely primarily on microscopic observation of water concentrates using either phase-contrast microscopy or an immunofluorescent technique (Suach, 1985), neither of which and also the gene probe technique (Abbaszadeagn et al., 1991) are not able to differentiate between viable and non-viable cysts. Current methods to determine the viability of cysts include infectivity of animal models (Belosevic et al., 1983), *in vitro* excystation procedures (Boucher et al., 1990), and the incorporation of vital dyes (Smith and Smith, 1989). These methods are costly, time-consuming and lack sensitivity because they require large numbers of cysts for the results to be statistically accurate.

As a result of possible public health threats, it is necessary to develop a rapid and sensitive assay for *Giardia* cysts and *Cryptosporidium* oocysts based on PCR-amplification of the mRNA of an inducible *Giardia* gene. The inducible gene chosen for this study is an hsp-70-like gene specific for *Giardia lamblia* and which produces a heat-stress protein.

Many environmental stresses are known to induce the production of specific proteins called stress proteins that help protect an organism from damage until the stress is removed. Temperature change is one environmental stress that is known to activate a specific set of genes called the heat shock genes which are associated with newly synthesized mRNA (Lindquist, 1986; Lindquist, 1980). Heat-shock mRNA appears in the cytoplasm of a viable cell within a few minutes of temperature elevation and is immediately translated with very high efficiency into a small number of highly conserved proteins, the heat-shock proteins (hsps) (DiDomenico et al., 1982; Lindley et al., 1988). As long as cells are maintained at a high temperature, hsps continue to be the primary products of protein synthesis. When cells are returned to a normal temperature, normal protein synthesis gradually resumes (Lindquist, 1986; Lindley et al., 1988).

If a cell is not viable it will not produce new heat-shock mRNA when it is exposed to elevated temperatures. The hsp70-like gene that is specific to *Giardia lamblia* has been identified and its promoter region determined (Aggarwal et al., 1988). By raising the temperature of the cysts this hsp-70-like gene can be induced to produce mRNA that codes for heat shock proteins.

The Polymerase Chain Reaction

Since its invention, PCR has become one of the most widely used biochemical assays. The speed, specificity and low cost of the procedure has led to its use in such fields as criminal and pathological forensics, genetic mapping, disease diagnosis, systematics and evolutionary studies, and environmental science.

PCR can be used to amplify, to detectable levels, nucleic acids associated with pathogens that may be present in low numbers in water samples. PCR assays must be able to detect viruses after concentration from large volumes (100 to 1,500

liters) of water. This is usually accomplished by a filter-adsorption and elution method, resulting in a concentrate containing microbes, and organic and dissolved solids. Compounds, such as humic substances, once concentrated, can interfere with the activity of the enzymes used in PCR assay.

PCR is a process in which target DNA, polymerase enzyme and the DNA subunits are combined in a test tube and subjected to the temperature changes needed for the DNA duplication to occur. By repeating this process many times, a large amount of DNA is generated. This reaction, termed the Polymerase ChainReaction (Mullis et al., 1987, Saiki et al., 1988), or PCR, can, under ideal conditions, generate millions of copies of a single DNA molecule in just 20 to 30 repetitions of the temperature cycle - each cycle requiring only a few minutes. The PCR assay can selectively amplify only a portion of the target DNA for diagnostic applications.

The advantages of PCR are numerous. When compared with techniques such as cell culture for the detection of viruses, the time required for the assay can be reduced from days or weeks to hours. Both the initial and recurring costs for PCR are much less than cell culture techniques and the technique is easily performed. Additionally, PCR can be used to identify a specific pathogen found in water. Standard PCR cannot, however, be used to detect the infectious state of an organism - only the presence or absence of pathogen-specific DNA or RNA. PCR assays have been applied to the detection of enteroviruses and other pathogens in clinical (Rotbart, 1990) and environmental samples (Abbaszadegan et al., 1993 & 1999; Pillai et al., 1991).

Cell Culture Methods

Conventional methodology for the detection of enteric viruses from the environment relies on a few established cell lines. The Buffalo Green Monkey (BGM) kidney cell line is the most commonly used for the detection of enteroviruses in the environment (Dahling et al., 1984). This cell is preferred over others, including primary cells, because it provides high sensitivity to natural isolates of enteroviruses (Dahling and Wright, 1986). Its sensitivity can be further enhanced by pretreatment of the cells with enzymes or other substances (Benton and Hurst, 1986). Unfortunately, the use of other cell lines is required to detect other groups of enteric viruses (Smith and Gerba, 1982). This can greatly increase the cost and time of the assay. While the cell culture assay can detect infectious viruses in environmental samples, without additional tests, no determination can be made as to the particular strain of virus present in a sample. Additionally, the length of time needed to detect infection in the cell culture can vary greatly, from a few days to several weeks, depending on the type and number of viruses present.

Discussion

Using different microbial detection methodologies we developed a comprehensive database on virus occurrence in the untreated source water of public ground water systems at the United States. In addition, we investigated various water quality parameters and the occurrence of microbial indicators in ground water and their possible correlation with the presence of human viruses. The analysis of the data (microbial, physical and chemical measurements) was used to develop a quantitative approach, which can be used to screen ground water systems to identify wells that are at risk to fecal contamination.

The results of microbial analyses of 250 samples indicate a risk of fecal contamination of some ground water sources as measured by two different microbial methods. Analyses using the PCR assays suggest that 38% of the samples contained viral contamination. It is important to note that PCR assay is indicative of the presence of viral nucleic acid, and not necessarily the infectious viral particle. Therefore, the PCR results should be interpreted as indicative of the possibility of virus transport within the aquifer and a potential risk of disease, rather than an absolute public health problem. It is noted that the total of the cultural indicators (coliforms, enterococci,bacteriophage) were positive in 35% of the samples tested, suggesting that the general overall indication of fecal contamination of ground water sources did not substantially differ between the molecular technique (PCR) and the cultural indicators. PCR,however, did reveal a greater level of viral contamination than did the cell culture assay. This could be due to the greater sensitivity of the PCR method for the detection of viruses in water samples, the ability of PCR to detect a wider variety of viruses than the cell culture method, and the possibility that PCR detected non-infectious viral nucleic acids.

Although no waterborne outbreaks were documented during this study, the high level of indicators of fecal contamination does suggest a mechanism by which illness could result in members of the community. The U.S. General Accounting

Office (GAO) reported that annually 3 to 6% of community water systems in a six state area exceeded total coliform levels during a four-year period (GAO 1997). Between 0.3 and 1.3% of the systems contained *E. coli* or fecal coliform bacteria. However, because the Total Coliform Rule requires samples to be collected from the distribution system, these rates may not reflect the occurrence of bacterial indicators in the sourcewater. Total coliform occurrence rates in private water systems (which are typically not disinfected) ranged between 15 and 59% (GAO 1997) and are consistent with the findings of this study.

All of the sites which tested positive (either by cell culture, PCR, or any of the cultural indicators) practiced disinfection, typically with injection of chlorine at the well head and maintenance of a chlorine residual in the distribution system. The seven sites tested that did not disinfect the well water all tested negative. It was the objective of this study to develop a sampling scheme that would represent the type of geological formations used for production of drinking water in the US. It was reasoned that the soil type would be indicative of the possibility of viral transport within the aquifer. However, there is some evidence that the placement (siting) of certain wells was made with regard to the level of treatment provided. For example, the majority of wells positive for culturable viruses were followed by full, conventional (coagulation, sedimentation, filtration, disinfection) treatment. This argues that the placement of the well near a surface water source (subject to contamination) was permitted because the treatment provided was adequate to handle the type of microbial contaminant that might be present. A similar line of reasoning could be applied to the disinfected wells examined in this study. The high occurrence rate of microbial indicators may be related to fact that disinfection was provided so that large setback distances were not required by the governmental agencies that permitted the placement of the well. However, the mean setback distance to surface water for sites in this study (3,400 ft) far exceeded the mean distance (462 ft) for the average community ground water system reported by the USEPA (USEPA 1997). Therefore, without additional research focused on exclusively non-disinfected systems, one cannot determine if the level of microbial occurrence found in this study is generally representative of both disinfected and non-disinfected wells. However, it may be prudent to provide protective barriers (including disinfection) until the possibility of contamination can be eliminated.

A drawback of this method is the lack of aviability or infectivity determination, and the health significance of viruses detected by this method is unknown. It is recommended that epidemiology studies and quantitative microbial risk assessment be conducted to assess the health effects of viruses detected by PCR methods in groundwater.

PCR Assay Compared With Cell Culture

The use of cell culture for virus detection and the PCR assay for viruses differ significantly in several ways. For cell culture, the minimum detection level of viruses in a sample is, by definition, one PFU per unit volume - a quantity of virus particles that may range from just a few or many more -at least some of which must be infectious. In addition, when a sample tests positive for viral infectivity using cell culture,the infectious agent is not necessarily known. The BGM cell line, routinely used for enterovirus assays, is susceptible to infection by many viruses, including reoviruses such as rotavirus, a pathogen often present in environmental samples in numbers greater than enterovirus (Puig et al., 1994). Cell culture protocols do not detect all human viruses present in the environment. Norwalk virus, for instance, has yet to be successfully grown in cell cultures, and therefore environmental samples cannot be assayed for this pathogen. Finally, since each environmental sample is unique, little is known regarding possible sample components that may inhibit the viral infectivity in culture. Cell culture, however, does offers the advantages of isolating an infectious a viral pathogen, and is widely accepted as the standard method for viral detection in water.

Conversely, RT-PCR is potentially much more sensitive assay for virus detection, in that it is possible to detect as little as a single molecule of RNA. The technique can detect less than one PFU of a virus (since some virus particles may not be infectious), and PCR can detect both infectious and noninfectious viruses.

The two techniques also differ in the amount of time and cost required. Most cell culture protocols call for a 14-day initial passage and for a 14-day secondary passage of the sample on cells, followed by a seven-day confirmation passage of putative positive samples. To test for different viruses, multiple cell lines must be maintained, different growth media must be purchased and stored, and different protocols followed. The cost of one sample

by cell culture assay in our lab is approximately $650.00. Conversely, a single RT-PCR virus assay can easily be accomplished in two to three days, including confirmation, and costs less than $200.00. This price includes the cost of a large volume 1MDS filter, elution, and concentration. A single RT-PCR reaction costs less than $20.00.

Given its increased sensitivity and ability to detect an intactvirus particle (Abbaszadegan et al. 1997), PCR analysis would be expected to reveal more positive results than cell culture analysis. Since either cell culture analysis or PCR can only reveal a “snapshot” of the quality of the groundwater being sampled, PCR would be a desirable rapid initial screening tool, in that the presence of even noninfectious or non-intact viruses would suggest that a groundwater supply may be subject to contamination.

While the detection of viral RNA does not show an infectious level of contamination, the presence of viral RNA does suggest a source of viral contamination and thus the potential for health risk. The most sensitive method of detection would be the most desirable, even without the ability to confirm infectivity of the sample contamination.

References

Abbaszadegan, M., C.P. Gerba, and J.B. Rose. 1991. Detection of *Giardia* cysts with a cDNA probe and applications to water samples. Appl. Environ. Microbiol. 57:927-931.

Abbaszadegan, M., M.S. Huber, C.P. Gerba, and I.L. Pepper. 1997. Detection of Viable *Giardia* Cysts by Amplification of Heat Shock-Induced mRNA. Appl. Environ. Microbiol. 63:324-328.

Abbaszadegan, M., M.S. Huber, C.P. Gerba, and I.L. Pepper. 1993. Detection of Enteroviruses in Groundwater with the Polymerase Chain Reaction. *Applied and Environmental Microbiology*. 59:1318-1324.

Abbaszadegan, M., P.W. Stewart, and M.W. LeChevallier. 1999. A Strategy for the Detection of Viruses in Water by PCR. Appl. Environ. Microbiol. 65:444-449

Aggarwal, A., P. Romans, Vidal F. de la Cruz and T.E. Nash. 1988. Conserved sequences of the HSP gene family in *Giardia lamblia*. In: Advances in *Giardia* Research. P.M. Wallis, and B.R. Hammond (ed.) Univ. of Calgary Press, pp. 173-175.

Akin, E.W., and W. Jakubowski. 1986. Drinking water transmission of giardiasis in the United States. Water Sci. Tech. 18:219-226.

Belosevic, M., G.M. Faubert, J.D. MacLean, C. Lau and N.A. Croll. 1983. *Giardia lamblia* infections in Mongolian gerbils: an animal model. J Inf. Dis. 147:222-226.

Benton, W.H. and C.J. Hurst. 1986. Induction of Cytopathogenicity in Mammalian Cell Lines Challenged with Culturable Enteric Viruses and its Enhancement by 5-Iododeoxyuridine. *Applied and Environmental Microbiology*. 51:1036-1040.

Boucher, S. E. M., and F. D. Gillin. 1990. Excystation of in vitro-derived *Giardia lamblia* cysts. Infect. Immun. 58:3516-3522.

Craun G.F. 1992. Waterborne disease outbreaks in the United States of America: causes and prevention. *World Health Statistics Quarterly*. Rapport Trimestriel de Statistiques Sanitaires Mondiales. 45(2-3):192-9.

Craun, G.F. 1988. Surface Water Supplies and Health. J Am Water Works Association. 80:40-52.

Craun, G.F. 1986. Waterborne diseases in the United States.CRC Press, Boca Raton, FL.

Craun, G.F. 1984. Health aspects of groundwater pollution, p. 135-179. In G. Bitton and C.P. Gerba (ed.), Groundwater Pollution Microbiology. John Wiley & Sons, Inc., New York

Dahling, D.R., and B.A. Wright. 1986. Optimization of the BGM Cell Line Culture and Viral Assay Procedures for Monitoring Viruses in the Environment. *Applied and Environmental Microbiology*. 51:790-812

Dahling, D.R., R.S. Safferman, and B.A. Wright. 1984. Results of a survey of BGM cell culture practices. Environ. Int. 10:309-313.

DiDomenico, B.J., G.E. Bugaisky and S. Lindquist. 1982. The heat shock response is self-regulated at both the transcriptional and post-transcriptional levels. Cell 31:593-603.

GAO. 1997. Drinking Water. Information on the Quality of Water Found at Community Water Systems and Private Wells. United States General Accounting Office, Washington, DC.

Gerba, C.P., and J.B. Rose. 1990. Viruses in source and drinking water, *In: Drinking Water Microbiology*, Spring-Verlag, New York, 1990, pp.380-395.

Keswick, B.H., C.P. Gerba, H.L. DuPont, and J.B. Rose. 1984. Detection of enteroviruses in treated drinking water. Appl. Environ. Microbiol. 47:1290-1294.

Lindley, T. A., P. R. Chakraborty, and T. D. Edlind. 1988. Heat shock and stress response in *Giardia lamblia*. Mol. Biochem. Parasitol. 28:135-144.

Lindquist, S. 1986. The heat shock response. Ann. Rev. Biochem. 55:1151-1191.

Lindquist, S. 1980. Translational efficiency of heat-induced messages in *Drosophila melanogaster* cells. J. Mol. Biol. 137:151-158.

Melnick, J.L. 1990. Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses, p. 549-605. In B.N. Fields (ed.), Virology. Raven Press, New York.

Moore, A.C., B.L. Herwaldt, G.F. Craun, R.L. Calderon, A.K. Highsmith, and D.D. Juranek. 1994. Waterborne disease in the United States, 1991 and 1992. J. Am Water Works Assoc. 86:87-99.

Mullis, K. B. and F. A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology*. 155:335-351.

Palmenberg. 1988. Personal communication.

Payment, P. 1981. Isolation of viruses from drinking water at the Pont-Viau water treatment plant. Can. J. Microbiol. 27:417-420.

Pillai, S.D., K.L. Josephson, R.L. Baily, C.P. Gerba, and I.L. Pepper. 1991. Rapid Method for Processing Soil Samples for Polymerase Chain Reaction Amplification of Specific Gene Sequences. *Applied and Environmental Microbiology*. 57:2285-2286.

Puig, M., J. Jofre, F. Lucena, A. Allard, G. Wadell, and R. Girones. 1994. Detection of Adenoviruses and Enteroviruses in Polluted Waters by Nested PCR Amplification. *Applied and Environmental Microbiology*. 60:2963-2970.

Roberts-Thomson, I.C., D.P. Stevens, A.A.F. Mahmoud and K.S. Warren. 1976. Giardiasis in the mouse: An animal model. Gastroenterol. 71:57-61.

Rotbart, H.A. 1990. Enzymatic RNA Amplification of the Enteroviruses. *Journal of Clinical. Microbiology*. 28:438-442.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science.* 239:487-494.

Sauch, J. F. 1985. Use of immunofluorescence and phase-contrast microscopy for detection and identification of *Giardia* cysts in water samples. Appl. Environ. Microbiol. 50:1434-1438.

Smith, E.M. and C.P. Gerba. 1982. Laboratory Methods for the Growth and Detection of Animal Viruses. In *Methods in Environmental Virology*. Edited by C.P. Gerba and S.M. Goyal. New York: Marcel Dekker.

Smith, A. L., and H. V. Smith. 1989. A comparison of fluorescein diacetate and propidium iodide staining and *Giardia intestinalis* cyst viability. Parasitology. 99:329-331.

Sobsey, M.D., P.A. Shields, F.S. Hauchman, A.L. Davis, V.A. Rullman, and A. Bosch. 1988. Survival and persistence of hepatitis A virus in environmental samples, p.121-124. In A.J. Zuckerman (ed.), Viral hepatitis and liver disease. Alan R. Liss, Inc., New York.

USEPA. 1997. Community Water System Survey, Volume II: Detailed Survey Result Tables and Methodology Report. EPA-815-R-97-001b, Office of Water (4607), Washington, DC.

Wolfe, M.S. 1984. Symptomatology, diagnosis and treatment. In: *Giardia* and giardiasis. S.L. Erlandsen and E.A. Meyer.(ed.) Plenum Press, NY. p. 147-161.