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Human Viruses in Water
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Chapter 9

The Detection of Waterborne Viruses

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Viruses in water are usually present in concentrations too low for detection by direct analysis. Virological investigation of water samples is therefore nearly always a multi-stage process involving concentration of viruses present followed by an appropriate detection procedure. The exception is analysis of sewage, where viruses may be present in sufficiently high numbers to be detectable without concentration.

The volume of water analysed and the degree of concentration required will depend on the number of viruses likely to be present and therefore on the origin of the sample. While viruses in sewage may require minimal concentration (or none at all) to render them detectable, those in treated drinking water or groundwater may require several thousand-fold concentration to make detection likely. It is often possible to find viruses in 100 ml of unconcentrated inlet (i.e. raw) sewage, whereas several hundred litres of drinking water may have to be processed. It is common, for instance, to take 10 l samples of water from recreational sites which may be subject to sewage effluent pollution and which will require concentration of about thousand-fold. The final volume of concentrate will be influenced by (a) the minimum volume achievable by the concentration technique and (b) the volume required by the detection procedure(s) and any replicates thereof. In practise, final concentrate volumes of about 5–10 ml are usually produced.

There are several approaches to detection of viruses. Part or all of the concentrate may be inoculated into cell cultures to detect infectious cytopathogenic virus, and if this is done in a quantitative fashion the virus can be enumerated, the count being reported as plaque-forming units (pfu), the tissue culture infectious dose (TCD_{50}), or most probable number (MPN) units. The virus may be isolated and identified from the cell cultures. Viruses that multiply without producing an identifiable cytopathic effect (c.p.e.) in culture may sometimes be detected by

immunoperoxidase or immunofluorescence staining. The concentrate may also be analysed by molecular biological procedures (usually polymerase chain reaction (PCR) or real-time-PCR (RT-PCR)). The problem then is that such techniques do not usually detect the infectious virus, and novel approaches have been made recently to meet this challenge.

Concentration methods

It is common for concentration to comprise at least two stages. The first stage will reduce the volume to between 100 and 400 ml, and the second stage will reduce it to 2–10 ml. Supplementary stages may be added to remove cytotoxic or PCR-inhibitory compounds.

Block and Schwartzbrod (1989) defined a number of criteria that an ideal concentration method must fulfil to be of practical use. The method should:

- be technically easy to accomplish in a short time;
- have a high virus recovery rate;
- concentrate a range of viruses;
- provide a small volume of concentrate;
- not be costly;
- be capable of processing large volumes of water; and
- be repeatable (within a laboratory) and be reproducible (between laboratories).

No single method fulfils all these requirements.

The properties of viruses are most often exploited in their concentration, and the general approaches to concentration derived from them are shown in Table 1. Numerous methods based on these approaches have been devised for the concentration of viruses from water and the principal ones are summarised in Table 2. These have been reviewed extensively by Wyn-Jones and Sellwood (1998) in respect of enteroviruses and by Wyn-Jones and Sellwood (2001) for other virus groups. The virology of waterborne disease is discussed in Percival et al. (2004).

Table 1

General approaches to virus concentration

Property	Technique applicable
Ionic charge	Adsorption/elution
Particle size	Ultrafiltration
Density and sedimentation coefficient	Ultracentrifugation

Table 2

Summary of concentration techniques for viruses in water and related materials

Technique	Method	Water quality	Initial volume	Relative virus content	Recovery	Capital cost	Recurrent cost	Secondary concentration required?	Comments
Adsorption/elution	Gauze pads	Sewage or effluent	Large	High	Low to medium	Nil	Very low	No	Not quantitative
	Electronegative membranes	All waters	1–1000 l	Low to medium	50–60% with practise	Medium	Medium	Yes	High volumes require dosing pumps
	Electropositive membranes	All waters	1–1000 l	Low to medium	50–60% with practise	Medium	High	Yes	No pre-conditioning required
	Electronegative cartridges	Any low turbidity	1–50 l	Low to medium	Variable: higher with clean waters	Low	Low	Yes	Clogs more quickly than membranes
	Electropositive cartridges	All waters	1–1000 l	Low to medium	Variable	Medium	High	Yes	Wide range of viruses
	Glass wool	All waters	1–1000 l	Low to medium	Variable	Low	Very low	Yes	No pre-conditioning required
	Glass powder	All waters	<100 l	Any	20–60%	Medium	Low	For volume >100 l	Special apparatus
Entrapment: ultrafiltration	Alginate membranes	Clean only	Low	High	Good	Low	Low	No	Very slow. Clogs rapidly if turbid
	Single membranes	Clean	Low	Any	Variable	Medium	Low	No	Slow
	Tangential (= cross) flow and hollow fibres	Treated effluents or better	High	Low	Variable	High	Medium	Sometime	Pre-filter for turbid waters
	Vortex flow	Treated effluents or better	High	Low	Unknown	High	Medium	Unknown	Undeveloped yet
Hydroextraction	PEG or sucrose	Any	Low	High	Variable (toxicity)	Negligible	Very low	No	High virus loss in wastewaters
Ultracentrifugation		Clean	Low	High	Medium	High	Medium	No	Wide range, but usually impractical
Other techniques	Iron oxide flocculation	All	Low	Any	Variable	Low	Low	No	
	Biphasic partition	All	<7 l	Any	Variable	Low	Low	No	Toxic to cells
	Immunoaffinity and magnetic beads	Unknown	Low	Low	High	High	Low	No	New method

Concentration based on ionic charge: adsorption/elution

The development of virus adsorption/elution methods, suitable for the recovery of viruses from waters, stems from the work of Melnick and his colleagues in Houston, TX (e.g. Wallis and Melnick, 1967a,b,c; Wallis et al. (1970). In general terms, a virus-containing sample is brought into contact with a solid matrix to which the virus will adsorb under specific conditions of pH and ionic strength. Once the virus is adsorbed, the water in which it was originally suspended is discarded. The virus is then released from the matrix by elution into a smaller volume of fluid, though this is usually still too large to be analysed directly. Choice of adsorbing matrix, eluting fluid and processing conditions will be influenced by the nature of the sample and by experience, but elution is commonly done using a solution containing beef extract or skimmed milk, both at high pH, which displaces the virus from the adsorbing matrix into the eluant. Eluants comprising basic amino acids (glycine, lysine) are also used. The USEPA Standard Method (2007) for the concentration of waterborne viruses is based on an adsorption/elution procedure, quoted in the Information Collection Rule (ICR) and at http://www.epa.gov/microbes/about.htm.

Adsorption to electronegative membranes and cartridges

The popularity of membranes, made of cellulose acetate or nitrate, is due to their availability in various pore sizes, configurations and compositions. The virus is bound to the filter by electrostatic attractive forces, and not by size exclusion. It is possible to get good recoveries of the virus accompanied by good flow rates and a minimum of filter clogging even from turbid waters, and many solids-associated virus can be recovered. In its simplest form, a virus-containing sample is passed under positive pressure or vacuum through a cellulose nitrate membrane 142 or 293 mm in diameter and of mean pore diameter 0.45, 1.2 or 5 μm (Fig. 1). For waters containing particulate material a pre-filter is used upstream of the membrane.

Since viruses and the filter materials are both negatively charged at neutral pH the water sample must be conditioned to allow electrostatic binding of virus particles to the filter matrix. The water sample is adjusted to pH 3.5 and Al^{3+} or Mg^{2+} ions may be added, though opinion is divided as to whether metal ions are needed at all when using cellulose nitrate membranes.

Negatively charged filters may also be used in tube form. Balston filters are epoxy resin-bound glass fibre filters with an 8 μm nominal pore diameter. They were originally used for concentration of viruses from tap water (Jakubowski et al., 1974) and have since been employed for concentration of viruses from river water (e.g. Morris and Waite, 1980) and other waters. Their recoveries are as good as membrane filters, they are less expensive and can be obtained in sterile cartridges in disposable form. However, they are prone to clogging, cannot be used with even moderately turbid water and according to Gerba (1987), cannot be used at high flow rates. Because of problems of clogging of membrane or tube filters, the

Fig. 1 Membrane filtration.

processing of seawater samples in this way is limited to a maximum of 20 l before filters have to be changed (Block and Schwartzbrod, 1989).

One way of overcoming the problem of clogging without having to change membranes or tubes frequently is to increase the surface area of filtration by the use of larger cartridge filters, where sheets of negatively charged pleated filter material approximately 25 cm wide are rolled and used in 30 cm cartridge holders. These were evaluated by Farrah et al. (1976) who used fibreglass membrane material in a pleated format. Seeded poliovirus was recovered from 378 l volumes of seawater with 53% efficiency. The authors reported that the filters could be regenerated up to five times by soaking for 5 min in 0.1 M NaOH.

Papaventsis et al. (2005) reported a modification of the use of negatively charged membranes wherein they were able to culture sewage-derived enteroviruses directly from the filter without elution, thus reducing the total time required for analysis.

Generally, recovery rates are as variable with negatively charged filter media as with any other kind. Block and Schwartzbrod (1989), citing Beytout et al. (1975) considered cellulose nitrate membranes relatively efficient insofar as they give 60% recovery of virus; the same authors recorded glass fibre filters giving a poor average yield from wastewater but 70% recovery with river water. Payment and Trudel (1979), using glass fibre filters, reported 38–58% recovery of 10^2–10^6 pfu seeded in 100 ml–1000 l volumes. Few studies have been done on recovery efficiencies from marine waters in a controlled way; however controlled studies have been done to evaluate the recovery efficiency of the method using drinking water.

It is not usually possible to conduct studies where the virus is deliberately added to water systems, however Hovi et al. (2001) in assessing the feasibility of environmental poliovirus surveillance added poliovirus type 1 into the Helsinki sewers and recovered it over a period of 4 days by taking samples at downstream locations and concentrating 100-fold by polymer two-phase separation.

Adsorption to electropositive membranes and cartridges

Positively charged filters adsorb virus from water and other materials without the need for prior conditioning of the sample. Initial work was done in the USA by Sobsey and Jones (1979) and by Hou et al. (1980). They adsorb virus in the pH range 3–6; at pH values above 7 the adsorption falls off rapidly, so the pH still needs to be carefully controlled. These properties make the use of positively charged filters attractive, not only for the convenience of not having to condition the sample but also because it makes possible the concentration of other viruses such as rotavirus and coliphages, which are sensitive to the low pH conditions needed for adsorption to negatively charged media. Keswick et al (1983) reported that type 1 poliovirus and rotavirus SA11 survived at least 5 weeks on electropositive filters at 4°C, which makes them useful for on-site concentration. They are used in the same way as electronegative materials. The virus is eluted from the filter and secondary concentration is carried out as for the electronegative types.

Recoveries from positively charged filters are similar to those from negatively charged ones; Sobsey and Jones (1979) reported 22.5% recovery using a two-stage procedure in the concentration of poliovirus from drinking water. The original positively charged material, Zeta-plus Series S, is made of a cellulose/diatomaceous earth/ion-exchange resin mixture. Sobsey & Glass (1980) compared these Virozorb 1 MDS filters with Filterite (fibreglass) pleated cartridge filters for recovery of poliovirus from 1000l tap water and obtained recoveries of about 30% with both types. The advantages of these filters lie in the large volumes they can handle without the need for conditioning the sample. Elution from the filter still needs to be carried out at pH 9 or above, which limits their use to viruses stable below that pH, though Bosch et al. (1988) successfully concentrated rotavirus in this way. Organic materials in the sample, especially fulvic acid, were reported to interfere more with virus recovery from Virozorb cartridges than from glass-fibre materials (Sobsey and Hickey, 1985; Guttman-Bass and Catalano-Sherman, 1986). Such filters are used extensively in the USA for concentration of many types of viruses from treated drinking water to sewage effluent (e.g. Sedmak et al., 2005). A different electropositive material (MK) is cheaper but its recoveries were reported to be not as good as 1 MDS in comparative tests (Ma et al., 1994). Improvements to poliovirus and norovirus recovery from tap water samples by coating of electropositive Zetapor filters by passage of $AlCl_3$ prior to filtration was reported by Haramoto et al. (2004).

During the 2002/2003 outbreak of severe acute respiratory syndrome (SARS) attention was focused on possible transmission of the SARS-corona virus (SARS-CoV) in sewage since SARS-CoV RNA had been found in the stools of affected

patients. Electropositive filters were used to concentrate the virus from sewage (Wang et al., 2005) and SARS-CoV RNA was recovered from sewage concentrates.

Advances in membrane technology have also resulted in charge-modified nylon membranes being available for concentration of viruses from water. Gilgen et al (1995, 1997) described the use of positively charged nylon membranes coupled with ultrafiltration for the concentration of a variety of enteric viruses prior to detection by RT-PCR. Other nylon membranes are also available which are made in various pore sizes, which would permit passage of the virus (0.45, 1.2 and 3 μm) and have a positive surface charge over the pH range 3–10, which would promote strong binding of negatively charged particles. Although nylon filters have been shown to bind viruses in freshwater samples, adsorption from marine samples is very poor and they would not be used for seawater (Sellwood, personal communication). Their low cost and ease of use suggest that further evaluative research should be done. Triple-layered polyvinylidene fluoride (PVDF) membranes and cartridges have been used in industry for the removal of polio and influenza viruses from pharmaceutical products (AranhaCreado et al., 1997), though whether the viruses can be recovered from the filter is not known.

A recent advance in the use of positively charged filters has been the use of membranes (disc or pleated) consisting of ''nano-alumina'' fibres approximately 2 nm in diameter bound into a support matrix of cellulose, polyester and glass fibre. Such filters carry a high electropositive charge and are claimed to bind $6\log_{10}$ MS2 phage in the pH range 5–9 with no conditioning of the water and to have a high flow rate. The virus may be eluted from the filter using a high-protein fluid such as beef extract at pH 9 (see below). Originally intended as water purification devices, these filters have been considered for use as filters to meet the USEPA drinking water standard. There are no reported peer-reviewed studies on their performance with animal viruses.

The need to determine the presence of *Cryptosporidium* and *Giardia* as well as viruses in water samples has led some workers to attempt the simultaneous concentration of both types of microorganism (e.g. Watt et al., 2002).

Adsorption to glass wool

Glass wool is an economic alternative to microporous filters. It is used in a column and provided it is evenly packed to an adequate density, adsorption of viruses appears at least as efficient as with other filter types. An advantage of the method is that the virus will adsorb to the filter matrix at or near neutral pH, and without the addition of cations, which makes it suitable for viruses sensitive to acid, however, elution still has to be done at high pH.

The technique was pioneered in France principally by Vilaginès and co-workers (e.g. Vilaginès et al., 1988), who applied it to the concentration of a range of viruses from surface, drinking and waste waters. Glass wool packed into holders (Fig. 2) at a density of $0.5\,g/cm^3$ is washed through in sequence with HCl, water, NaOH and

Fig. 2 Glass wool filtration.

finally with water again to neutral pH before the sample is passed through the filter. Different sizes of filter can be prepared according to the type of water and flow rate.

In the French studies sample sizes ranged from 100 to 1000 l for drinking waters, 30 l for surface waters and 10 l for wastewaters. The only pre-treatment necessary was dechlorination of drinking waters. Surface water samples were filtered at 50 l/h in a 42 mm diameter filter holder. The virus was eluted from the filter with 0.5% beef extract solution and secondary concentration done by organic flocculation.

Recovery efficiency of approximately 10^2 pfu poliovirus seeded into 400 l drinking water averaged 74% (SD 18.9%). For surface waters the recovery rate was 63% and 57%, respectively. Clogging of the filters was reduced by lowering the flow rate to 50 l/h.

Other viruses were also concentrated during field evaluation of the method; adenoviruses and reoviruses were also recovered, though as expected enteroviruses predominated. Vilaginès et al. (1993) also reported a survey of two rivers over a 44-month period and concluded that the technique was robust enough to be used for routine monitoring of surface waters.

Glass wool has been used in many other laboratories; Hugues et al. (1991) found it more sensitive than the glass powder method; it was used by Wolfaardt et al. (1995) to concentrate small round-structured viruses (SRSVs, now noroviruses) from spiked sewage and polluted water samples prior to detection by RT-PCR, by Ehlers et al. (2005) for concentration of enteroviruses from sewage

and treated drinking water, and by van Heerden et al. (2005) to recover human adenoviruses from 200 l treated drinking water samples and 25 l river water samples. Adsorption to the filters was done on site and the filters transported to the laboratory for elution.

Adsorption to glass powder

Glass beads constitute a fluidised bed and so have the advantage that the filter matrix cannot become clogged as with glass-fibre systems. Sarrette et al. (1977) first developed this technique, which was extended by Schwartzbrod and Lucena-Gutierrez (1978). The method gives a low eluate volume, which may not need secondary concentration prior to further analysis. A disadvantage is the complexity of the apparatus.

Other adsorbents

A range of viruses can be concentrated from different waters using talc (magnesium silicate) mixed with celite (diatomaceous earth) (e.g. Sattar and Westwood, 1978; Ramia and Sattar, 1979; Sattar and Ramia, 1979).

Baggi and Peduzzi (2000) reported a simple and inexpensive (though relatively insensitive) method for concentration of rotaviruses from surface waters and sewage, which involved addition of 200 µl (*sic*) SiO_2 per litre of conditioned water sample, settling or centrifugation of the silica and elution of virus from the pellet.

Dahling et al. (1985); Lahke and Parhad (1988) and Chaudhiri and Sattar (1986) used powdered coal as an adsorbent with a view to transferring the virus concentration and water purification technology to developing countries.

The same kind of matrix in a more refined state was used as granular activated carbon by Jothikumar et al. (1995) for the first stage concentration of enteroviruses, hepatitis E virus (HEV) and rotaviruses. Using RT-PCR as a detection method, these authors reported 74% recovery of poliovirus 1.

Entrapment

Entrapment, or size exclusion, refers to those techniques in which the virus in a sample is bound to a filter matrix principally by virtue of its size rather than by any charges on the particle, though in practice electrostatic effects can also exert an effect.

Ultrafiltration

Variations in technique involve passing the sample through capillaries (e.g. Rotem et al., 1979), membranes (e.g. Divizia et al., 1989a,b) and hollow fibres (Belfort et al., 1982) with pore sizes that permit passage of water and low molecular mass solutes but exclude viruses and macromolecules, which become concentrated on the

membrane or fibre. Most laboratories use membranes or fibre systems with cut-off levels of 30–100 kDa. In systems in which the fluid passes directly through the filter, non-filterable components quickly clog the filter or precipitate at the membrane surface, thus this type of filter is only useful for small volumes (<1000 ml). Some ultrafilters employ tangential flow or vortex flow (VFF), which reduces clogging. Tsai et al. (1993) used VFF for processing inshore water samples in Southern California. Fifteen litres of each sample were concentrated to 100 ml using a 100 kDa cut-off membrane and the samples were further concentrated to 100 µl using Centriprep and Centricon units at $1000 \times g$.

The minimum "dead" volume (e.g. 10–15 ml, Divizia et al., 1989a) is the final volume of concentrate. If this is small enough then it may be analysed or it may have to be further processed by secondary concentration. Hill et al. (2005) showed that it was possible to concentrate viruses and other microorganisms simultaneously using hollow fibre technology, and used sodium polyphosphate to minimise adhesion of organisms to the filter. Rutjes et al. (2005) used a membrane ultrafilter of 10 kDa cut off for secondary concentration of enteroviruses following primary concentration by adsorption/elution; the starting primary concentrate volumes were approximately 650 ml (raw sewage) and 1800 ml (river water).

Some workers have experienced binding of the virus to the membrane rather than just the prevention its passage through it. In these cases the virus was eluted by backwashing with glycine buffer or beef extract and the eluate reconcentrated by organic flocculation. Some authors have even reported differences in binding between related viruses. Divizia et al. (1989b) for example noted that hepatitis A virus (HAV) was recovered with 100% efficiency though poliovirus was recovered very poorly under standard conditions, but this improved if the membranes were pretreated with different buffers. Further, recovery was best if the virus was eluted with beef extract at neutral (not high) pH.

The advantages of ultrafiltration are principally that the sample requires no pre-conditioning and that a wide range of viruses can therefore be recovered, including those sensitive to the pH changes necessary in most adsorption/elution procedures, and also bacteriophages (e.g. Nupen et al., 1981; Urase et al., 1994). Efficiency of recovery is usually good, though as with all methods it is variable. Surface water samples may take a long time to process if they are turbid; Nupen et al. (1981) were able to filter 50 l volumes but this took about 40–72 h depending on the sample. Systems have high capital cost, though disposable cartridges have recently become available. The technique is sometimes seen as an advance on the adsorption/elution technique (e.g. Grabow et al., 1984; Muscillo et al., 1997).

Ultracentrifugation

Ultracentrifugation is a catch-all method capable of concentrating all viruses in a sample provided sufficient *g*-force and time are used. Differential ultracentrifugation allows separation of different virus types. A number of studies have been reported, including one in which virus from a polluted well was recovered (Mack et al., 1972),

and one where viral numbers in natural waters were as high as 2.5×10^8/ml, 10^3–10^7 times as high as had been found by plaque assay (Bergh et al., 1989). However the limited volumes that can be processed, even using continuous flow systems, together with the high capital costs and lack of portability of the equipment, limit its usefulness in concentrating viruses directly from natural waters. It does find a use as a secondary concentration method however. Murphy et al. (1983), in an investigation of a gastroenteritis outbreak associated with polluted drinking water, concentrated 5 l samples of borehole water to 50 ml using an ultrafiltration hollow fibre system and followed this by ultracentrifugation to pellet the virus for electron microscopical examination. They were thus able to detect rapidly rotaviruses, adenoviruses and SRSVs (noroviruses), as well as enteroviruses, which were confirmed by cell culture.

In an investigation to detect HEV in sewage, Pina et al. (1998) concentrated viruses and removed suspended solids from 40 ml samples by differential ultracentrifugation; Vaidya et al. (2002) used the same protocol to detect HEV and HAV in sewage samples. Le Cann et al. (2004) concentrated astroviruses from sewage samples by ultracentrifugation and extracted the RNA from the pellets.

Other methods

Many other methods exist, though none satisfies all the requirements given above by Block and Schwartzbrod (1989). These include hydroextraction with hygroscopic solids (Wellings et al., 1976; Ramia and Sattar, 1979), iron oxide flocculation (Rao et al., 1968; Bitton et al., 1976), two-phase separation (Lund and Hedstrom, 1966) and freeze-drying (Bosch et al., 1988; Kittigul et al., 2001).

Affinity columns were used by Schwab et al. (1996) in a broad-based antibody-capture technique for a variety of viruses and Myrmel et al. (2000) described the separation of noroviruses in this way. An important attribute of this method is that it acts as a clean-up stage to remove RT-PCR inhibitors. Cromeans et al. (2004) reported the preparation and use of a soluble Coxsackie virus-adenovirus (sCAR) receptor immobilised to magnetic beads for the concentration of Coxsackie and adenoviruses from water sample concentrates. The receptor, which neutralised Coxsackie virus B3, also reacted with other Coxsackie B types. The group also reported the use of a neutralising monoclonal antibody for immunocapture of the same viruses.

Secondary concentration

Where proteinaceous eluant fluids are used, the most commonly used secondary concentration technique is that of Katzenelson et al. (1976); the pH of the primary eluate is reduced to 3.5–4.5, which causes isoelectric coagulation (flocculation) of the protein. The virus adsorbs to the floc, which is deposited by centrifugation and dissolved in 5–10 ml neutral phosphate buffer. If the concentrate is to be inoculated into cell cultures it is common to filter it through a 0.22 μm pore diameter filter to remove contaminating bacteria.

Secondary concentration can also be accomplished using two-phase separation, usually with polyethylene glycol (PEG)/NaCl, or PEG and dextran T40. Rutjes et al. (2006) compared two-phase separation (PEG and dextran T40) with ultrafiltration for secondary concentration of noroviruses from water following primary concentration by adsorption/elution, and found ultrafiltration to be better, the techniques being assessed by estimation of the recovered norovirus RNA.

If molecular biological analysis is to be done, the volume may be reduced to about 1 ml by dialysis, in spin-columns or microconcentrators with a M_r cut-off of 100,000 KDa.

Gilgen et al. (1997) developed a protocol for analysis of bathing waters and drinking water which used filtration through positively charged membranes followed by ultrafiltration as a secondary concentration step, and Huang et al. (2000) used positively charged membranes followed by beef extract elution and PEG precipitation for the concentration of caliciviruses in water.

Table 2 summarises the methods for virus concentration from different water types.

Detection and enumeration of waterborne viruses

Detection and enumeration are conveniently considered together since for many viruses they are performed simultaneously. Detection may be done by infectivity-based methods where the virus undergoes at least partial multiplication in cell culture, or it may be done by techniques based on properties other than infectivity. Most important in this latter category are the molecular biological techniques, especially the PCR. Enumeration by molecular means may be semi-quantitative, such as end-point dilution assays or, increasingly, by real-time PCR for enumerating genome copies of a target virus, though the relationship between numbers of infectious units and genome copies depends on many variables.

Detection of virus infectivity is traditionally done by inoculating cell cultures with part or all of the concentrate and allowing the virus to multiply in the cells so that they are killed. The c.p.e. of many enteroviruses and some other types is visible to the naked eye. If a range of cell cultures is inoculated under liquid assay it should be possible to detect polio, Coxsackie B, echo viruses, as well as some adenoviruses and reoviruses. HAV may also be detected this way but only after prolonged incubation of cultures, and it is therefore not an approach used in routine waterborne HAV detection.

Cell culture

The line most favoured for enumeration of water-associated enteroviruses is the Buffalo green monkey (BGM) line first described in a water context by Dahling et al. (1974). This was reported to give higher plaque assay titres of poliovirus, Coxsackie viruses B, some echovirus and reoviruses than obtained in rhesus or grivet monkey kidney cells. Morris (1985) examined ten cell lines for their ability to grow

enteroviruses isolated from wastewater effluent. Eighty-two percent of isolates were positive in BGM cells, 73% in rhabdomyosarcoma (RD) cells and 64% in chimpanzee liver cells. BGM was also the most sensitive in the number of plaques counted.

Dahling and Wright (1986) carried out an extensive set of experiments to optimise the BGM line in respect of a number of assays for waterborne viruses, and made recommendations in respect of many cell culture and assay parameters, as well as doing a comparative virus-isolation study involving BGM cells and nine other cell lines. This work has become the accepted basis for many standard methods on detection of water-associated viruses.

Other cell lines have been investigated for their ability to support the growth of enteric viruses. Most of these studies have been directed at growing the more fastidious agents like rotaviruses and astroviruses, but Patel et al. (1985) carried out a large survey on the susceptibility of a range of lines to different enteroviruses, including all 31 serotypes of echovirus; they found that two lines, HT-29 and SKCO-1, had a markedly wider sensitivity for enteroviruses than primary monkey kidney (PMK) or RD cell cultures. They require a high seed density and do not grow quickly however, and perhaps this is why they have not found greater favour, along with CaCO2 cells (Fogh et al., 1977), which are of similar origin, in the detection of waterborne enteric viruses generally. This latter line, along with RD, BGM and human epithelial type 2 (HEp-2) cells, were used by Sedmak et al. (2005) in the detection of infectious reoviruses, enteroviruses and adenoviruses in a range of water types.

A549 cells, derived from human lung tissue, support the growth of some adenoviruses derived from water; they have also been used in the integrated cell culture-PCR technique (see below) for rapid detection of infectious adenoviruses (Greening et al., 2002).

There are two approaches to the enumeration of virus infectivity, plaque assay and liquid culture assay.

Plaque assay

The plaque assay is most frequently used for the enumeration of infectious waterborne enteroviruses. All the concentrate should be tested. In both cases plaques develop following incubation and may be counted as they become visible, in the case of enteroviruses usually after about 3 days. One plaque is taken as being the progeny of one infectious unit of the virus; this may be the same as one virus particle, but is unlikely given the aggregation of virions and their association with both organic and inorganic particulate matter.

Monolayer plaque assay

The virus concentrate is inoculated on to preformed monolayers in petri dishes or flasks and the cells are reincubated under an agar overlay until a c.p.e. is seen.

Plaques are counted daily starting at day 2. Since viruses multiply at different rates counting is continued after the first appearance of plaques. Echoviruses, for example, take longer to form plaques, if they do at all. The UK (Standing Committee of Analysts SCA, 1995) method recommends counting plaques for 2–5 days; the USEPA (2007) method suggests counting should continue for 12 days or until no new plaques appear between counts; Block and Schwartzbrod (1989) recommended 6–14 days.

Suspended-cell plaque assay

The suspended-cell assay (Cooper, 1967) increases the sensitivity of the ordinary plaque assay by five to eight times (Dahling and Wright, 1988). Five times as many cells are used, suspended in the agar instead of being in a layer underneath it and thus many more adsorption sites are available to any virus present. No prior establishment of monolayers or fluid changes are required since cells and concentrate are added to the culture vessels at the same time. It can only be used where the virus is liberated into the medium. The USEPA method recommends that the suspended-cell assay should be used where the level of indigenous virus is likely to be less than 5 pfu/ml.

Liquid assays

Cells under liquid media may support the growth of more viruses than cells growing in or under agar. Many enteroviruses, especially some echoviruses, do not form plaques and so will not be detected under agar; some viruses take a long time to produce a c.p.e. and agar cultures may have deteriorated too far to be useful. In these cases cells growing under liquid medium are used. Virus multiplication produces cell degeneration and often a c.p.e. characteristic of the infecting virus, so some idea may be gained of the agent at hand.

Most probable number assay

Lee and Jeong (2004) analysed source, finished, and tap water samples for enteroviruses and adenoviruses in a comparative study of MPN titres, obtained by normal observation of c.p.e. and MPN titres, obtained by integrated cell-culture PCR (ICC-PCR, see page 196). They found that by normal observation of c.p.e. 15% of cultures were positive, all from source water samples, and that titres ranged from 3.3–21 MPN/100 l water. In contrast, MPN by ICC-PCR gave 21% cultures positive and a narrower range of titres for source waters (4.5–10.2 MPN/100 l water). Target viruses were also found in the finished and tap waters (0–0.9 MPN/100 l). The range of viruses detected by ICC-PCR will be limited by the primers used, and in this study re-resting of the c.p.e.-positive dishes with reovirus-specific primers revealed 89% of cultures positive. The MPN approach can thus be

extended beyond the simple scoring of c.p.e.-positive cultures, but the limits of the detection system need to be kept in mind.

End-point dilution assay (TCD_{50})

Serial dilutions of the concentrate are inoculated into cell cultures and each culture is scored positive or negative after incubation. The titre is calculated (e.g. by the method of Reed & Muench, 1938) as the logarithm of the dilution of the virus producing a c.p.e. in 50% of the cultures. Though the method is simple and economic, its precision is difficult to evaluate. It is the least favoured of the three methods described.

Choice of assay method

Table 3 shows a comparison of assay methods in agar and under liquid media. It will be seen that there is no clear-cut best method. Plaque assays have greater advantages of individualising the pfu and providing entities (plaques), which are countable and directly related to the number of viruses (or aggregates). For many users this is an easier concept to grasp than the more abstract MPN or TCD_{50}. The MPN is more reliable than the others provided the number of cultures inoculated per dilution exceeds 30 (Block and Schwartzbrod, 1989).

Several comparative studies have been done on methods for the detection of enteroviruses in water. Morris and Waite (1980), for example, concluded that

Table 3

Characteristics of cell-culture assay methods

Attribute	Liquid	Agar
Range of viruses detected	Wide range possible	Non-plaquing viruses not detected
Blind passage	Blind passage possible to increase titres to detectable levels	Faster-growing viruses in a mixture overgrow slower ones, which are not isolated
Sensitivity	Greater sensitivity (especially than monolayers)	Sensitivity improved using suspended cell assay
Sub-culture	Sub-culturing easy	Sub-culturing difficult (impossible without c.p.e.)
Virus separation	Impossible to separate virus types	Separation of viruses possible by plaque picking
Statistical precision	Bad precision, large bias where few replicates used (as is usual)	Good, especially where all concentrate tested in one assay

monolayers were the least sensitive system, tube cultures were of intermediate sensitivity (for MPN determination, though only four tubes were set up per dilution) and the suspended-cell assay was the most sensitive. BGM cells gave the best recoveries and RD cells were variable. RD cells have been reported susceptible to Coxsackie virus A strains (Block and Schwartzbrod, 1989) though they are less sensitive than suckling mice, which is the only other system that supports growth of this group of viruses.

Virus infectivity may also be determined by immunofluorescence or immunoperoxidase techniques, which are particularly useful where limited replication occurs and a distinct c.p.e. is not produced. It may also be determined by molecular biology techniques such as the detection of virus-specific mRNA.

Identification

Viruses may be identified by the serum neutralisation test (SNT), immunoassay (Payment et al., 1982; Pandya et al., 1988), immunoperoxidase (Payment and Trudel 1985, 1987) or by genome-sequence analysis.

Flow cytometry has been used by Abad et al. (1998), Baradi et al. (1998) and Bosch et al. (2004) to sort rotavirus-infected $CaCO_2$ and MA-104 cells automatically.

Detection of viruses by molecular biology

The use of molecular biological detection techniques has permitted faster detection times and, in many cases, increases in sensitivity. It is particularly useful in the detection of viruses which do not multiply in cell culture and, since most of the gastroenteritis viruses fall into this category, this is an important development.

Techniques were first validated against cell-culture methods, which led to the development of molecular biology-based detection methods for enteroviruses in environmental concentrates, which were then taken forward in the development of methods for the detection of enteric pathogens.

Gene probes were the first approach made in the molecular biological detection of enteric viruses, and have been widely used (Dubrou et al., 1991; Enriquez et al., 1993; Margolin et al., 1993; Moore and Margolin, 1993). However they lack sensitivity and they have largely been superseded. Richardson et al. (1991) reviewed the water industry application of gene probes.

The PCR reaction (Saiki et al., 1988) overcomes these problems. Ease of use and increased sensitivity has made the technique commonplace in many laboratories. Problems encountered with PCR include the possible presence of fulvic and humic acids in the concentrates which inhibit the RT and/or polymerase reactions, and different solutions have been found including adsorption of the extracted RNA to silica (e.g. Shieh et al., 1995). Pallin et al. (1997) devised a method for recovering all the virus in a concentrate into a single PCR tube, which allowed direct comparisons of sensitivity with cell-culture methods where the whole of the concentrate is tested at one time.

The polymerase chain reaction

Numerous investigations have been done using RT-PCR to detect enteroviruses in different environmental samples, including river and marine recreational waters (e.g. Kopecka et al., 1993; Gilgen et al., 1995; Wyn-Jones et al., 1995), ground waters (Abbaszadegan et al., 1993; Regan and Margolin, 1997) and sludge-amended field soils (Straub et al., 1995). Detection of enteroviruses is a practical proposition since the picornavirus group contains well-conserved nucleotide sequences at the 5′ end of the genome, which are used to prepare pan-enterovirus primers, which are the starting reagents in the PCR. The technique has been extended to cover other virus groups present in water including adenoviruses (Puig et al., 1994), HAVs (Graff et al., 1993), astroviruses (Marx et al., 1995) and rotaviruses (Gajardo et al., 1995). Van Heerden et al. (2005a) compared two nested PCR methods for detection of adenoviruses in river and treated drinking water; the same group investigated swimming pools for the presence of adenoviruses by nested PCR (van Heerden et al., 2005b) and Jiang and Chu (2004) investigated rivers and coastal waters.

Lodder and de Roda Husman (2005) investigated the incidence of noroviruses, rotaviruses, enteroviruses and reoviruses in source waters and sewage. They developed a quantitative approach by analysing 10-fold serial dilutions of the extracted RNA and found noroviruses between 4 and 4900 "PCR-detectable units" per litre of river water. Higher titres were found in sewage. The Lordsdale strain of norovirus GGII was the most prevalent. Other viruses were also found. This approach to quantitation was extended and supported by statistical estimation by Westrell et al. (2006) who found norovirus titres up to 1700 (mean 12) PCR detectable units per litre in source water samples from the River Meuse. Borchardt et al. (2004) used a similar approach to estimate viruses transported by river water infiltrating municipal wells. Half the well water samples tested were positive for one or more of a range of enteric viruses, though no infectious virus was found.

Refinement of the RT-PCR and restriction enzyme analysis of amplicons has permitted the differentiation of virus types within the enterovirus group. Hughes et al. (1993) compared the nucleotide sequences of six Coxsackie virus B4 (CB4) isolates from the aquatic environment with those of four CB4 isolates from clinical specimens and found that the isolates fell into two distinct groups not related to their origin, and Sellwood et al. (1995) reported a system using restriction fragment length polymorphism analysis to discriminate between wild and vaccine-like strains of poliovirus.

Many (RT-)PCR-based analyses relate to outbreak investigations. Yeats et al. (2002) described an outbreak of illness in about 90 children followed by their attendance at a summer camp. Analysis by RT-PCR of stool specimens and drinking and swimming pool water samples revealed the presence of an enterovirus, later typed as echovirus 3 (EV3), in several of each kind of sample. Parshionikar et al. (2003) investigated an outbreak of gastroenteritis at a tourist saloon in the US and by RT-PCR found norovirus GGI.3 in both stool specimens and well water samples; Hoebe et al. (2004) conducted an epidemiological and virological

investigations into an outbreak of gastroenteritis in children who had played in a recreational fountain, and found the same norovirus sequences in the stool samples as in the water samples. During investigations into the outbreak of SARS, Wang et al. (2005) used semi-nested RT-PCR followed by sequencing to detect and identify SARS-CoV RNA in sewage.

The persistence of HAV in many communities (and therefore the local environment) led Morace et al. (2002) to develop a rapid method for monitoring its environmental presence at sewage treatment plants in Southern Italy. RT-PCR was used to detect HAV in sewage and effluent, and the sensitivity could be refined by the use of an antigen-capture stage. Grimm and Fout (2002) developed an RT-PCR method for the detection of HEV in spiked water samples.

Most (RT-)PCR methods focus on the polymerase-gene sequence of the virus. However, it is often necessary to refine the analyses to discern different strains of viruses (e.g. noroviruses). Bon et al. (2005), in a molecular epidemiological study of calicivirus cases and outbreaks over a 6-year period found it important to target the capsid gene region as well as the polymerase region in order to discriminate between strains in outbreaks where more than one strain was involved. It is likely that this approach, where capsid-gene sequence can be related to serological information, will become increasingly useful in molecular epidemiological studies.

In further modifications designed to reduce the analysis time, Papaventsis et al. (2005) developed a method for culturing enteroviruses directly on the filter following adsorption, then further analysed by RT-PCR, restriction fragment length polymorphism and sequencing. Coxsackie A, B, and polioviruses were found.

Multiplex PCR methods have been developed by several investigators, but must be employed with caution and the appropriate controls. Egger et al. (1995) devised a multiplex PCR for the differentiation of polioviruses from non-polioviruses, which made an important step in the accumulation of public health information, and multiplex (RT-)PCR reactions have been described by Fout et al. (2003), Formiga-Cruz et al. (2005), Denis-Mize et al. (2004) and Li et al. (2002) for a range of viruses in several different aquatic matrices.

There is an important use for RT-PCR in the screening of samples for enteroviruses; negative ones can be discarded and positives investigated further for presence of infectious virus.

Real-time-PCR

Real-time PCR provides the possibility to quantify the number of specific sequences in a sample and has been applied to a number of environmental virology investigations. Choi and Jiang (2005) used it to estimate human adenoviruses in 114 river water samples; 16% were positive, each containing between 10^2 and 10^4 adenovirus genomes per litre. Plaque assays on A549 and HEK-293 cultures were negative, suggesting that the viruses detected by quantitative PCR (QPCR) were non-infectious. The group went on to develop a TaqMan® assay for Ad40 in a variety of environmental samples (Jiang et al., 2005). Pusch et al. (2005) detected a

wide range of viruses in samples taken downstream of a waste-water treatment plant. By QPCR they estimated the range of titres of astroviruses to be $3.7 \times 10^3 - 1.2 \times 10^8$ and of noroviruses to be $1.8 \times 10^4 - 9.7 \times 10^5$ "genome equivalents" per litre. Laverick et al. (2004) devised a QPCR for noroviruses and used it in an in-depth 14-months surveillance of sewage, marine and riverine recreational waters. Absolute quantitation of template was obtained from a standard curve constructed using quantitative standards produced by cloning a modified sequence of the norovirus forward primer. Le Cann et al. (2004) devised a real-time RT-PCR for astrovirus in sewage, and reported mean values of 4.1×10^6 "astrovirus genomes" per 100 ml inlet sewage and 1.04×10^4 genomes in the effluent. HAV in polluted seawater was estimated by Brooks et al. (2005) to contain 90–523 copies of HAV per litre at one location and 347–2656 copies per litre at another, the range at each site being attributed to the variation in rainfall.

Nucleic acid sequence-based amplification

Nucleic acid sequence-based amplification (NASBA) amplifies target RNA at a single temperature (usually 41°C) and provides an alternative approach to the amplification of DNA sequences at varying temperatures. One advantage of this is that thermal stressing of blocks or carousels is avoided, another is that the time of the overall process is reduced compared with PCR. The progress of the reaction may still be monitored in real-time. The technique and its application to food and environmental materials have been reviewed by Cook (2003). Jean et al. (2002) used a NASBA coupled to an ELISA reaction for the detection of rotavirus in seeded sewage effluent samples, and Abd el-Galil et al. (2005) developed a NASBA reaction coupled to a molecular beacon for real-time detection of HAV in seeded surface water samples. The technique was used by Rutjes et al. (2005) for the detection of enteroviruses in surface water samples, though it was slightly less sensitive in detecting target virus sequences than RT-PCR. Rutjes et al. (2006) also developed a broadly reactive NASBA reaction for the detection of waterborne noroviruses and found it to be more sensitive than RT-PCR and, further, that the reaction was unaffected by inhibitors in the sample.

Molecular biology and virus infectivity

The principal drawback of molecular detection methods is that in their native form they give no indication of infectivity. Although knowledge of the structure of the target virus and some knowledge of how it behaves in the environment can lead to inferences about its infectivity, there is no direct indication of this in the data obtained from examination of an agarose gel or thermal cycler printout. This has led to much (mostly inconclusive) debate about the relationship between infectivity assay and molecular data. Difficulties in interpretation have arisen since the two kinds of information are not really comparable, being based on different properties of the virus. A number of approaches have been made to overcome this.

Integrated cell culture– PCR

Combination of cell culture with PCR has permitted detection of infectious virus even where it normally fails to produce a c.p.e., or where the c.p.e. takes a long time to appear. This technique, integrated cell culture–PCR (ICC-PCR, or ICC/RT-PCR for RNA viruses) has been used by several groups. Reynolds et al. (1996) and Murrin and Slade (1997) inoculated BGM cultures with concentrates and tested the supernatants at intervals up to 10 days. Virus was detectable by RT-PCR as early as 1 day post-inoculation, instead of more than 3 days by normal visualisation of c.p.e. Lee and Jeong (2004) compared ICC–PCR with total culturable virus assay for detection of enteroviruses, adenoviruses and reoviruses in water and found the ICC–(RT)PCR applicable as long as the limitations of the primers used were recognised; Spinner and Di Giovanni (2001) applied the technique to reovirus detection in drinking water sources. Jiang et al. (2004), investigating HAV in water, refined the technique in developing an integrated cell culture/strand-specific RT-PCR procedure capable of distinguishing between infectious and non-infectious HAV in spiked water samples. This involved initial propagation of infectious virus in cell culture followed by detection of the negative-strand RNA of the replicative intermediate using strand-specific RT-PCR. Greening et al. (2002) were able to detect naturally-occurring infectious enteroviruses and infectious adenoviruses in three days and five days respectively by ICC-(RT)PCR, compared with five days and 10 days if plaque assays or immunofluorescence were used. Cromeans et al. (2004) used a similar approach for the detection of HAV in water. The detection of the double-stranded replicative form of RNA viruses in cultured cells permits the conclusion that the virus is actually replicating and that it is not the sample inoculum which is being detected.

Detection of virus-specific mRNA

DNA viruses that do not replicate well in cell culture may be detected by the detection of virus-specific mRNA. This is particularly a useful approach in the detection of adenoviruses in water sample concentrates, particularly Ad40 and 41, which do not produce a clear c.p.e. Adenoviruses have a high particle/infectious virion ratio in culture (Brown et al., 1992), which is important when estimating the infectious viruses in a sample. Ko et al. (2003) developed a method for detection of infectious Ads2 and 41 in culture by detecting virus-specific mRNA, which is only produced during virus replication. The mRNA of Ad2 was detected as soon as 6 h after infection, and of Ad41 as soon as 24 h after infection of A549 cell cultures. This is in contrast to the development of up to 10 days for environmental isolates of "culturable" adenoviruses and several weeks (if at all) for the growth of Ad41 in culture. The group went on to develop the technique for use in detecting Ads in water sample concentrates and found they could detect as little as two infectious units (IU) Ad2 and 10 IU Ad41 in sample concentrates inoculated into cell cultures (Cromeans et al., 2004).

The combination of real-time PCR and detection of components produced only by replicating virus has significant meaning for the progress of detection of enteropathogenic viruses in aquatic matrices and the understanding of the significance of enteric viruses in the environment.

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