Pro’s and Con’s of Methods of Detection for Viruses in Foods

Dr Alvin Lee (Institute of Food Safety and Health, US)
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Alvin Lee, Ph.D.
Center Director / Associate Professor
Center for Processing Innovation
Institute for Food Safety and Health

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Human Enteric Viruses

*Caliciviridae* (Norovirus)
*Picornaviridae* (Hepatitis A virus)
*Astroviridae* (Astrovirus)
*Reoviridae* (Rotavirus)
*Hepeviridae* (Hepatitis E virus)
Norovirus

- First discovered in fecal samples after outbreak in Norwalk, Ohio (1968)
- Originally named “Norwalk virus”
- Transmission via human feces and vomit
  - ~30 million virions shed in one vomiting episode
- Infectious dose very low – ~18 virions
- Viron size – 27 nm
Typical Methods of Human Norovirus Detection

• Electron microscopy
  – Specialized equipment & technical expertise
  – Must have relatively high ($10^6$ or greater) virus titers
  – Cannot distinguish between genogroups/genotypes/strains

• RT-PCR
  – Relatively rapid and distinguishes between genogroups
  – Can detect low virus titers
  – Need for technical expertise and equipment

• EIA- Enzyme immunoassay
  – Rapid & minimal technical skill required
  – Current methods lack the sensitivity for low virus titers
  – Not broadly reactive
Laboratory Diagnosis of Enteric Viruses

• Food
  – Often present at low levels (<100 virions for infection)
  – PCR techniques have improved detection in past decade
  – Purification methods very important

• Stools
  – Best chance if specimen taken 48 to 72 h after onset of symptoms. Good results even 5 days after symptom onset (if tested)
  – Previously direct and immune electron microscopy often performed

Still no standardized method!
Recovery from Lettuce

Inoculated Leaf

Virus Elution with DMEM or 3% Beef Extract or Stripping Solution

Stomacher Bag with 25 ml of Eluent

Stomacher 1 min at 230 RPM

Transfer onto ice

Enumeration by Plaque assay

Centrifuge Tube with 25 ml of Eluent

Pusifier 1 min

Vortex 1 min

Stripping solution (0.04% KH₂PO₄, 1.01% Na₂HPO₄, 0.1% Triton X-100)
Recovery from Berries

60 ml of elution buffer (50 mM Glycine, 100 mM Tris, 1% beef extract) at pH 9.5 to 15 g of berries or vegetables

Agitation for 15 min

Filtration through a cell strainer

Centrifugation at 3500 x g for 15 min

Concentrate the virus particles by centrifugation at 3500 x g for 15-45 min using a centrifugal filter (100K NMWL)

RNA extraction and real-time RT-PCR

Plant RNA Isolation Aid

Pectinase treatment

Butot et al., 2007 AEM
ISO/CEN Method – ISO 15216-1:2017

• Replaces ISO/TS 15216-1:2013

• Quantification of HAV, NoV GI and GII RNA

• Applicable to foodstuffs (soft fruit, leaf, stem and bulb vegetables, bottled water, bivalve molluscan shellfish) and food surfaces.

• After viruses release from the test sample, viral RNA extracted by lysis with guanidinium thiocyanate and adsorption on silica.

• Target sequences within the viral RNA amplified and detected by real-time RT-PCR.
## ISO/CEN Method

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major viruses and food matrices included</td>
<td>Improvements of method may be halted</td>
</tr>
<tr>
<td>Simple set-up with detailed protocols on reagent and equipment</td>
<td>Does not include methods for processed food matrices</td>
</tr>
<tr>
<td>Increases confidence on the results due to use of controls and details on how to interpret results</td>
<td>High number of controls increases costs</td>
</tr>
<tr>
<td>International recognition of ISO method leading to increased implementation</td>
<td>Availability of commercial controls</td>
</tr>
<tr>
<td>Enables the formulation of guidelines</td>
<td>May lead to non-detection of low levels of virus in some specific matrices</td>
</tr>
<tr>
<td>Possibility to compare and evaluate results from different labs</td>
<td>Cannot distinguish between infectious and non-infectious particles</td>
</tr>
<tr>
<td>Facilities accreditation of laboratories for virus testing</td>
<td>Method complexity</td>
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</table>
Organization of the HuNV and MNV

Quantification and Confirmation

• Uses in outbreak investigations and provide data for risk assessments
• Quantification can be done by RT-qPCR with a standard curve
• Low levels of viruses can lead to variation of up to 1 log
• Viruses in foods are not evenly distributed
• No regulatory microbiological criteria
• Short amplicons may not be suitable for typing
Quantification and Confirmation

Pros

• Routine quantification provides data on baseline levels of viruses in food and will inform implementation of acceptable levels

• Systematic confirmation of RT-qPCR results by sequencing provides information on virus strain epidemiology

Cons

• Quantification by RT-qPCR is sensitive to inhibitors and has an unreliable accuracy for low levels of viruses

• Confirmation of RT-qPCR positive results by sequencing is difficult due to low sensitivity

• Quantification and confirmation increase cost

• Time consuming
Detection of Intact Virus Capsids

• Use of RNase treatments
• Photoreactive DNA binding propidium monoazide dye (PMA)
• Nucleic acid aptamers and phage display
• Histo-blood group antigen glycans
• Monoclonal and polyclonal antibodies
Traditional Ligands for Human Norovirus Recognition

• Monoclonal antibodies
  – Very specific but cumbersome to produce
  – Traditionally require selection and preparation of hybridoma and animal inoculation
  – Creating a cocktail that recognizes all norovirus genotypes and strains is difficult

• Polyclonal sera
  – Antigen expression and animal inoculation
  – Cross-reactive between genotypes
  – Lacks the specificity if genotype/stain identification is required
Monoclonal Human Single Chain Antibodies (scFv)

• Phage display of a large combinatorial library of synthetic scFv
  – Tomlinson I + J phage
• Biopanning and selection of those recognizing human norovirus Virus-Like Particles (VLPs)
• Bound phages can be eluted by trypsin addition or
  – Using HBGAs to select sequences that are at or near the HBGAs-binding site of the norovirus VLPs
• scFv’s of interest can be easily and rapidly produced, concentrated and purified for use in EIA
  – $\sim 10^{13}$ PFU from a 20 ml preparation of *E. coli*
Identification of human single-chain antibodies with broad reactivity for noroviruses

Wanzhi Huang¹, Moumita Samanta¹, Sue E.Crawford², Mary K.Estes²,³, Frederick H.Neil², Robert L.Atmar²,³ and Timothy Palzkill¹,²,⁴
Phage Detected in Clinical Samples Containing NV or NV VLPs
Phage Display of Peptides and scFv Human Monoclonal Antibodies

• Advantages
  – Easy and inexpensive to produce large quantities
  – $\sim 10^{13}$ PFU per 20 ml preparation of E. coli
  – Mutagenized clones can be produced to have
    • Greater binding affinity and modified specificity
  – Broadly reactive clones can be selected
  – M13 phages can be used directly for NoV capture

• Disadvantages
  – Stool matrix can interfere with ligand binding
  – Peptide ligands are not soluble- limits application
  – M13 phage may be present in stool- false positives
Aptamer Candidates

SMV-19
Kd = 191nM
ΔG = -8.41Kcal/Mol

SMV-21
Kd = 131nM
ΔG = -8.08Kcal/Mol

SMV-25
Kd = 232nM
ΔG = -7.36Kcal/Mol

SMV-26
Kd = 281nM
ΔG = -6.46Kcal/Mol
Binding of aptamer 25 to HuNoV Diluted Stool Specimens Derived From Outbreaks

T/N = Ratio of absorbance of test sample versus negative control

NVF = HuNoV-negative stool

HoV VLP = GII.4 Houston VLP

* Statistically significant differences between the ratios obtained from the positive stool specimens and the NVF (p<0.05); n=3.
Aptamer M6-2
Aptamer SMV-19

Moore et al., 2014, 2016
Porcine gastric mucin binds to recombinant norovirus particles and competitively inhibits their binding to histo-blood group antigens and Caco-2 cells

P. Tian, M. Brandl and R. Mandrell

Produce Safety and Microbiology Research Unit, Western Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Albany, CA, USA

Histo-Blood Group Antigen Assay for Detecting Noroviruses in Water

Jennifer L. Cannon and Jan Vinje

Department of Environmental Science and Engineering, University of North Carolina, Chapel Hill, North Carolina; Gastroenteritis and Respiratory Viruses Laboratory Branch, Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; and Atlanta Research and Education Foundation, Decatur, Georgia
**Figure 6:** Comparison of porcine gastric mucins or saliva coated magnetic beads capture of Norwalk virus-like particles with NV3901 coated magnetic bead with either rabbit sera as the primary detection mechanism. Legend indicates VLP concentration (micrograms/ml). Error bars represent the standard error within the means.
Magnetic Bead Capture of Native NV using PGM, Saliva or mAB in the Context of Food Samples

Figure 13: Capture of native virus (GI.1) using saliva, porcine gastric mucin-, or NV3901 coated magnetic beads suspended in neutral elution buffers with either lettuce or beef food extract. Amine and Tosyl labels are shown representing an uncoated bead. Legend indicates log genome copies/ml. Error bars represent the standard error within the means.
<table>
<thead>
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<th>Pros</th>
<th>Cons</th>
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<tbody>
<tr>
<td>Reduces overestimation of the number of infective particles</td>
<td>Broad range of reagents need to be develop</td>
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<td></td>
<td>Needs careful evaluation of protocols according to type of virus</td>
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<tr>
<td></td>
<td>Controls for infective and non-infective particles</td>
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<td>Increased costs compared to standard PCR method</td>
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Infectivity Assays

Replication assays
ICC RT-PCR
Digital PCR
NOROVIRUS, CULTURED.

A 48 YEAR MYSTERY SOLVED

Dr. Mary Estes and her Lab at Baylor College of Medicine have successfully cultured human norovirus in intestinal cells.

Scientists have been trying to culture the virus since the first norovirus outbreak was described in 1968.

The lack of an in vitro culture system has long been considered the single greatest barrier to norovirus research.

HISTORY OF NOROVIRUS RESEARCH

1929 RUMORED
Dr. John Zahorsky, a pediatrician, gives the name "winter vomiting disease" to a common childhood illness that causes vomiting, diarrhea, and a fever.

1972 VISUALIZED
The Norwalk virus is first seen by Dr. Albert Kapikian and his team at NIH using immune electron microscopy (IEM).

1992 CREATED
Empty shells of norovirus proteins (capsids) are artifically created by the Estes Lab. These virus-like particles are not infectious and enable studies of the capsid.

1968 DESCRIBED
An elementary school in Norwalk, OH experiences an outbreak of "winter vomiting disease." A virus is suspected.

1990 CLONED
The Norwalk virus genome is cloned, paving the way for an era of molecular studies.

2016 CULTURED
Human noroviruses are successfully cultured by Dr. Mary Estes and her team at Baylor College of Medicine.

WHAT IS NOROVIRUS?

- It is a tiny (≈27nm), spherical virus belonging to the Caliciviridae family.
- It is the most common cause of diarrhea in the world and the most common cause of foodborne illness in the United States.
- An estimated 1 in 15 Americans experience the virus each year, amounting to around 20 million cases.

Laboratory growth requires host cells (mammalian tissue culture cells)

• But human NoV does not grow; HAV takes 2 weeks
• Specialized staff and equipment required

CRFK  FRhK
Recovery of Viruses From Foods

**Elution** at high pH or alkaline environment

Mix and centrifuge to **remove** food debris

**Concentrate** viruses using either ultrafiltration techniques or 10% PEG6000 + 0.3M NaCl precipitation

**Analysis** – plaque assay or harvest RNA for qRT-PCR
Fomite Recovery Method

Surface Inoculation

Recovery Tools & Elution

Recovery Method

Cotton Swab  Composite Tissue  Sterile Sponge
Attributes of The Ideal Detection Method for Noroviruses

- Sensitive and specific
- Yet broadly reactive - detects all human genotypes
- Can be used for detection and genotyping
- Rapid or realtime results
- Low detection limit
- Easy to use, portable and no specialized equipment required
- Works on a variety of sample types (clinical, food or water) and with adopted sampling protocols
- Can distinguish between infectious and non-infectious virus
### Summary and Conclusions

#### Attributes of a good detector

<table>
<thead>
<tr>
<th>Attribute</th>
<th>scFv</th>
<th>peptide</th>
<th>aptamer</th>
<th>PGM</th>
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<tbody>
<tr>
<td>Sensitive</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Specific</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>❌</td>
</tr>
<tr>
<td>Broadly reactive across genogroups/types</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Can be used for genotyping</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>❌</td>
</tr>
<tr>
<td>Rapid &amp; cheap production &amp; modification</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>❌</td>
</tr>
<tr>
<td>Commercially available currently</td>
<td>❌</td>
<td>❌</td>
<td>❌</td>
<td>✔</td>
</tr>
<tr>
<td>Tested in a variety of sample matrixes</td>
<td>❌</td>
<td>❌</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Low limit of detection with complex matrix</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>~</td>
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<tr>
<td>Integration in a realtime sensor platform</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Infectivity discrimination</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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</table>

- ✔: Available
- ❌: Not available
- ?: Unknown
Acknowledgements

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Thank you & Questions

Alvin Lee
allee33@iit.edu