식품중 노로바이러스 분리 및 농축기술

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Norwalk virus(NV)와 Norwalk-like virus(NLV)에 의한 식중독은 전 세계적으로 가장 빈번히 일어나는 식품기인성 질환이며 국내에서도 최근 이러한 노로바이러스에 의한 식중독 발생이 급증하고 있는 추세이다. 여러 선진국에서 패류 식품의 생산, 유통, 소비의 전 단계에 대하여 A형 간염바이러스와 노로바이러스 신속검출법 개발 및 불활성화 기술 확보를 위한 연구가 활발히 이루어지고 있으며, 여러 종류의 바이러스의 검출기법 중 RT-PCR(Reverse Transcription-Polymerase Chain Reaction)법이 가장 검출 민감도가 좋은 것으로 판명되었으나, 아직까지 문제가 되고 있는 식품군에 직접 적용하기에는 해결되어야 할 문제점이 많다. 국내의 경우 장염 바이러스가 급증하면서 환자의 가검물에서는 노로바이러스가 검출되고 있으나, 추정되는 문제 식품군에서의 검출은 거의 이루어지지 못하고 있는 실정이다.

바이러스성 식중독의 경우 식중독 사고 건수 당 환자수가 대규모인 경우가 많으므로 이에 대한 적극적인 예방 및 관리 대책이 더욱 중요하다. 바이러스성 식중독의 예방을 위하여 보균자의 관리 다음으로 가장 중요한 것은 오염된 식품에서 신속 정확하게 식중독 바이러스를 검출하는 것이다. 특히 식품 내에 매우 낮은 농도로 존재하는 바이러스 입자를 분리하고 농축하는 기술의 확보는 식중독성 바이러스 검출법 개발에서 필수적인 요소라 할 수 있다. 특히 패류 식품은 filter feeding의 특성 상 소화기내에 바이러스를 축적하여 문제가 되고 있으며, 살균처리 없이 샐러드나 쌈 등으로 소비되는 신선야채 등의 경우 보균자에 의한 오염이 직접적으로 문제를 일으킬 수 있다. ELISA법의 경우 바이러스 입자 검출한계가 10^5particles per gram이므로 노로바이러스 감염이 10^2 이하의 숫자로도 이루어질 수 있다는 연구결과를 감안할 때, 현재로서는 RT-PCR법과 같은 유전자 검출법이 식품 중에 낮은 농도로 존재하는 바이러스를 검출하기 위한 최적의 방법이라고 볼 수 있다. RT-PCR법의 정확성을 높이기 위해서는 먼저 식품에 존재하는, RT-PCR 효소 반응에 저해를 나타내는 간섭물질들이 최대한 제거된 상태의 순수한 바이러스 입자만을 분리하고 농축하는 것이 중요하다. 세균과는 달리 enrichment 단계가 불가능하므로 식품 중 존재하는 바이러스입자를 최대한 손실 없이 회수하는 것도 필수적이다. 또한 RT-PCR법은 이미 불활성화 되어 감염력이 없는 바이러스 입자에 대해서도

false-positive를 나타내는 결점이 있는데, 이에 대한 극복 방안도 필요하다.

식품 중에 존재하는 바이러스를 검출하기 위한 첫 번째 단계는 liquefaction 단계로, 식품으로부터 바이러스 입자를 떼어내는 단계이다. 바이러스입자가 식품 내부에 존재하는지 아니면 외부에 존재하는지에 따라 grind, homogenize 또는 rinse 하는 등의 방법이 사용된다. 두 번째는 식품 현탁액을 제거하고 바이러스 입자를 분리하는 *clarification* 단계이다. 이를 위하여 filtration과 cetrifugation을 주로 이용한다. 세 번째 단계는 concentration 단계로서 전체 volume을 PCR을 수행할 정도의 양으로 줄이는 것이다. Chemical precipitation, hydroextracton, ultracentrifugation, ultrafiltration, 그리고 antibody capture (immunomagnetic separation 포함) 등의 다양한 방법이 이용될 수 있다. 마지막 단계는 최종 detection 단계로서 infectivity assay, electron microscopy, immunofluorescent confocal scanning microscopy, enzyme immunoassay, hybridization assay, RT-PCR, NASBA(Nucleic Acid Sequence Based Amplification) 등의 방법으로 해당 바이러스의 존재를 확인한다.

식품에 오염된 노로바이러스는 매우 낮은 개수로도 섭취 시 전염될 수 있으며 상당 기간 감염활성을 유지하므로, 식품 내에 극미량 존재하는 바이러스 입자를 분리하고 농축하여 정확하게 검출하는 기술의 개발은 식품위생학적인 관점에서 매우 중요하다. 노로바이러스는 host cell line이 없어서 배양이 불가능하므로 정밀하게 바이러스 농도에 따른 분리 농축 실험을 수행하는 것이 용이하지 않다. 그러므로 노로바이러스를 식품으로부터 검출하는 최적의 방법을 확보하기 위한 기반 실험으로서, *Caliciviridae* family에 속하며 그 중에서 배양이 가능한 Feline Calicivirus (FCV)를 surrogate model로 하는 연구는 매우 효과적이다. 식품으로부터 노로바이러스를 분리하고 농축하는 기술 개발 연구의 첫 단계로 Crandell-Reese feline kidney(CRFK) 세포주에서 FCV배양, plaque assay, RT-PCR 검출 조건 등을 확립한 후에, 다양한 바이러스 농축법 중에서 가장 간편하게 현장 적용이 가능한 PEG법, Organic Flocculation법, Filter-adsorption-elution법 등을 이용하여 virus titer 별 FCV 농축효율을 분석하는 실험이 필요하다. Filter-adsorption-elution법 중에서 가장 널리 쓰이는 양전하를 띄는 1MDS filter(CUNO, Meriden, Conn. USA)를 사용하는 방법에서 elution buffer 농도 및 침전 조건 등을 최적화하고, 향후 filter의 국산화 방안 등이 모색되어야 하며, 진행된 연구 결과를 종합하여 우선 가장 문제가 되고 있는 생굴에서 노로바이러스를 효율적으로 검출하는, 현장적용이 간편한 검출방법이 개발되어야 한다.

이러한 연구를 기반으로, 농축산물 및 해산물의 수입이 급증하고 있는 시기에 발맞추어 수입 식품 내에 존재하는 식중독성 바이러스를 신속 정확하게 검출하는 기술을 국내실정에 맞게 개발함으로서 국민 보건을 증진시키는데 기여할 것이며, 현장적용이 용이하고 간편한 검출기술을 개발함으로서 국내 현실에서도 패류에 대한 위해미생물 검사 항목에 바이러스를 추가하는데 일조할 것으로 기대한다. 바이러스성 식중독을 차단하기 위해서는 패류 식품뿐만 아니라 다양한 비가열식품에 대하여 유통, 소비 등 여러 단계에서의 오염 상태에 대한 기본 통계자료를 확보하는 것이 국가적 연구 차원에서 필요하다.

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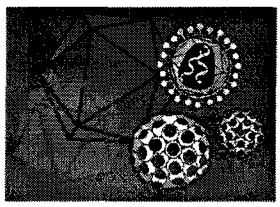
Contents

Viruses transmitted via foods

VIRUS

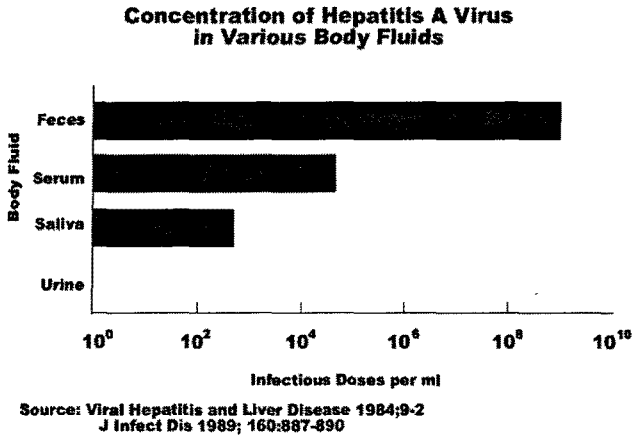
A group of infectious agents characterized by their inability to reproduce outside of a living host cell. Viruses may subvert the host cells' normal functions, causing the cell to behave in a manner determined by the virus.



Transmission of foodborne viruses

- A wide range of diseases in humans
- Typical host range and cell preference
- Viruses can be transmitted by
  - droplets generated by infected person
  - fecal contamination
  - contact with blood
  - contact with infected animal
  - vectors (e.g. foods)

Fecal oral transmission



Source: Viral Hepatitis and Liver Disease 1984;9-2
J Infect Dis 1989; 160:887-890

Genome & Physical Characteristics			Virus Family
Type	Nucleic Acid Description	Envelope	Name
DNA	ds	enveloped	Baculoviridae
			Herpesviridae
			Iridoviridae
			Poxviridae
			"African Swine Fever Viruses" (unnamed family)
		nonenveloped	Adenoviridae
			Caulimoviridae
			Myoviridae
			Phycodnaviridae
			Tectiviridae
			Papovaviridae
	ss	nonenveloped	Circoviridae
			Parvoviridae
	ds/ss	enveloped	Hepadnaviridae

Genome & Physical Characteristics					Virus Family
Type	Nucleic Acid Description			Envelope	Name
RNA	ds	positive	nonsegmented	enveloped	Cystoviridae
			segmented	nonenveloped	Birnaviridae
					Reoviridae
	ss	positive	nonsegmented	enveloped	Coronaviridae
					Flaviviridae
					Togaviridae
					"Arterivirus" (a floating genus)
				nonenveloped	Astroviridae
					Caliciviridae
					Picornaviridae
					Potyviridae
			DNA step in replication	enveloped	Retroviridae
		negative	segmented	enveloped	Orthomyxoviridae
			nonsegmented	enveloped	Filovoviridae
					Paramyxoviridae
					Rhabdoviridae
		Negative & ambisense	segmented	enveloped	Arenaviridae
					Bunyaviridae

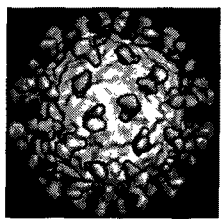
Classification of most foodborne viruses

Particle diameter (nm), nucleic acid strands	Nucleic acid type	
	RNA	DNA
25-35, single	Astroviruses Caliciviruses Picornaviruses Hepatitis E virus	Parvoviruses[a]
70-90, double	Reoviruses Rotaviruses	Adenoviruses

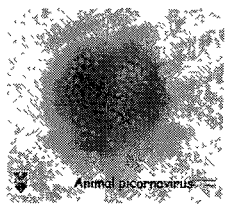
[a]Rarely or never foodborne

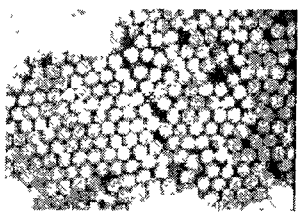
Picornaviruses

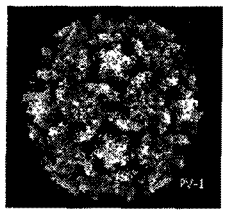
- Hepatitis A (HAV)
- Poliovirus type 1 (PV-1)



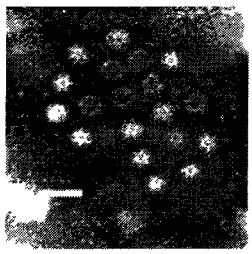
Rhinovirus 14 complexed with the ICAM-1 receptor, as solved by cryo-electron microscopy and image reconstruction (right)

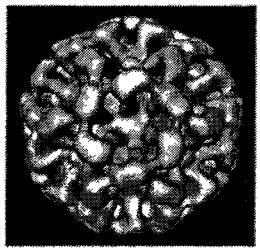


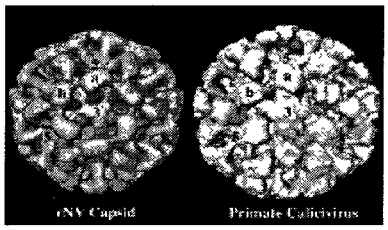




Feline Calicivirus (FCV) as surrogate for NLVs







Foodborne disease outbreaks, United States, 1993-1997[a]

Etiology	Outbreaks		Cases		No. of deaths
	No.	%	No.	%	
Bacterial	655	23.8	43,821	50.9	28
Chemical	148	5.4	576	0.7	0
Parasitic	19	0.7	2,325	2.7	0
Viral	56	2.0	4,066	4.7	0
Confirmed etiology	878	31.9	50,788	59.0	28
Unknown etiology	1,873	68.1	35,270	41.0	1
Totals	2,751	100.0	86,058	100.0	29

[a]CDC, Surveillance for foodborne-disease outbreaks-United States, 1993-1997, MMWR, 2000, 49(SS-1):1-62

Estimated foodborne illnesses, hospitalization, and deaths in the United States from viral pathogens[a]

Viral pathogens	Illnesses	Hospitalization	Deaths
Norwalk-like viruses	9,200,000	20,000	124
Rotavirus	39,000	500	0
Astrovirus	39,000	125	0
Hepatitis A	4,170	90	4
Subtotal, viral	9,282,170	20,715	128
Grand total foodborne[b]	13,814,924	60,854	1,809

[a]Sources : Mead *et al.*, Food related illnessand death in the United States, *Emerg. Infect. Dis* 1999, 5:607-625

[b]Foodborne illness, all causes.

Statistics of known Foodborne pathogens*

Agent	Estimated cases (million)	%
Bacterial	5.2	13.5
Parasitic	2.5	6.5
Viral	30.9	80
Total	38.6	100

*Center for Diseases Control and Prevention (CDC) 1999

Foodborne Viral infection vs Bacterial infection

- Smaller numbers are needed to produce illness
- Higher numbers are shed in the stools
- Virus cannot replicate in food or water
- Foodborne viruses typically are quite stable outside the host

Characteristics of Norwalk-like viruses

Characteristic	Observation	Consequences
Low infectious dose	$<10^2$ viral particles	Permits droplet or person-to-person spread, secondary spread, or spread by foodhandlers
Prolonged symptomatic shedding	≤ 2 weeks	Increased risk for secondary spread or problems with control regarding foodhandlers
Environmental stability	Survives ≤ 10 ppm chlorine, freezing, and heating to 60°C	Difficult to eliminate from contaminated water; virus maintained in ice and steamed oysters
Substantial strain diversity	Multiple genetic and antigenic types	Requires composite diagnostics; repeat infections by multiple antigenic types; easy to underestimate prevalence
Lack of lasting immunity	Disease can occur with reinfection	Childhood infection dose not protect from disease in adulthood; difficult to develop vaccine with lifelong protection

Source : Daniels N. A. *et al.*, J Infect Dis, 2000, 181:1467-1470

Outbreak examples of gastroenteritis caused by Norwalk-like viruses

Year	Setting	Vehicle	Situation	Unique Feature
1993	Multistate and Louisiana	Oysters	Multistate outbreak traced to oysters in Louisiana	Sequence analysis linked outbreaks in multi states; virus found in oysters
1996	United States and Canada	Well water	Multiple outbreaks among American tourists visiting a bus stop in Canada	Sequence analysis linked tourists to common water source; first detection of outbreak strain in both patients and water
1998	Europe and Canada	Raspberries	International outbreak in five countries traced to raspberries from Slovenia	Outbreak followed distribution of contaminated product
1999	United States	Delicatessen meal	Diners ill from delicatessen meal; foodhandler implicated	First detection of implicated virus on food surface; same virus detected among patients

Source : Daniels N. A. *et al.*, Foodborne outbreak of gastroenteritis associated with Norwalk- like viruses, J Infect Dis, 2000, 181:1467-1470

Detection of foodborne viruses

Detection of foodborne viruses

1. Sampling
2. Liquefaction of solid foods
3. Clarification of food suspension
4. Concentration of food extracts
5. Removal of contaminants and Detection

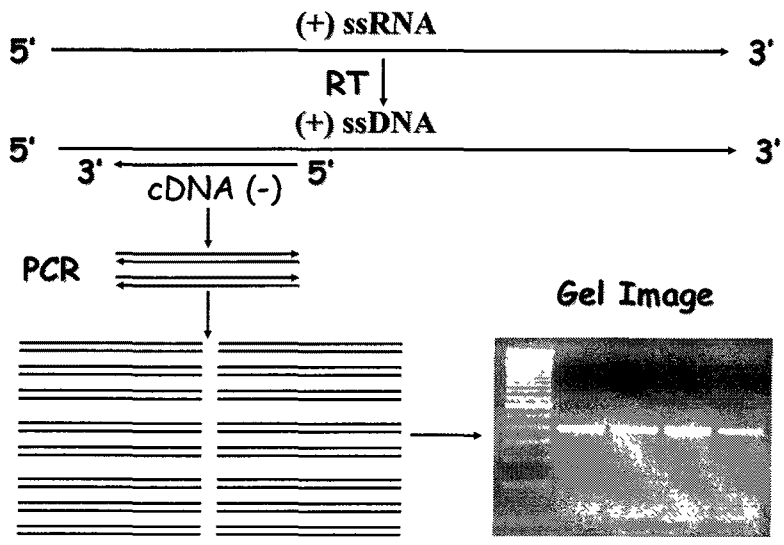
Detection methods of foodborne viruses

- Infectivity assay
  - ~ highest sensitivity
  - ~ specific host cells
  - ~ cytopathic effect (CPE)
- Alternative assays
  - ~ morphology
    - * Electron microcopy (EM)
    - * Immune confocal microscopy
  - ~ Enzyme immunoassays (ELISA)
  - ~ nucleic acid
    - * RT-PCR
    - * NASBA

Detection limits of the assay methods

Principle of assay	Example	Infectivity test	Detection limit (particles per gram)
Visualization of particles	EM	No	10^{5-6}
Detection of viral protein	ELISA, latex test	No	10^{5}
Detection of genome	Probe hybridization	No	10^{4}
Detection of genome	RT-PCR	No	10^{1-3}
Infectivity on living cells	Cell culture isolation	Yes	10^{0-1}
Measurement of exposure	Antibody assays	Yes	Varies by type of antibody

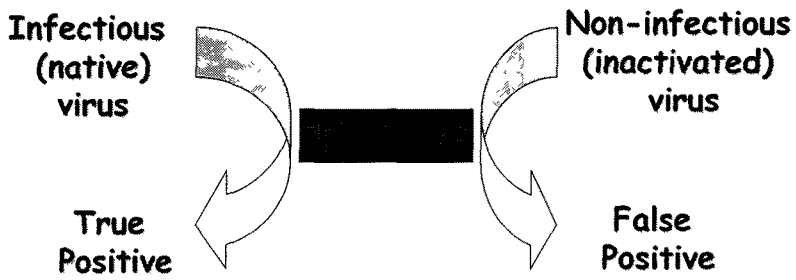
Reverse transcription – polymerase chain reaction



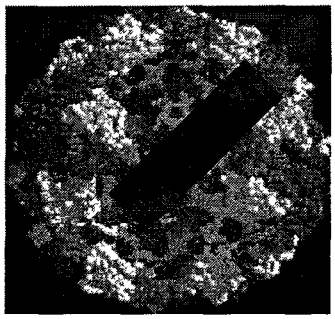
Problems of detecting FBVs

- Burdensome, costly, unavailable, unattempted
- Low sensitivity for clinical specimens & large volume of food or water
- No laboratory host cells for HAV and NLVS
- RT-PCR is a leading detection method
  - ~ fast, specific, sensitive
  - ~ cannot differentiate infectious and non-infectious virus

RT-PCR cannot differentiate whether detected virus represents threat to health !



Virion particle = Capsid + RNA



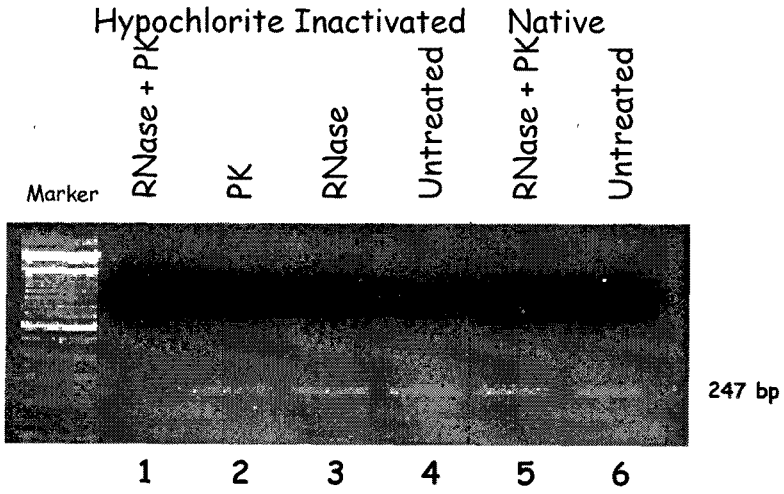
Inactivated

Capsid + RNA

Non-functional

False positive RT-PCR

Hypochlorite inactivation of hepatitis A virus

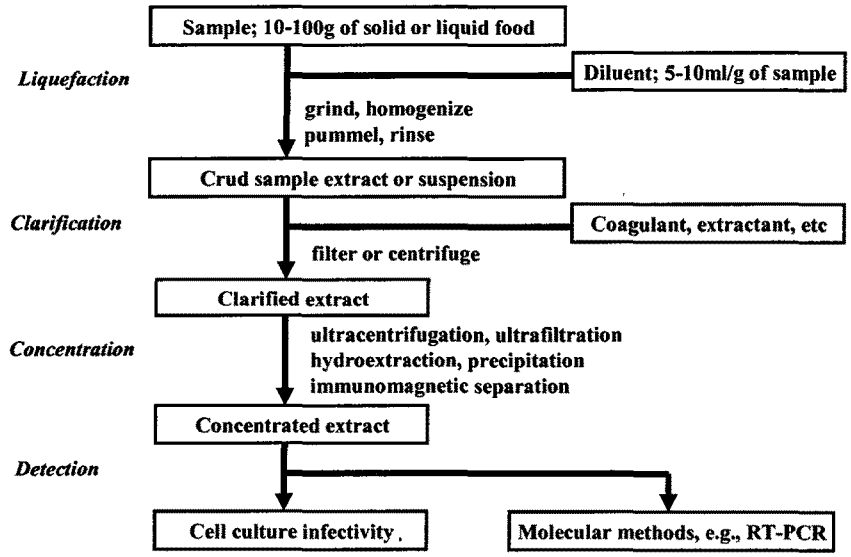


Separation and Concentration of virus particles from foods

Detection of foodborne viruses

1. Sampling
2. Liquefaction of solid foods
3. Clarification of food suspension
4. Concentration of food extracts
5. Removal of contaminants and Detection

Extraction and Concentration of Viruses from Foods



Elution-Precipitation Method for Extraction of Viruses from Foods

	Purpose of Step
Food Sample (25 to 100g)	
↓	
Dilute sample 1:5 to 1:10 with 50-90mM glycine-NaOH plus 0.8% NaCl, pH8.8-9.5 Homogenize or mix for 1 to 2 min at 0-22°C	To elute viruses from foods at high pH
↓	
Add Cat-Floc to 0.1 to 0.5%, Let settle for 20 min Centrifuge at 10,000xg for 15 min, Retain supernatant	To remove extraneous food solids by flocculation
↓	
Add beef extract, meat extract, or skim milk Adjust pH to 3.5 to 4.5 Mix slowly for 15 min Centrifuge at 10,000xg for 15 min	To add carrier protein and acidity to precipitate protein and absorbed viruses
↓	
Resuspend pellet in 15 to 20 ml of 0.1-0.15 M Na_2HPO_4, pH 9.0	To resuspend viruses at alkaline pH
↓	
Treat with antibiotics or filter	To remove non-viral contaminations
↓	
Assay for viruses	

Adsorption-Elution-Precipitation Method for Extraction of Viruses from Foods

	Purpose of Step
Food Sample (25 to 100g)	
↓	
Dilute sample 1:7 to 1:10 with water Homogenize for 2 min at 0-22°C Adjust pH 4.5 to 5.0	To adsorb viruses to solids at low pH
↓	
Reduce conductivity to <2g/L NaCl Centrifuge at 2,000xg for 15 min	To pellet viruses, protein, food solids
↓	
Resuspend pellet in 7 to 10 times the original sample volume of 50-90 mM glycine plus 0.8% NaCl, pH 7.5 to 9.5 Centrifuge at 2,000xg for 15 min and retain sediment	To elute viruses from the solids
↓	
Adjust to pH 4.5 with 1N HCl, Mix for 15 min Centrifuge at 2,000xg for 15 min and retain sediment	To acidify suspension and precipitation proteins and attached viruses
↓	
Resuspend in 15 ml 0.15 M Na_2HPO_4, Adjust to pH 7.2 to 7.5 with 1N HCl, Add Cat-Floc to 0.1 to 0.5% Centrifuge at 2,000xg for 15 min	To adjust pH and clarify
↓	
Treat with antibiotics or filter	To remove non-viral contaminations
↓	
Assay for viruses	

HAV concentration method using urea arginine phosphate buffer (UAPB)

I. Elution:

1. Filter 100 ml sample using positively charged membrane filter (Cuno64085-02-1MDS)
2. Discard the filtrate
3. Add 10 ml elution buffer (UAPB)
4. Collect the filtrate

II. Precipitation:

1. Add 200 ul of 1M $MgCl_2 \cdot 6H_2O$ to 10 ml of collected filtrate and mix
2. Centrifuge at 4500 rpm for 15 min.
3. Discard the supernatant and collect the pellet

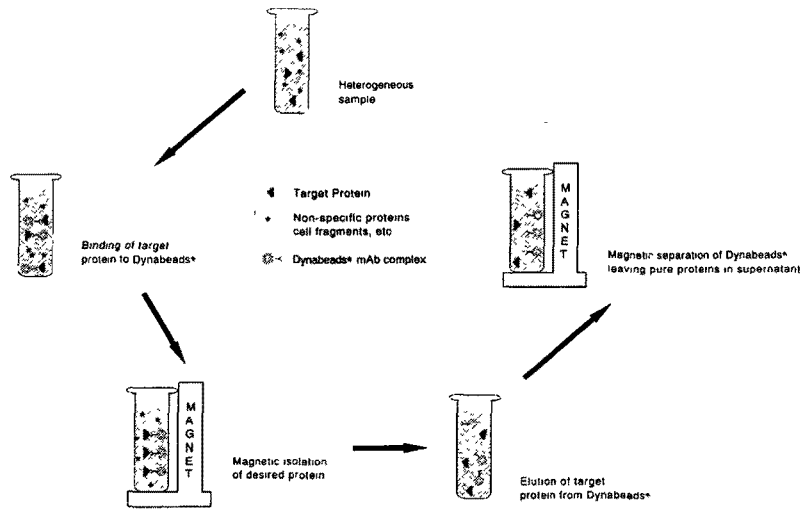
III. Dissolution:

1. Add 1 ml McIlvaine's buffer, pH 5.0 to the pellet and shake it

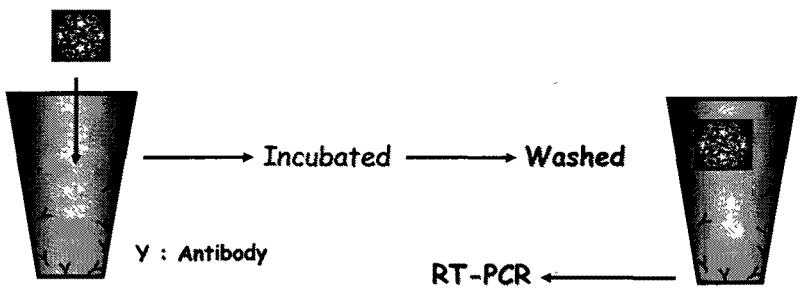
IV. RT-PCR

(http://www.epa.gov./nerlcwww/chap6.htm)

Immunomagnetic separation of virus particles



Antigenic specificity



Antigen capture-polymerase chain reaction (AC-PCR)

Prevention and Inactivation of foodborne viruses

CBNU

Prevention and Inactivation

- **Preventing contamination**
  - * Human fecal contamination
  - * Good personal hygiene
  - * Vaccination
- **Inactivation of viruses in foods**
  - * HACCP
  - * GMP

Survival of foodborne viruses

(Data from Enriquez et al 1995 kurdziel et al 2001 Mbithi et al 1991, and Ward and Irving 1987)

Two essentials for the prevention of foodborne viruses

- **Water used in the preparation of food should be of drinking water quality.**
- **Guidelines specifically aimed at the reduction of viral contamination are needed**

Inactivation of foodborne viruses

CBNU

Functions of capsid (food virology standpoint)

- Protection of RNA genome
- Specific attachment to receptors of host cell
- Antigenic specificity

Inactivation methods

Ultraviolet light (UV)
- **Low pressure mercury lamp**
- **254 nm (germicidal)**
- **UVX radiometer**
- **up to 120 mWs/cm^2**

Hypochlorite
- **NaOCl -> Free chlorine**
- **1.20-1.25 ppm 5°C**
- **DPD calorimetry**
- **$Na_2O_2S_3$**

Heat
- **37°C & 72°C**
- **PBS**
- **Preheated**
- **Prechilled**

Homologous cells for virus attachment

Virus	Cell culture
HAV	FRhK-4 (Fetal rhesus monkey kidney cell)
PV-1	FRhK-4 (Fetal rhesus monkey kidney cell)
FCV	CRFK (Crandell reese feline kidney Cell)

The plaque assay technique

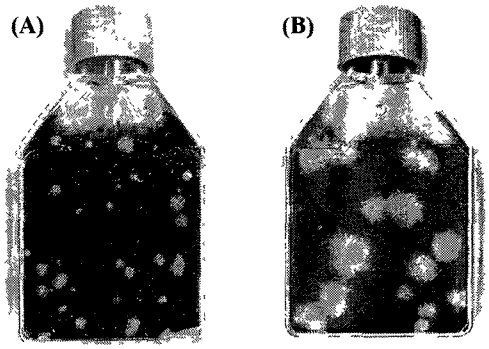
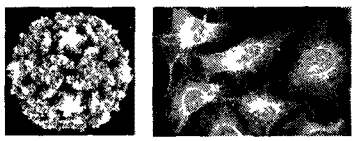


Fig. The plaque technique in 25cm² polystyrene tissue culture flask – feline calicivirus (FCV) in Crandell Reese feline kidney(CRFK) cell (A) and poliovirus type I (PV-1) in fetal rhesus monkey kidney(FRhK-4) cell (B). Infected cells were incubated for 2 days and 3 days, respectively, under agarose overlay, and then fixed with formaldehyde and stained with crystal violet.

Specific attachment to receptors of host cell



Incubated

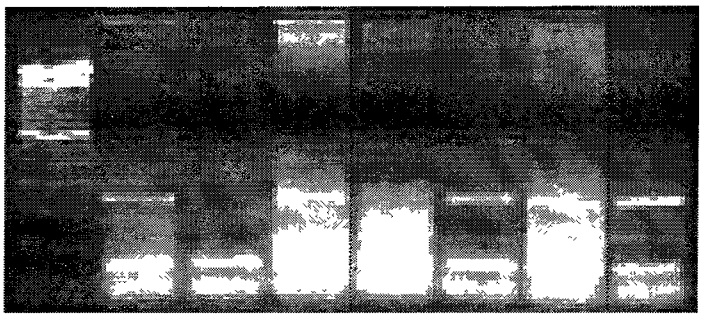
Washed

RNA extraction

RT-PCR of viruses

Specificity of HAV attached to the homologous cell monolayer

Cell culture	Homologous			Heterologous			
Lysate	+	-	+	+	-	+	RT-PCR Control
Washed	+	-	-	+	-	-	
Wash-off	-	+	-	-	+	-	



247 bp

M 1 2 3 4 5 6 7

Food processing and remaining risks

Process	Example of food product	Virus inactivation (log10)	Risk of infection of consumer if viruses are present before processing	Likelihood of presence after processing
Drying (spray and freeze drying)	Dried milk, instant dried soups, dessert mixes, chocolate	HAV, FeCV<1 (Doultry *et al.*, 1999, Mbithi *et al*, 1991)	High	Unlikely
Freezing	Ice-cream, frozen desserts (containing fruit)	HAV, PV, FeCV<1 (Hollinger *et al*, 1996)	High	Possible
Acidification	Fruit juices, still fruit drinks	NLV pH 2.7, 3h incomplete (Dolin *et al.*, 1972) HAV pH 1, 5h incomplete (Hollinger *et al*, 1996)	Medium	Possible
Depuration of oysters and mussels	Oysters and mussels	NLV incomplete (Grohmann *et al*, 1981)	High	Likely
High hydrostatic pressure (600Mpa, 1h)	Liquid food	PV<1 (Wilkinson *et al*, 2001)	High	

Conclusions

Future considerations for foodborne virus research

- **Development of efficient detection methods**
- **Standardization of virus survival stability and inactivation**
- **Development of cell culture system for various foodborne viruses**
- **Information on virus survival in various foods**
- **More information on virus shedding by infected person**