New methods for the detection of viruses: call for review of drinking water quality guidelines

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Abstract
Drinking water supplies which meet international recommendations for source, treatment and disinfection were analysed. Viruses recovered from 100 L–1,000 L volumes by in-line glass wool filters were inoculated in parallel into four cell culture systems. Cell culture inoculation was used to isolate cytopathogenic viruses, amplify the nucleic acid of non-cytopathogenic viruses and confirm viability of viruses. Over a period of two years, viruses were detected in 23% of 413 drinking water samples and 73% of 224 raw water samples. Cytopathogenic viruses were detected in 6% raw water samples but not in any treated drinking water supplies. Enteroviruses were detected in 17% drinking water samples, adenoviruses in 4% and hepatitis A virus in 3%. In addition to these viruses, astro- and rotaviruses were detected in raw water. All drinking water supplies had heterotrophic plate counts of <100/mL, total and faecal coliform counts of 0/100 mL and negative results in qualitative presence-absence tests for somatic and F-RNA coliphages (500 mL samples). These results call for a revision of water quality guidelines based on indicator organisms and vague reference to the absence of viruses.

Keywords
Viruses; drinking water; quality; guidelines; cell culture; polymerase chain reaction

Introduction
Drinking water supplies have a long history of association with a wide spectrum of viral infections (Grabow, 1996). Evidence of waterborne transmission is predominantly based on epidemiological data and has only in exceptional cases been confirmed by direct viral detection in drinking water supplies. This is because most viruses typically transmitted by water (enteric viruses) are not detectable by methods used in the past. Largely as a result of shortcomings in technology for virus detection, guidelines and specifications for drinking water are generally based on indicator organisms and vague reference to the absence of viruses (EC, 1980, 1998; EPA, 1989; WHO, 1996, 1997; SABS, 1999) where the “absence of viruses” probably refers to viruses detectable by cytopathogenic effect in cell cultures as this was the detection method used at the time when the guidelines were formulated.

Recent progress on virus detection in water has placed water quality analysis into a new perspective. These techniques are largely based on molecular procedures using principles of the reverse transcriptase-polymerase chain reaction (RT-PCR) (Metcalf et al., 1995; Reynolds et al., 1996; Gantzner et al., 1999; Grabow et al., 1999b; Soule et al., 2000). Theoretically, these are capable of detecting any known cytopathogenic as well as non-cytopathogenic viruses. In addition, they are highly specific being based on the detection of specific nucleotide sequences of selected viruses. The techniques are also highly sensitive because detection is based on the amplification of nucleotide sequences of a single viral genome. Disadvantages include the failure of conventional PCR techniques to distinguish between viable and non-viable viral particles (Sobsey et al., 1998). Additionally, detection is qualitative and restricted to known viruses for which reliable PCR procedures are available.

The viability of viruses detected by molecular techniques may be confirmed by inoculating test samples into cell cultures prior to detection. Prior cultivation in cell cultures has the additional advantage of increasing sensitivity because infected cells amplify the nucleic acid
of viruses thus facilitating subsequent detection by molecular techniques. However, detection by molecular techniques remains essential because, in many cases, infection and replication of viral nucleic acid does not lead to a cytopathogenic effect (Sobsey et al., 1998; Grabow et al., 1999a; Vivier et al., 2000). The ability of viruses to infect cell cultures and to replicate their nucleic acid implies that they are also capable of infecting the natural human host which, as a rule, is much more susceptible to viruses than presently available cell culture systems. Exceptions in this regard include caliciviruses that are often transmitted by water but fail to infect presently available cell cultures (Wolfart et al., 1997). Detection of these viruses in water is restricted to direct PCR procedures (Kukkula et al., 1999).

Although the detection of viruses in drinking water has been reported by a number of laboratories, there is no meaningful information on routine monitoring of drinking water supplies by technology presently available. This study deals with routine monitoring of drinking water supplies over a period of two years. Monitoring was based on in-line recovery of viruses from large volumes of water using glass wool adsorption–elution procedures followed by parallel inoculation of four cell culture systems and ultimate detection of selected viruses by RT-PCR. In previous studies the glass wool adsorption–elution procedure proved highly efficient (Vilaginès et al., 1997) and the cell culture systems proved to be the most susceptible to a variety of viruses that were presently available (Taylor et al., 1997; Grabow et al., 1999a).

Materials and methods
Drinking water (413 samples) collected at regular intervals over a period of two years was analysed. The supplies were derived from acceptable quality surface water sources using treatment processes that conformed to international specifications for the production of safe drinking water (WHO, 1996, 1997). Disinfection was by chlorination based on corresponding specifications for concentration levels and conditions of application. Glass wool filters were used for the on-site and in-line recovery of viruses from 100 L–1,000 L volumes of water (Vilaginès et al., 1997). Viruses eluted from the glass wool were inoculated in parallel into triplicate flasks containing cultures of: (a) BGM monkey kidney, PLC/PRF/5 human liver and CaCo-2 human colon carcinoma cell lines and (b) primary vervet monkey kidney cells. After two passages, cell cultures were homogenised and analysed by RT-PCR for a variety of enteric viruses (Grabow et al., 1999a,b). The procedures used detected positive strand RNA of enteroviruses, hepatitis A virus and astroviruses (Agnès et al., 1994). Corresponding samples of water were analysed for microbial indicators of water quality using internationally accepted techniques and principles (Grabow, 1996; Grabow et al., 1999b). A total of 224 raw water samples collected from rivers and a dam were likewise analysed.

Results
Viruses were detected in 23% of the 413 drinking water samples and 73% of the 224 raw water samples. Cytopathogenic viruses were detected in 6% of raw water samples but not in any treated drinking water supplies. Enteroviruses were detected in 17% of drinking water samples, adenoviruses in 4% and hepatitis A virus in 3%. In addition to these, astroviruses and rotaviruses were detected in the raw water. All the drinking water supplies had heterotrophic plate counts of <100/mL, total and faecal coliform counts of 0/100 mL and negative results in qualitative presence-absence tests for somatic and F-RNA coliphages in 500 mL samples.

Discussion
The detection of viruses in treated drinking water supplies was in agreement with reports from other parts of the world (Rose et al., 1986; Grabow, 1996; Payment et al., 1997). The
results also supported epidemiological data which revealed low level transmission of viruses by drinking water supplies which had been treated and disinfected by standard procedures and met quality specifications for indicator bacteria (Keswick et al., 1984; Payment et al., 1985, 1997; Zmirou et al., 1987; Bosch et al., 1991; Rose et al., 1986; Grabow, 1996).

The results for heterotrophic plate counts, coliform bacteria and coliphages supported earlier evidence on shortcomings of conventional indicators for assessment of the virological quality of drinking water (Payment et al., 1985, 1997; Moore et al., 1994; Grabow, 1996). The failure of even sensitive tests for somatic and F-RNA coliphages in 500 mL samples to reflect the presence of viruses suggested that the shortfall of indicator procedures may be substantial. The sensitivity of microbial indicator tests may, therefore, have to be upgraded by a wide margin to reflect the virological safety of drinking water supplies. Results for the detection of viruses reported here are considered reliable because every effort has been made to exclude false positive results due to contamination by viruses or PCR products. Internationally accepted standard operating procedures for the detection and identification of viruses were strictly adhered to (Reynolds et al., 1996).

However, there is sound reason to believe that the results reported here represented an underestimate of the true incidence of viruses in the raw and treated drinking water supplies investigated. For instance, analyses excluded members of the family Caliciviridae. This family comprises a wide spectrum of viruses including the Norwalk-like viruses. Many of these viruses are known to be excreted by gastroenteritis patients in the study area (Wolfaardt et al., 1997), the viruses have been associated with transmission by water and they have been detected in water supplies (Kukkula et al., 1999). The number of rotaviruses detected would appear to be lower than may be expected in view of the incidence of rotavirus infections in the study area (Wolfaardt et al., 1997) and reports on rotaviruses in water environments elsewhere (Soule et al., 2000). In addition, the water was not analysed for other enteric viruses such as the hepatitis E virus (Grabow, 1997) and many other human viruses that may occur in water environments (Grabow, 1996; Bofill-Mas and Girones, 2000).

The procedures for the detection of viruses described here were designed for the reliable and sensitive qualitative detection of viruses in drinking water. The reason for this approach was that most guidelines for viruses in water refer to the qualitative presence or absence of viruses. The only exception is SABS (1999) referred to in detail below. The specification implies that quantitative enumeration of viruses would be required only in a small percentage of samples. Unacceptable quality and treatment of drinking water, which contains 10 enteric viruses/100 L, is generally also indicated by conventional microbial quality indicators, sanitary surveillance of water sources and failure of treatment and disinfection processes (Grabow, 1996). The first priority would, therefore, be sensitive and reliable qualitative detection. If indeed necessary, these methods could be converted into most probable number procedures for quantitative results. This would be rather labour, time and cost intensive.

Test samples were inoculated into cell cultures prior to RT-PCR screening to amplify viral nucleic acid and to contribute to the removal of RT-PCR inhibitory compounds. Amplification of viral nucleic acid increases the sensitivity of RT-PCR detection of viral nucleic acid. In addition, it gives an indication of the viability and infectivity of viruses because in vivo amplification of nucleic acid requires infection of host cells and activation of the replication cycle. In the techniques used here, positive strand RNA sequences of entero-, hepatitis A and astro-viruses were detected. This confirmed the production of new viral nucleic acid sequences which may be interpreted as meaningful evidence of the infectivity of the viruses concerned (Agnès et al., 1994; Pintó et al., 1996; Reynolds et al., 1996).
Theoretically, it is possible that viruses, or their nucleic acids, in the test samples may pass through the cell culture cycle to be detected eventually by the RT-PCR tests without having infected the host cells. The procedure would not confirm the viability of these viruses. However, this possibility appears negligible because the drinking water supplies under investigation contained extremely low numbers of viruses and it would appear most unlikely that these viruses could pass through two cell culture cycles and still be present in sufficient numbers to be detected by RT-PCR. This was confirmed by the failure to detect viruses in the original test samples prior to cell culture amplification. The possibility of naked viral nucleic acid sequences to pass through the system to be detected eventually by RT-PCR appears even less likely. Reasons are that only viral capsids are known to attach to glass wool and there is no evidence that naked nucleic acid sequences were recovered by the glass wool adsorption-elution procedure. This implied that glass wool concentrates contained only intact virions. In addition, naked viral nucleic acid is known to be unstable in water environments and is rapidly degraded. The viruses detected in this study would, therefore, seem to constitute a meaningful health risk.

The detection of viruses in treated drinking water implied that the supplies concerned failed to meet quality guidelines for viruses recommended by authorities (Table 1).

The above guidelines have a number of shortcomings for meaningful assessment of the virological quality of drinking water.

1. In some guidelines there is no indication whether or not the viruses referred to should be viable or not (EPA, 1989; SABS, 1999). The reference to “pathogenic microorganisms” (EC, 1980, 1998) and “waterborne viral disease” (WHO, 1996) would appear to suggest that these guidelines refer to viable viruses but this is not categorically confirmed and no indication is given as to how viability is expected to be confirmed. Although non-viable viruses may be considered not to constitute a health risk, there are sound reasons to expect drinking water supplies not to contain unacceptable levels of even non-infectious viruses. For instance, the presence of non-infectious viruses reflects shortcomings in treatment such as removal by filtration, flocculation or sedimentation. In addition, the presence of non-infectious viruses suggests that other viruses may also be present, some of which may be viable.

**Table 1** Recommended levels and action for viruses in drinking waters

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<th>Guideline authority</th>
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| United States Environmental Protection Agency (EPA, 1989) | (a) “Maximum contaminant level goals for microbiological contaminants: Viruses: zero”  
(b) “Filtration and disinfection, general requirements: at least 99.99% (4-log) removal and/or inactivation of viruses ...” |
| European Communities (EC, 1980) | “Water intended for human consumption should not contain pathogenic organisms. If it is necessary to supplement the microbiological analysis of water intended for human consumption, the samples should be examined not only for the bacteria referred to in Table E but also for pathogens including: enteroviruses ...” |
| European Communities (EC, 1998) | “... ensure that there is no potential danger to human health arising from the presence of pathogenic microorganisms, e.g. Cryptosporidium.” |
| World Health Organization (WHO, 1996) | “It is essential that drinking-water supplies should be essentially free from human enteric viruses so that the risk of transmission of waterborne viral disease is negligible.” |
| South African Bureau of Standards (SABS, 1999) | Microbiological requirements: enteric viruses, count/100 L: 95% min. – not detected; 4% max. – 1/100 L; 1% max. – 10/100 L” |
2. With the exception of SABS (1999), the guidelines give no indication of the volume of drinking water from which viruses are expected to be absent. This implies that the guidelines serve no purpose for routine monitoring or for confirmation of acceptable virological quality of drinking water supplies.

3. The viruses referred to are poorly defined in the guidelines. For instance, EPA (1989) merely refers to “viruses” and directly proving the absence of all possible viruses which may find their way into water sources and supplies is for practical reasons virtually impossible. The vague reference to “pathogenic microorganisms” in EC (1998) may likewise be interpreted to imply any possible viruses. The “enteroviruses” referred to in EC (1980) is probably an error because the group of viruses classified as “enteroviruses” represents a minor component of viruses associated with waterborne transmission. The reference to “human enteric viruses” (WHO, 1996) and “enteric viruses” (SABS, 1999) is technically more correct but a clear definition of “enteric viruses” would improve the practical value of the guidelines.

4. None of the guidelines gives an indication as to how viruses are expected to be recovered from drinking water supplies and how they are expected to be detected. Technology and expertise available today makes it possible to give at least an indication of basic principles of procedures and techniques expected to be used.

5. The recommended “4-log” reduction in numbers of viruses by treatment processes (EPA, 1989) is a valuable guideline but gives no indication as to what viruses it refers to and how the viruses are expected to be detected. For instance, if the recommendation refers to cytopathogenic viruses, compliance may be readily achieved. However, if viruses were to be detected by molecular techniques this recommendation may not be readily achieved as suggested by the results reported here.

The revision of quality guidelines for viruses in drinking water warrants urgent attention because many drinking water supplies analysed in various parts of the world using the latest technology fail to meet current quality guidelines. Even though most, if not all, of the guidelines referred to are not enforceable by law, water supply utilities endeavour to meet the quality recommendations and consumers expect drinking water supplies to meet international quality recommendations. Implications are that the treatment of drinking water supplies to meet the recommended quality may require extensive upgrading of treatment and disinfection processes which may have major financial and practical implications (Clark et al., 1993; Regli et al., 1993).

The need for meaningful guidelines regarding viruses in drinking water supplies is underlined by WHO guidelines (WHO, 1996) which, in an evaluation of waterborne pathogens, state that the health significance of viruses in water is high, that the persistence of viruses in water is long and that the relative infective dose of viruses in water is low. The need is furthermore emphasised by many epidemiological studies referred to earlier in which viral infections have been associated with drinking water supplies that meet other guidelines for treatment and quality. Additional considerations include data on the public health and financial impact of waterborne viral diseases (Clark et al., 1993; Hughes, 1993; Payment, 1993; Regli et al., 1993; Pegram et al., 1998) and the increasing component of consumers with elevated susceptibility to waterborne diseases, notably the elderly and immunocompromised (Hughes, 1993; Gerba et al., 1996; Grabow, 1996). A number of other reasons for improving water quality monitoring procedures and techniques for the detection of aetiological agents have been outlined (Hughes, 1993; Ford and Colwell 1996; Kramer et al., 1996). The time and effort invested by water quality authorities into formulating guidelines also reflects perceptions about the need for such guidelines.

Expertise for meaningful assessment and modelling of the risk of viruses in drinking water based on dose response curves and the incidence of viruses in drinking water supplies
is available (Regli et al., 1991, 1993; Haas et al., 1993; Macler and Regli, 1993; Sobsey et al., 1993; States and Sykora, 1995). Progress is being made with the formulation of acceptable risks for drinking water based on risk assessment and burden of disease data (Haas et al., 1999; Havelaar et al., 2000; Haas and Eisenberg, 2001; Prüss and Havelaar, 2001). The proposed level of one infection/10,000 consumers/year is widely accepted as at least a valuable point of departure for acceptable risks (Macler, 1993; Regli et al., 1993; Hunter and Fewtrell, 2001). However, further details for refinement of practical and meaningful guidelines based on acceptable risks are desirable and authorities such as the World Health Organisation are actively engaged in work along these lines (WHO, 2001).

Conclusions
The detection of enteric viruses in a substantial percentage of drinking water supplies which meet international specifications for treatment, disinfection and conventional indicator organisms, reveals shortcomings in guidelines which vaguely recommend the absence of viruses. The results obtained by means of new techniques based on cell culture amplification and detection of viruses by molecular procedures indicate that most drinking water supplies may fail current specifications for viruses. This has major implications for the water industry and calls for a revision of quality specifications to accommodate the latest techniques for the detection of viruses.

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References


