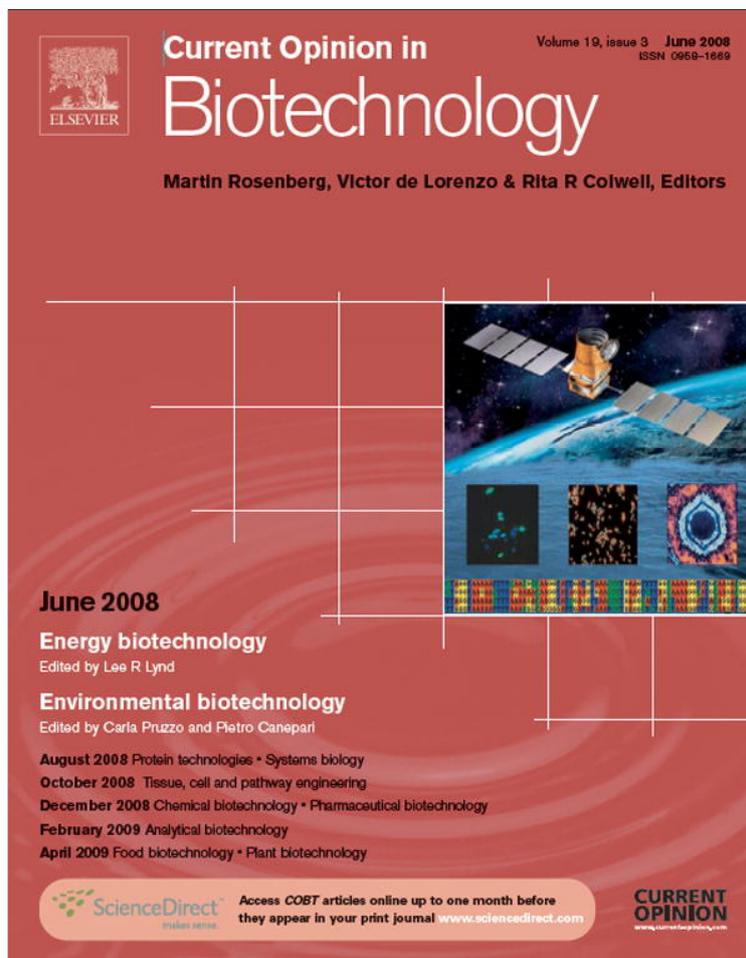


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New tools for the study and direct surveillance of viral pathogens in water

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Half a century ago scientists attempted the detection of poliovirus in water. Since then other enteric viruses responsible for gastroenteritis and hepatitis have replaced enteroviruses as the main target for detection. However, most viral outbreaks are restricted to norovirus and hepatitis A virus, making them the main targets in water. The inclusion of virus analysis in regulatory standards for viruses in water samples must overcome several shortcomings such as the technical difficulties and high costs of virus monitoring, the lack of harmonised and standardised assays and the challenge posed by the ever-changing nature of viruses. However, new tools are nowadays available for the study and direct surveillance of viral pathogens in water that may contribute to fulfil these requirements.

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Introduction

As stated by one of the seminal names in water virology, WOK Grabow, virological analysis of water is required for a number of purposes that include research on the incidence and behaviour of viruses in the water environments, assessment of the presence of viruses and the risk of infection, as well as evaluation of the efficiency of treatment and disinfection processes and routine quality monitoring to test the compliance of water quality with guidelines and specifications [1] and in essence the public health impact of waterborne viral infections.

Additionally, identification of strains isolated from the water environment may also be used as a tool for the study of the epidemiology of waterborne viruses providing an overview of all the viruses circulating in the community,

including viruses causing both symptomatic and asymptomatic infections [2,3,4].

However, the tasks required to fulfil the aforementioned goals are easier said than done. Main difficulties to overcome for virus detection and characterisation in water samples encompass viral diversity, occurrence of low particle numbers, particularly in drinking water, and the technical challenges of virus assays.

Waterborne viruses

A wide variety of different viruses may be found in human sewage (Table 1). Some of these viruses are shed in extremely high numbers, that is, patients suffering from diarrhoea or hepatitis may excrete up to 10^{13} and 10^{10} virus particles, respectively, per gram of stool [5,6,7]. Since current water treatments do not ensure their complete removal they become contaminants of the water environment in numbers high enough to represent a public health threat, although low enough to pose serious difficulties for their detection. Figure 1 illustrates the possible routes of waterborne transmission of enteric viruses. Enteric viruses can be transmitted by a variety of routes including person-to-person contact, zoonotic and/or vehicle transmission.

Poor water quality continues to pose a major threat to human health. Billions of cases of gastrointestinal illness occur annually worldwide. The World Health Organisation (WHO) declared that diarrhoeal disease alone contributes to an estimated 4.1% of the total DALY (disability adjusted life years) global burden of disease and is responsible for the deaths of 1.8 million people every year [8]. It was figured that 88% of that burden is attributable to unsafe water supply, sanitation and hygiene and it is mostly concentrated on children in developing countries. A significant amount of disease could be prevented especially in developing countries through better access to safe water supply, adequate sanitation facilities and better hygiene practices.

Viruses are a major cause of water-related disease. It is now well recognised that the most common viral gastrointestinal illness are rotavirus and norovirus diarrhoea in the infantile and adult population, respectively. However, most well documented waterborne outbreaks of viral gastroenteritis are related to noroviruses [9,10]. Other gastro enteric viruses, such as rotaviruses [11] and astroviruses [12] have also occasionally been implicated in waterborne outbreaks.

Table 1

Human viruses documented to be found in the water environment

Genus (genome)	Popular name	Disease caused
<i>Enterovirus</i> (ssRNA)	Poliovirus	Paralysis, meningitis, fever
	Coxsackie A, B virus	Herpangina, meningitis, fever, respiratory disease, hand-foot-and-mouth disease, myocarditis, heart anomalies, rash, pleurodynia, diabetes
	Echovirus	Meningitis, fever, respiratory disease, rash, gastroenteritis
<i>Hepatovirus</i> (ssRNA)	Hepatitis A virus	Hepatitis
<i>Reovirus</i> (segmented dsRNA)	Human reovirus	Unknown
<i>Rotavirus</i> (segmented dsRNA)	Human rotavirus	Gastroenteritis
<i>Norovirus</i> (ssRNA)	Norovirus	Gastroenteritis
<i>Sapovirus</i> (ssRNA)	Sapporo-like virus	Gastroenteritis
<i>Hepevirus</i> (ssRNA)	Hepatitis E virus	Hepatitis
<i>Mamastrovirus</i> (ssRNA)	Human astrovirus	Gastroenteritis
<i>Coronavirus</i> (ssRNA)	Human coronavirus	Gastroenteritis, respiratory disease, SARS
<i>Orthomyxovirus</i> (segmented ssRNA)	Influenza virus	Influenza, respiratory disease
<i>Parvovirus</i> (ssDNA)	Human parvovirus	Gastroenteritis
<i>Mastadenovirus</i> (dsDNA)	Human adenovirus	Gastroenteritis, respiratory disease, conjunctivitis
<i>Polyomavirus</i> (dsDNA)	Polyomavirus	Progressive multifocal leucoencephalopathy, diseases of urinary tract
<i>Circovirus</i> (ssDNA)	TT (Torque Teno) virus	Unknown, hepatitis

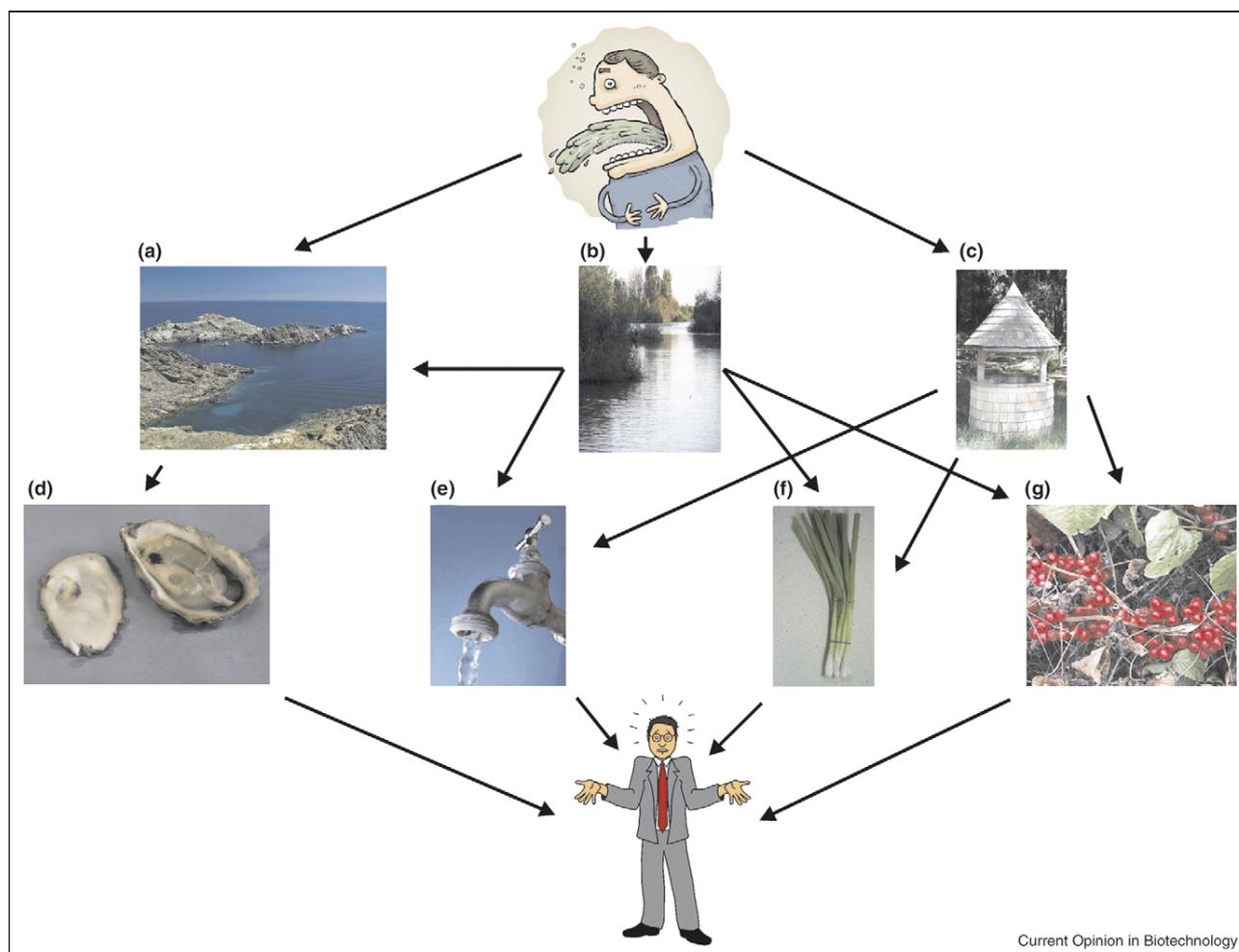
Another major waterborne disease is hepatitis that can be a serious debilitating disease progressing from a non-specific illness with fever, headache, nausea and malaise to vomiting, diarrhoea, abdominal pain and jaundice. Hepatitis A represents worldwide around 50% of the total hepatitis cases and although is self-limiting and rarely causing death may incapacitate patients for several months. The causative agent is the hepatitis A virus that has been linked to several waterborne outbreaks [13[•]]. Hepatitis E, although less frequent than hepatitis A, has a higher mortality rate, particularly in pregnant women. It is the most important or the second most important cause of acute clinical hepatitis in adults throughout Asia, the Middle East and Africa. By contrast, hepatitis E is rare in industrialised countries, but antibody (anti-HEV) is found worldwide. Hepatitis E is principally the result of a waterborne infection in developing countries and is thought to be spread zoonotically (principally from swine) in industrialised countries [14[•]].

The significance to human health of many of the non-human animal viruses present in environmental samples is less well understood although it is remarkable that zoonotic viruses infecting humans continue to be discov-

ered or appear to re-emerge as important human pathogens. One example of an emerging disease is the severe acute respiratory syndrome or SARS, reported in November 2002. Although several coronaviruses are known to be spread by the faecal–oral route, there is no current evidence that this mode of transmission plays a key role in the transmission of SARS in spite of the considerable shedding of the virus in stools [15]. Another important zoonotic issue is the case of influenza viruses. Despite human influenza viruses replicate primarily in the respiratory tract, avian influenza viruses, such as the highly pathogenic H5N1, cause generalised infection in birds with replication in the gastrointestinal duct and virus shedding in faeces. However, their potential waterborne transmission remains controversial [16[•],17^{••}].

Water-related diseases are associated with exposure to water environments in many ways. These include not only waters used for drinking and recreation purposes but also those used for agricultural purposes such as crop irrigation, and food processing, eventually resulting in foodborne outbreaks [18,19]. Additionally, shellfish grown and harvested in polluted waters is a well-docu-

Figure 1



Possible routes of waterborne transmission of enteric viruses. Viruses are shed in extremely high numbers in the faeces and vomit of infected individuals. Pathogenic viruses are routinely introduced into the environment through the discharge of treated and untreated wastes, since current treatment practices are unable to provide virus-free wastewater effluents. In consequence viral pathogens contaminate the marine environment **(a)**, fresh water **(b)** and ground water **(c)**. Mankind is exposed to enteric viruses through various routes: shellfish grown in polluted waters, contaminated drinking water and food crops grown in land irrigated with sewage contaminated water and/or fertilised with sewage. Surface and ground waters are employed for public consumption **(e)** and have been implicated in waterborne outbreaks of gastroenteritis and hepatitis. Foods susceptible to be contaminated at the pre-harvest stage such as bivalve molluscs **(d)**, particularly oysters, clams and mussels; salad crops **(f)**, as lettuce, green onions and other greens; and soft fruits **(g)**, such as raspberries and strawberries have also been implicated in outbreaks of viral diseases.

mented cause of gastroenteritis and hepatitis outbreaks [20,21]. While drinking water may not be considered a major public health problem in developed communities, prevention of water-related virus contamination of food remains a perennial challenge both in developing and developed societies owing to its global trade.

Water sample processing for virus analysis

One of the challenges to overcome in the virological analysis of water is the need to recover the low number of viruses from large volumes of sample. This is particularly important when molecular micro-methods are applied. Methods for virus concentration from water

samples are depicted in Table 2 and reviewed elsewhere [22]. A good concentration method should fulfil several requirements: it should be technically simple, fast, provide high virus recoveries, be adequate for a wide range of enteric viruses, provide a small volume of concentrate, and be inexpensive. No single method meets all these requests. Criteria based on the experience and expertise of the user on a given method should be employed to select the most appropriate system. Positively charged filters [23] and glass wool [24] based methods are still among the best possibilities. Sampling large volumes requires a two-step concentration procedure, with polyethylene glycol precipitation [25] and ultrafiltration [26]

Table 2

Procedures for the concentration of viruses from water samples			
Method	Principle	Pros	Cons
Adsorption–elution methods	Ionic charge	Good recoveries	Requires sample preconditioning
Negatively charged filters			
Positively charged filters			
Glass powder	Chemical precipitation	Cheap. Good recoveries	Fragile apparatus
Glass wool		Good recoveries	
Precipitation methods		Efficient for dirty samples or as secondary concentration	
Organic flocculation			
Ammonium sulfate			
Polyethylene glycol	Physical sedimentation	Efficient for dirty samples or as secondary concentration	Inhibitory to RT-PCR enzymes Intra-assay variability
Ultracentrifugation			
Lyophilisation		Freeze-drying	
Ultrafiltration			
Magnetic beads	Immunoaffinity		Good recoveries for clean samples
		Good recoveries from small volumes	

as preferred procedures for reconcentration of the primary eluates. Additionally, PEG [25] as well as lyophilisation [3] may be used for direct virus concentration in heavily polluted medium size samples, for example, sewage, having this latter method the added advantage of removing substances inhibitory to RT-PCR enzymes if this method is employed for virus detection [27].

Specific virus detection assays employed in water analysis: infectious vs. non-infectious virus detection

When virus detection procedures are mentioned the recurrent issue of detecting infectious or physical particles comes into discussion. Whenever possible,

infectious assays coupled with identification methods are preferred for direct assessment of human health risk. The detection of infectious enteroviruses and even astroviruses or rotaviruses may be achieved by cell culture techniques with the appropriate cell line. Despite recent reports of cell lines allowing the growth of wild-type hepatitis A virus [28^{••}] and norovirus [29^{••}], issues related to assay complexity, cost-effectiveness and validity for the detection of a broad spectrum of isolates make their use a difficult and unrealistic approach for hepatitis A virus and norovirus detection, respectively.

Nucleic acid amplification techniques are currently the most widely used methods for detection of viruses in

water, which also enable to gather information of the virus genotypes occurring in the environment, thus providing most relevant epidemiological information, particularly important for the implementation and follow-up of vaccination programmes [2,3,4*].

Although nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) techniques have been reported as highly sensitive and specific, respectively [30,31], PCR and RT-PCR remain as the current gold standard for virus detection. A further improvement comes from real-time RT-PCR, which enables not only qualitative determination but also, and particularly, quantitative diagnostic assays [7**,32–34]. However, the significance of a genome copy remains controversial since the virus-specific infectivity or infectious/physical particle ratio is highly variable in environmental samples. Several studies show uncoupling between the number of genome copies and infectivity in environmental studies [35–37]. Nevertheless, as stated above, no alternative to molecular detection analysis exists for highly health significant waterborne viruses such as human norovirus and hepatitis A virus.

Future developments and challenges

Most health-significant waterborne viruses have RNA genomes (Table 1), and a major challenge in the development of molecular techniques for the diagnostic of RNA viruses derives from the facts that they depend on error-prone polymerases that generate high mutation rates and the occurrence of recombination events that altogether lead to complex mutant genome populations or quasispecies [38*,39–41,42**]. It is needless to say that this genome variability implies a careful selection for highly conserved sequences targeting primers and probes, particularly when quantification is the objective. RNA regions containing complex multidomain structures involved in essential functions such as translation or replication are highly conserved and therefore are good candidates for this purpose.

An accurate quantification of genome copies demands the control of crucial steps such as the efficiency of the virus/nucleic acid extraction procedures and of the enzymes involved in the molecular amplification, particularly reverse transcriptase. While for the quantification of RNA viruses in clinical samples the use of an internal control based on the detection of the expression of a housekeeping gene is a clear first choice, this is obviously not valid for water samples leading to compromise in the use of an external control. The best candidate for this latter purpose is the use of an encapsidated RNA, such as RNA animal viruses, RNA bacteriophages or armoured RNAs that are pseudoparticles made of target RNAs packaged into MS2 coliphage coat protein [7**,43]. However, it is best to avoid the use of viruses that might be present as contaminants in the assayed samples, thus

ruling out the use of coliphages or human enteric viruses. The control of a RT-PCR reaction should rely on a ssRNA molecule as similar as possible to the viral target and amplifiable with the same pair of primers under exactly the same conditions and with the same efficiency [7**,44*,45]. Raw values of genome copies must then be adjusted accordingly to the figured efficiencies for better accuracy of the virus titre. Addressing these quality control and quality assurance (QC/QA) issues and harmonising the molecular techniques are required before virus analysis could be included in water quality standards.

The inclusion of the control measures above described calls for the development of multiplex approaches with the aim to analyse several pathogens in a single assay without increasing the economic cost. Multiplex formats may be based on real-time amplification or PCR-microarray systems [46,47]. A logical step forward is the development of chip/biosensors capable to be used in laboratory as well as in field settings [48].

Viruses will undoubtedly remain a major health threat, and the key scientific issues will have to be addressed to detect and characterise rapidly evolving old and new viral pathogens. One example is the potential emergence of diverse polioviruses from C-cluster coxsackie A viruses [49**] and its implication in a poliovirus-free world with a poliovirus-antibody naïve population. Such a situation could be a fertile ground for a poliovirus-like agent to emerge by mutation. Environmental virologists should be ready to handle this kind of threat through the rapid identification of the new pathogens by the use, among other tools, of whole-genome amplification and high-throughput sequencing techniques [42**,50**].

Conclusions

The availability of methods for accurate quantitative virus detection enables a sensible prospective water safety approach based on the identification and prevention of hazards that could cause waterborne illnesses. This is the basis of the Hazard Analysis and Critical Control Point (HACCP) principles that must be applied to ensure the virological safety of the water environment.

The actual burden of waterborne viral infections is still hard to figure owing to technical limitations in pathogen detection, scarce data on environmental epidemiology, difficulties in determining the source of infection and occurrence of unapparent infections, among others. These shortcomings may be already hard to overcome for well-known pathogens and awesome for newly emerging agents. The overall picture appears even more complicated owing to issues related with susceptibility to infection of malnourished or immunocompromised hosts and the influence of geographical, socioeconomic and seasonal factors in a global warming situation.

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