Detection methods of enteric viruses in a large volume of water

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1. Introduction

One of the most important roles of water supply and sewerage system is to protect public health from waterborne pathogens. At least, historically it was most important, and it is in some of the places in the world still now.

*Escherichia coli* are one of the best indicators to monitor the safety of the water, yet it cannot cover variety of pathogens existing in water. Especially enteric viruses are causing serious risk, whose fates are different from the classic indicator.

To determine the public health risk caused by human enteric viruses in water, a reliable, sensitive, and practical method for detecting small concentrations of viruses is needed.

2. Literature review of virus concentration method

A virus concentration method from water was firstly developed in 1940's. The first method reported was a pad method, where a pad was dipped in water overnight, followed by recovery of viruses adsorbed on the pad. This method is a practical method to detect viruses in water, but lacks information about amount of water.

A virus concentration method using a negatively charged membrane was developed by Wallis et al.(1967). Viruses are adsorbed onto negatively charged membrane under 25-50mM Mg$^{2+}$ condition, then eluted with beef extract solution at pH 9.5. Volume of water was available and increased. This negatively charged membrane method was modified using acid in place of Mg$^{2+}$ (Sobsey et al., 1973).

Positively charged membrane method was developed by Sobsey et al. (1979). Viruses are adsorbed onto positive charged membrane without addition of either Mg or acid. Viruses are eluted with beef extract solution. This method does not require any pretreatment to be used for a large volume of fresh water.

All these method depends on the mechanism of virus adsorption to elution from
membranes. Other methods developed include the cellulose coagulation method (Yano et al., 1993), glass wool and glass powder methods used as the adsorbent of viruses. In all these methods, whichever the adsorbents was, elution of viruses was done either by beef extract solution (3%, 1%) at pH 9~11 or sometimes by glycine buffer.

The elution process was based on the assumption that viruses are adsorbed due to hydrophobic interaction. These methods extract viruses by competition of adsorption sites with viruses.

The use of beef extract is good for following cultivation with mammalian cell but has inhibitory effect on PCR detection of viruses. Inorganic eluant is preferred for following detection of PCR so that the electrostatic interaction should be considered.

The electrostatic interaction was also working and considered in the adsorption step in these methods. Under neutral pH condition, viruses are negatively charged but are changed by multivalent cation. Viruses are positively charged under acid condition.

3. Development of a virus concentration method

A new virus concentration method using acid and alkali condition was developed by Katayama et al. (2002). Briefly, a sample with 25mM MgCl₂ was passed through an HA filter (Millipore), which was then rinsed with 200ml of 0.5mM H₂SO₄ (pH 3.0), followed by elution of viruses with 10 ml of 1mM NaOH (pH 10.8). The filtrate was recovered in a tube and neutralized, followed by centrifugation using a Centriprep YM-50 (Millipore) at 2500rpm for 10 min.

The recovery yields of the developed method was examined as well as the conventional method using 1MDS positively charged membrane using poliovirus as the model virus. As shown in Table 1, NaOH showed almost as high recovery as beef extract, where acid rinse promoted the recovery yields. This method showed high virus recovery from seawater as well. These phenomena can be explained by simple electrostatic interaction between viruses and the membrane.

This method was further modified by Haramoto et al. (2004). Pretreatment of membrane with AlCl₃ was applied for the membrane to have positively charged. Pre-sorption of Al enable viruses adsorb onto the membrane without addition of Mg. Acid rinse works in a same manner as Mg method.
Table 1. Recovery (%) of Poliovirus in water

<table>
<thead>
<tr>
<th>Type of Water</th>
<th>Positively charged filter (1MDS)</th>
<th>Negatively charged filter (HA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid</td>
<td>no</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>50%</td>
<td>83%</td>
</tr>
<tr>
<td>(50mM MgCl2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seawater</td>
<td>6%</td>
<td>94%</td>
</tr>
</tbody>
</table>

4. Detection of viruses in a large volume of water

The method developed was applied to the detection of NVs in tap water in Tokyo, Japan, from January 2002 to February 2003 (Haramoto et al., 2004). In total, 98 samples were collected whose volume ranged 100-500 L. The samples were concentrated using the Al pre-sorption method and subjected for virus detection. Virus genome was detected by real time PCR.

Norovirus genome was found in 10 out of 98 samples. Assuming the random sampling, the norovirus genome was found in tap water at a geometric mean concentration of 1/2800L. It should be noted that these genomes does not cause infectious risk directly because the water contained a good level of chlorine (0.6-1.0 mg/L) and the detection method of PCR does not reflect infectivity of viruses (Masago et al., 2006).

5. Discussion

The level of norovirus genome was very low in the tap water samples. The removal efficiency of viruses by the water treatment process is very important from a view point of risk management. Monitoring of the endpoint level of viruses is not a practical approach. Virus risk in the water supply industry should be based on the removal requirement of viruses in the treatment process and the monitoring of the source water. Effective methods to assess the virus removal in the real treatment plants should be developed for this purpose.
References


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Today’s Topic
1. Importance of viruses in water safety
2. Literature review of virus concentration method
3. Development of a virus concentration method
4. Detection of viruses in a large volume of water
1. Importance of viruses in water safety

Life cycle of pathogens of Fecal-oral infection

- Feces
- Flies
- Water
- Foods
- Hands

Increase in a warm-blooded animal body

Diffuse and Decrease in Environment

Water supply

Immune system

Sewer system

Wastewater Treatment

Heat

Water treatment

10^{10} - 10^0
Enteric Viruses

- φ 20～100nm in size, RNA or DNA coated with protein
- Propagate in animal intestine, emitted as feaces
- More than 100 types identified human as a host.
- Symptoms: Diarrhea, Vomit, fever, head ache, (Hepatitis, paralysis)
- Route of Infection: Food (oyster, etc), Drinking water, Swimming, Contact with infected individual

2. Literature review of virus concentration method
History of Virus Concentration Method from Water

- Pad method
  - Dipping a pad in water overnight, recover viruses adsorbed on the pad.

- Negatively charged membrane method
  Wallis et al., 1967
  - Viruses are adsorbed onto negatively charged membrane under 25-50mM Mg\(^{2+}\) condition, then eluted with beef extract solution pH 9.5
  - Volume of water increased, known amount.

Cont’d

- Negatively charged membrane method using acid in place of Mg\(^{2+}\) (Sobsey et al., 1973).
- Positively charged membrane method (Sobsey et al., 1979)
  - Viruses adsorbed to positive charged membrane without addition of either Mg or acid.
  - Viruses are eluted with beef extract solution.
Adsorption to Elution from

- Cellulose coagulation method (Yano et al., 1993)
- Glass wool and glass powder are also used as the adsorbent of viruses.
- Membranes are also used as the adsorbent.

Elution of viruses

- Beef extract solution (3%, 1%) pH 9～11
  - Sometimes glycine buffer
- Based on adsorption due to hydrophobic interaction.
- Extracting viruses by competition of site with viruses.
- Good for following cultivation with mammalian cell.
Mechanism of adsorption and desorption of viruses

- Applied and Theoretical aspects of Virus Adsorption to Surfaces (Gerba C.P. 1984)
  - Hydrophobic interaction for elution of viruses
    - Competitive extraction with beef extract
  - Electrostatic interaction for adsorption of viruses
    - Under neutral pH condition, viruses are negatively charged.
    - Viruses are positively charged under acid condition
    - Multivalent cation can change the surface charge of viruses

Reconcentration and purification

- Acid precipitation for beef extract eluate
  (Katzenelson et al., 1976)
- PEG precipitation
- Pro-Cipitate for selective precipitation of viruses.
- Ultrafiltration method using centrifugation
- Fleon/chloroform purification
- Gel filtration (Sephadex, or Sephacryl)
- Antigen-antibody purification method
Needs of new virus concentration method

- Inhibitory effect of beef extract eluate on PCR detection of viruses.
- Inorganic eluant for following detection of PCR.
- Electrostatic interaction should be considered.
  - Resulted in good recovery from seawater

3. Development of a virus concentration method

and Its Application to Detection of Enterovirus and Norwalk Virus from Coastal Seawater
State of the art of virus concentration methods

- Adsorption is easy, elution is difficult
- Lack of consideration of following virus detection by PCR
  - More severe condition can be applicable because of no need of maintaining virus culturability.
  - Avoid use of beef extract due to inhibitory effect on PCR
  - New acid or alkali condition for elution from membrane?

New Virus Concentration Method

<table>
<thead>
<tr>
<th>Sample (+Mg^{2+}, 25mM)</th>
<th>Acid rinse, pH 3.0(H_{2}SO_{4}) 200ml</th>
<th>Alkaline elution, pH 10.5(NaOH) 5ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negatively-charged membrane Millipore HA, pore size 0.45mm, diameter 47\mu m</td>
<td>Neutralization with Conc. TE buffer 0.05ml and H_{2}SO_{4} 0.025ml</td>
<td></td>
</tr>
</tbody>
</table>

How acid rinse works?

Adsorption

Acid rinse

Alkarine Elution

Recovery (%)

<table>
<thead>
<tr>
<th>Virus type</th>
<th>+ve filter (1MDS)</th>
<th>-ve filter (Millipore HA, 0.45 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beef extract</td>
<td>NaOH</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Acid</td>
</tr>
<tr>
<td>Pure water</td>
<td>Qβ</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Polio</td>
<td>50</td>
</tr>
<tr>
<td>Sea water</td>
<td>Qβ</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Polio</td>
<td>6</td>
</tr>
</tbody>
</table>
Improvement of the virus concentration method

- Pretreatment of membrane with AlCl₃, for the membrane positively charged.
  - Pre-sorption of Al enable viruses adsorb onto the membrane without addition of Mg.
  - Acid rinse works in a same manner as Mg method.

- Modification for field survey.
  - Concentrate viruses outside of laboratory.
  - Increase the area of the membrane
    - Disk filter to cartridge filter.

1. Al³⁺ Sorption on Membrane
2. Virus Adsorption
3. Acid rinse to remove Al³⁺
4. Elution of Virus in Alkali

\[ \text{AlCl}_3 \rightarrow \text{Al}^{3+} \rightarrow \text{Viruses adsorbed} \rightarrow \text{NaOH (pH 10.5)} \rightarrow \text{Concentrate} \]
Other methods developed

- Cultivate viruses without elution from membrane
  - Coliphage detection (Sobsey et al., 1995)
    - on host bacteria E. coli.
  - Enteric virus detection (Papageorgiou et al., 2000)
    - on mammalian cell layer

- Concentrate viruses, bacteria and protozoa at once by UF (Hill et al., 2007)
  - PCR inhibition is problem

Research work of Katayama’s

- Development of virus concentration method from water (Katayama et al., *Applied and Environmental Microbiology*, 68: 1033-1039, 2002)

- Field survey
  - Tap water and river water ( *Applied and Environmental Microbiology* 70: 2154-2160, 2004.)
  - Southeast Asian Water Environment ( *Water Science & Technology* Vol 54 No 3 pp 203–210, 2006.)
4. Detection of viruses in a large volume of water

Occurrence of Norovirus genomes in Tap water in Tokyo
Applied and Environmental Microbiology 70: 2154–2160, 2004

Outline of Tap Water Survey

- Sample Volume: 100-500 L
- 98 samples
- from January 2002 to February 2003
- All the concentrate was subjected for virus detection (9 portion each).
- Virus genome was detected by real time PCR.
Noroviruses were found in Tokyo tap water

<table>
<thead>
<tr>
<th>Season</th>
<th>Positive/tested NV-G1</th>
<th>Positive/tested NV-G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring</td>
<td>0/29 (0.0%)</td>
<td>3/29 (10.3%)</td>
</tr>
<tr>
<td>Summer</td>
<td>1/18 (5.6%)</td>
<td>0/18 (0.0%)</td>
</tr>
<tr>
<td>Autumn</td>
<td>0/19 (0.0%)</td>
<td>3/19 (15.8%)</td>
</tr>
<tr>
<td>Winter</td>
<td>3/32 (9.4%)</td>
<td>1/32 (3.1%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>4/98 (4.1%)</strong></td>
<td><strong>7/98 (7.1%)</strong></td>
</tr>
</tbody>
</table>

Norovirus genome was detected from tap water in Tokyo. PCR tubes were never opened to prevent carry-over contamination. Average concentration should be 1 genome /2800L. Infectious risk was calculated to be 1 infection / 200 people-year in the worst scenario, though there are a lot of unknown factors.

Norovirus concentration in Japan

<table>
<thead>
<tr>
<th></th>
<th>(E. coli) (CFU/ml)</th>
<th>(NV) (PDU/ mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td>0</td>
<td>(10^{-6.5})</td>
</tr>
<tr>
<td>Raw sewage</td>
<td>(10^5)</td>
<td>(10^3 \pm 1)</td>
</tr>
<tr>
<td>Treated wastewater</td>
<td>(10^3)</td>
<td>(10^1 \pm 1)</td>
</tr>
<tr>
<td>Coastal seawater</td>
<td>(10^1 \pm 1)</td>
<td>(10^{-2} \pm 2)</td>
</tr>
</tbody>
</table>
## Virus concentration in wastewater in Asian countries

<table>
<thead>
<tr>
<th></th>
<th>(CFU/mL)</th>
<th>(PDU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>NV G1</td>
</tr>
<tr>
<td>Japan (sewage)</td>
<td>$10^5$</td>
<td>$10^{-4}$</td>
</tr>
<tr>
<td>Beijing, China (sewage)</td>
<td>$10^{4-5}$</td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>Ho Chiminh, Vietnam (canal)</td>
<td>$10^3$</td>
<td>$10^1$</td>
</tr>
<tr>
<td>Jakarta, Indonesia (Flood water)</td>
<td>$10^4$</td>
<td>$10^{-1}$</td>
</tr>
</tbody>
</table>

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## Transport of Pathogen via Water

- Comprehensive Approach for Risk Management-

- Monitoring
- From Upstream
- Risk from drinking water
- By human, from human
- Barrier against Pathogens
- WWTP
- WTP
- zoonosis
- Recreational activity
- Risks via fishery