Journal of Food Protection, Vol. 67, No. 10, 2004, Pages 2315–2319

Research Note

Round-Robin Comparison of Methods for the Detection of Human Enteric Viruses in Lettuce

FRANÇOISE S. LE GUYADER,[1]* ANNA-CHARLOTTE SCHULTZ,[2] LARISSA HAUGARREAU,[1] LUCIANA CROCI,[3] LEENA MAUNULA,[4] ERWIN DUIZER,[5] FROUKJE LODDER-VERSCHOOR,[5] CARL-HENRIK VON BONSDORFF,[6] ELIZABETHA SUFFREDINI,[3] WIM M. M. VAN DER POEL,[5] ROSANNA REYMUNDO,[7] AND MARION KOOPMANS[5]

[1]IFREMER, Laboratoire de Microbiologie, BP 21105, 44311 Nantes cedex 03, France; [2]Danish Institute for Food and Veterinary Research, Department of Microbiological Food Safety, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark; [3]Laboratorio Alimenti, Istituto Superiore di Sanita, Viale Regina Elena 299, 00161 Rome, Italy; [4]Helsinki University Central Hospital, Division of Virology, Haartmaninkatu 3, 00290 Helsinki, Finland; [5]National Institute for Public Health and the Environment, Diagnostic Laboratory for Infectious Diseases and Perinatal Screening and Microbiological Laboratory for Health Protection, LIS, Pb 22, P.O. Box 1, 3720 BA, Bilthoven, The Netherlands; [6]Haartman Institute, Department of Virology, Helsinki University, Finland; and [7]Centro Nacional de Microbiologia, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid, Spain

MS 04-117: Received 12 March 2004/Accepted 30 April 2004

ABSTRACT

Five methods that detect human enteric virus contamination in lettuce were compared. To mimic multiple contaminations as observed after sewage contamination, artificial contamination was with human calicivirus and poliovirus and animal calicivirus strains at different concentrations. Nucleic acid extractions were done at the same time in the same laboratory to reduce assay-to-assay variability. Results showed that the two critical steps are the washing step and removal of inhibitors. The more reliable methods (sensitivity, simplicity, low cost) included an elution/concentration step and a commercial kit. Such development of sensitive methods for viral detection in foods other than shellfish is important to improve food safety.

The importance of foodborne transmission in outbreaks of viral origin is increasingly recognized *(6, 8, 16, 18).* At present, diagnosis of such outbreaks relies mostly on epidemiological investigations coupled with identification of the causative pathogen in persons with health complaints following consumption and, occasionally, in food handlers thought to be the source of infection. Final confirmation by detection of the pathogens in food still remains a challenge for various reasons. First, few methods have been developed for detection of viruses in foods, and methods that isolate and detect viruses in complex food matrices are limited *(7, 9, 27, 28).* Second, most viral foodborne outbreaks are caused by noroviruses. These RNA viruses, which cannot be propagated in cell culture, are antigenically diverse, making the use of immunological methods difficult, and these methods are probably not sensitive enough to detect viruses in foods *(2, 11, 25).* Moreover, the genetic diversity of these viruses has made the selection of a consensus primer set, and hence the use of reverse transcriptase PCR (RT-PCR), more difficult *(2, 30, 31).*

Various types of food have been implicated in outbreaks: shellfish, vegetables, fruits, delicatessen foods, and bakery products *(6, 16–18).* Foods served raw can be a transmission risk for viruses; lettuce, which is vulnerable to contamination, has been implicated in gastroenteritis and hepatitis A virus outbreaks *(12, 15, 24, 26).* Contamination can occur anywhere from the field to the table, and food handler hygiene is an important parameter throughout *(8, 12, 16, 28).* A simple contact with soiled hands is sufficient for contamination with high levels of virus, and the common enteric viruses can stay infectious for quite a long time, even under modified atmosphere or after disinfection *(3, 4, 7, 14).* Finally, the dose required for infection can be as low as a few particles for noroviruses *(13).* Combined, these factors provide a clear explanation for the great transmissibility via food of noroviruses and other nonenveloped enteric RNA viruses, such as hepatitis A virus.

To improve microbial monitoring of food quality and to assess the real role of food in viral transmission, standardized methods need to be developed for use in reference laboratories. Although significant progress has been made in detection of enteric viruses in shellfish, much needs to be done for other foods before this goal can be reached. The aim of this study was to compare methods for virus detection in a high-risk food item for virus transmission under controlled circumstances, mimicking multiple contaminations observed after sewage contamination. The main criteria used to evaluate and compare these tests were sensitivity, reproducibility, time, cost, and equipment needs. Lettuce was selected as a high-risk food item because it is eaten raw, it is handled extensively during harvest and preparation, its leaves have a large surface area, and it can become contaminated with soil or by irrigation with sewage water.

* Author for correspondence. Tel: 33 2 40 37 40 52; Fax: 33 2 40 37 40 73; E-mail: sleguyad@ifremer.fr.

TABLE 1. *Overview of the different methods used to eluate and concentrate the enteric viruses and to extract and purify the nucleic acids*[a]

Method	Elution	Concentration	RNA extraction
A	PBS-Vertrel	PEG	TRIzol, RNAMatrix
B	Beef extract	Ultracentifugation	Guanidium, CsCl cushion
C	Glycine-chloroform-butanol	PEG	RNeasy Plant Minikit
D	PBS-Vertrel	Ultracentifugation	RNeasy Plant Minikit
E	Glycine buffer	Filtration	RNeasy Plant Minikit

[a] PEG, polyethylene glycol precipitation; CsCl, cesium chloride.

MATERIALS AND METHODS

Viruses. Three different types of viruses were used: poliovirus Sabin strain type 2 (EV; kindly provided by A. Bosch, University of Barcelona, Spain); canine calicivirus strain no. 48 (CaCV; kindly provided by E. Duizer, RIVM, Bilthoven, The Netherlands), which can be titrated by cell culture; and a human stool RT-PCR positive for norovirus genogroup 2 (NoV GGII; kindly provided by P. Pothier, CHU Dijon, France). Viruses were extracted with the QIAamp Viral RNA Minikit (Qiagen, Courtaboeuf, France) and titrated by RT-PCR (endpoint dilution). The lowest dilution of extract that yielded a detectable product by RT-PCR confirmed by hybridization was considered 1 RT-PCR unit.

Artificial contamination of lettuce. Sterile water (800 ml) was artificially contaminated with the CaCV, EV, and NoV inocula. Approximately 80 g of freshly purchased lettuce (*Lactuca sativa*) was cut into small pieces (~5 cm^2) and immersed into the contaminated water for 20 min at room temperature. Then the lettuce was strained and left out to dry for about 1 h under laminar flow. The lettuce was weighed, divided into 10 replicates of 8 g each, and immediately analyzed by five different methods in duplicate.

To evaluate sensitivity of one method, the NoV-positive stool suspension was diluted in sterile water, and 100 μl were spread directly onto 10 g of lettuce. After a contact time of 15 min under laminar flow, the extraction was performed as described. Replicate experiments were done to evaluate the reproducibility of the method, including negative control lettuce samples.

Virus extraction and nucleic acid purification. The five methods evaluated are summarized in Table 1. Methods were developed independently in the laboratories indicated and were brought to the IFREMER laboratory for a workshop on comparative evaluation. Each workshop attendee performed his own protocol.

Method A. The viruses were eluted by addition of 6 ml of phosphate-buffered saline to the lettuce samples in a 50-ml polypropylene (pp) centrifuge tube (Falcon Corp., Franklin Lakes, N.J.) and shaking for 5 min by hand. This step was repeated, and one volume of Vertrel (1,1,1,2,3,4,4,5,5,5-decafluoropentane, DuPont, Paris, France) was added before shaking for 5 min and centrifuged for 10 min at 7,000 rpm and 4°C. Lettuce was eliminated from the supernatant, and polyethylene glycol (PEG) 6000 (Sigma, St. Louis, Mo.) at a final concentration of 10% and 0.3 M NaCl were added. The mixture was incubated for 2 h at 4°C and centrifuged for 30 min at 9,500 rpm and 4°C in a 50-ml pp tube. The pellet was suspended in 2 ml of TRIzol (Invitrogen, Cergy-Pontoise, France), incubated at room temperature for 5 min, and centrifuged for 20 min at 12,000 rpm and 4°C. The aqueous phase was adsorbed onto RNAMATRIX (Bio101, Carlsbad, Calif.), mixed for 1 h at room temperature, and centrifuged for 2 min at 3,000 rpm. The matrix was washed three times with 400 μl of a wash solution and dried overnight. The RNA was eluted with 100 μl of RNase-free water by heating for 10 min at 65°C.

Method B. The viruses were eluted by addition of 21.3 ml (1:3, wt/vol) of beef extract (3%, pH 9.5) and mixed for 20 min at room temperature in a 50-ml pp tube. The aqueous phase was collected (about 16.5 ml), distributed into four ultracentrifugation tubes (polyallomer centrifuge tubes, Beckman, Palo Alto, Calif.), and ultracentrifuged for 2 h at 200,000 × *g*. The pellet was suspended in 1 ml of sterile distilled water. For nucleic acid extraction, 334 μl (equivalent to one-third of the sample) was mixed with 666 μl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% [wt/vol] sodium lauryl sarcosinate, and 0.1 M β-mercaptoethanol) *(1)*, and 100 μl of CsCl (5.7 M solution of CsCl in 25 mM sodium acetate, pH 5, refractive index = 1.4000) was added (Baker Analyzed Reagent, J. T. Baker, Deventer, Holland). After centrifugation for 20 min at 12,000 × *g*, the pellet was suspended in 1 ml of 70% ethanol and centrifuged for 5 min at 12,000 × *g*. The pellet was then dried and suspended in 100 μl of RNase-free water.

Method C. The viruses were eluted by addition of 4 ml of glycine (0.05 M)–NaCl buffer (9 g/liter, pH 9.5) and vortexed for 1 min before adding 3 ml of chloroform-butanol (1:1, vol/vol) and 0.5 ml of Cat-Floc (Calgon Corp., Ellwood City, Pa.) in a 50-ml pp tube. After vortexing, the mixture was centrifuged for 20 min at 12,000 × *g* and 4°C. PEG 6000 was added to the supernatant to a final concentration of 8% (0.4 M NaCl) and rocked for 1 h at 4°C. After centrifugation for 30 min at 10,000 × *g* and 4°C, the pellet was suspended directly in the lysis buffer of the RNeasy Plant Minikit (Qiagen) and extracted according to the manufacturer's protocol. The nucleic acid was eluted twice in 50 μl of RNase-free water.

Method D. The viruses were eluted by addition of 5 ml of phosphate-buffered saline in a 50-ml pp tube, vortexed, and incubated and rotated for 5 min. After adding 5 ml of Vertrel (DuPont), the mixture was rocked for 30 min at room temperature and centrifuged 20 min at 13,000 × *g* and 4°C. The aqueous phase was then ultracentifuged for 2 h at 200,000 × *g*. The pellet was suspended in 200 μl of phosphate-buffered saline, and the nucleic acid was then extracted by the RNeasy Plant Minikit. The nucleic acid was eluted twice with 50 μl of RNase-free water.

Method E. The viruses were eluted for 15 min by addition of 5 ml of glycine buffer (pH 8.5) in a 50-ml pp tube. After gravity settling of large particles, the supernatant was collected and clarified by centrifugation for 20 min at 13,500 × *g*. The supernatant (3 ml) was then concentrated with a microconcentrator (Amicon, Millipore Corp., Bedford, Mass.) until volume was reduced to 200 μl. The nucleic acid was extracted with the RNeasy Plant Minikit and eluted twice in 50 μl of RNase-free water.

TABLE 2. *Detection of inhibitors and inoculated viruses on lettuce samples with five virus recovery and nucleic acid extraction methods*[a]

Method	Replicate	Inhibitor detection[b]	Enterovirus[c]	Canine calicivirus[d]	Norovirus[c]
A	1	+ (−)	+ (+)	+ (+)	+ (−)
	2	+ (−)	− (+)	+ (−)	+* (−)
B	1	− (−)	+ (−)	− (−)	− (−)
	2	− (−)	+ (−)	− (−)	− (−)
C	1	− (−)	+ (+)	+ (+)	+ (+)
	2	− (−)	+ (+)	+ (+)	+ (+)
D	1	+ (−)	+ (+)	+ (+)	+ (+)
	2	+ (−)	+ (+)	+ (+)	+ (−)
E	1	− (−)	+ (+)	+ (+)	+ (+)
	2	− (−)	+ (+)	+ (+)	+ (+)

[a] Two replicates were analyzed for each method and the results reported are for the undiluted nucleic acid extract and after a 1-log dilution (in parentheses).

[b] The inhibitor removal was evaluated by amplification of 10 RT-PCR units of an internal control (IC) mixed with 1 μl of the nucleic acid extract undiluted and after a 1-log dilution (in parentheses). The presence (+) of inhibitors means no amplification of the IC amplification; the absence (−) of inhibitors means amplification of the IC, detected after gel electrophoresis.

[c] The enterovirus and the norovirus were detected by RT-PCR and hybridization as described in ''Materials and Methods.'' All positive results were detected both after gel electrophoresis and confirmation by hybridization, except one (+*), which was positive only after hybridization.

[d] The canine calicivirus was detected by RT-PCR.

RT-PCR. RT-PCR was performed as previously described according to the instructions of the reverse transcriptase and *Taq* polymerase supplier (Perkin-Elmer Corp., Foster City, Calif.) *(21).* Primers used were P1-PV444 for EV *(5),* P110-NI for NoV *(20),* and CaCV-3–CaCV-4 for the CaCV *(10).* Extracts were tested undiluted (2 μl) and after a 1-log dilution (0.2 μl). For each primer set, all nucleic acid extracts were assayed in one run, including negative controls. RT-PCRs were performed according to the instructions of the murine leukemia virus reverse transcriptase and *Taq* polymerase suppliers (Applied Biosystems, Foster City, Calif.). The reverse transcription was done for 30 min at 42°C, and the PCR amplification was performed for 40 cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s) with final extension at 72°C for 7 min in a thermocycler (9600 or 2400 Cycler, Perkin-Elmer). PCR products were visualized by electrophoresis on a 9% polyacrylamide gel and stained with ethidium bromide *(21).* Positive and several negative controls were included in each RT-PCR run.

Hybridization. PCR products of EV and NoV were hybridized by the dot-blot method and chemiluminescence detection (Roche Applied Science, Mannheim, Germany) *(21).* Specific probes (P357 and NVp117, respectively) were used *(5, 20).*

Detection of inhibitors. Each nucleic acid extract, undiluted and after a 1-log dilution, was coamplified with 10 RT-PCR units of a calibrated single-strand RNA internal control (IC) (constructed from the EV genome) *(21).* Briefly, 1 μl of an IC dilution was mixed with 1 μl of each nucleic acid solution and subjected to RT-PCR as described above. Detection of inhibitors after gel electrophoresis meant no amplification of the IC dilution; the absence of inhibitors meant amplification of the IC.

TABLE 3. *Detection of norovirus directly spread onto lettuce samples at various concentrations by RT-PCR and hybridization after extraction by method C (PEG precipitation and RNeasy Plant Minikit extraction)*

Stool inoculum (RT/PCR units/g)	Positive samples[a]	
	Undiluted nucleic acid	1-log dilution nucleic acid
100	2/5	5/5
10	6/10	9/10
1	0/3	0/3

[a] Results are indicated as number of positive replicates/number of spiked samples tested.

RESULTS

Artificial contamination. A titer was calculated for each virus stock at 2.5×10^7 RT-PCR units per ml for the poliovirus Sabin strain type 2, 1×10^7 RT-PCR units per ml for the canine calicivirus strain, and 1×10^5 RT-PCR units per ml for an NoV GGII–positive stool. The water used for lettuce contamination was seeded at a final concentration of 3×10^4 RT-PCR units per ml for the CaCV strain, 1×10^5 RT-PCR units per ml for EV, and 1.2×10^2 RT-PCR units per ml for NoV. A control, done after the lettuce was removed, showed that less than 1% of viruses can be detected in the water (data not shown).

Inhibitor removal. The IC could not be amplified in undiluted nucleic acid extracted by methods A and D, indicating the presence of residual inhibitors. However, no inhibition persisted after a 1-log dilution of the nucleic acid. The three other methods (B, C, E) seemed to be more efficient in eliminating inhibitory compounds because the IC was amplified in undiluted nucleic acid extracts (Table 2).

Virus detection. The EV strain was detected in all nucleic acid extracts (Table 2). However, method B detected the strain only in the undiluted extract and method A only after dilution (confirming inhibitor detection). All results observed on the gel were confirmed after hybridization.

For CaCV detection, positive results were observed after gel electrophoresis for all methods except B and for one replicate after 1/10 dilution for method A. Unfortunately, no probe was available for this RT-PCR to increase sensitivity and confirm specificity.

For the NoV strain, in all instances except one, product could be detected by both gel electrophoresis and hybridization. In one case (A2), product could not be detected by electrophoresis but was indeed confirmed by subsequent hybridization, and in an other case (D2), the virus was not detected after a 1-log dilution. (Table 2).

Method C sensitivity study. On the basis of results obtained for virus detection and evaluation criteria, method C was selected for further evaluation. To evaluate reproducibility and sensitivity, several replicate experiments were done using stool sample dilutions spread directly onto lettuce aliquots (Table 3). Of the 10 experiments that used an inoculum of 10 RT-PCR units per g, 9 were positive and 1 failed to detect the virus. Among the nine positive ex-

periments, inhibitors persisted in four, and the virus could be detected only after dilution. The ability to detect virus after a 1-log dilution suggests that the sensitivity of the amplification method was about 1 RT-PCR unit. However, this does not take into account the effect of inhibitors on the overall detection limit of the assay, which is a function of the combined effect of extraction and amplification efficiency.

DISCUSSION

Comparison of methods for food analysis is a difficult challenge because of the scarceness of naturally contaminated food and the variability of food matrices and contamination. For in vitro studies, artificially contaminated samples are more convenient to use provided they mimic the natural mode of contamination. Lettuce contamination is likely to be related to the use of sewage-contaminated water in the fields or to washing before consumption. Similarly, in the case of contamination by a food handler, the virus is likely to be on the surface. Therefore, the immersion of lettuce in water contaminated with different virus strains at various concentrations, as was done here, can be considered to mimic the real situation.

Multiple viruses can be found in food contaminated by sewage, and the predominant strain is not always that detected in a patient's stool *(17, 22, 23, 29).* Because an efficient method should be able to detect a variety of pathogenic viruses, we used poliovirus and human (NoV) and animal caliciviruses (CaCV) for the seeding experiments, with comparatively lower doses for the human norovirus because of its high infectivity.

To optimize the reliability of the experiment and to avoid any discrepancy in the results, possibly from storage conditions, it was necessary to contaminate all the lettuce samples in the same batch and to do all the extractions at the same time. Therefore, all the extractions were done simultaneously in one laboratory, with all participants using their own reagents. To eliminate any variation that could interfere with the results, one person from the organizing laboratory did the RT-PCR and hybridizations immediately after extraction.

All methods proposed by the different laboratories consisted of an elution step, followed by a concentration step that used polyethylene glycol precipitation, ultracentrifugation, or ultrafiltration. Whereas polyethylene glycol precipitation can be done very easily in any laboratory, the ultracentrifugation step needs a more expensive apparatus and a suitable rotor. This method was found efficient in a previous study *(7),* which highlights the importance of comparing different methods under standardized conditions in the same laboratory.

For nucleic acid extraction, three methods were proposed. One method based on a cesium chloride cushion was quite easy and fast but needed an experienced technician and required the use of a toxic product (cesium chloride). TRIzol lysis and nucleic acid purification by adsorption used in method A is similar to the RNeasy kit principle but is more time consuming and complicated to perform and yielded a lower sensitivity. The RNeasy Plant Minikit included in three methods is simple, user friendly, efficient in eliminating inhibitors, and gave the best results. Residual RT-PCR inhibitors hamper most published methods, as reviewed by Sair et al. *(27),* who reported that this kit was less efficient than TRIzol with the QIAshredder homogenizer but more sensitive than TRIzol alone or the guanidium isothiocyanate extraction method *(27).* Comparison of sensitivity is difficult for noroviruses because it depends on the strain and the primer set used. However, the detection of about 10 RT-PCR units per g of lettuce obtained in this study, with good reproducibility, is better than or similar to previously described methods for lettuce analysis. For example, Leggitt and Jaykus *(19)* and Dubois et al. *(9)* reported about the same sensitivity, (i.e., $\geq 1.5 \times 10^3$ RT-PCR units per 50-g food sample and 1.5×10^3 RT-PCR units per 30-g food sample), whereas Sair et al. *(27)* reported a sensitivity of 50 PCR units per 6 g of food sample for NoV.

On the basis of the experiments described here, our preferred methods for further evaluation are C and E. Both methods (one elution per concentration step and a standardized commercial available product) are convenient and, by minimizing error factors, are promising for the future development of a competent viral quantification by real-time RT-PCR. More work is needed to establish the virus recovery more precisely, to extend the process to other enteric viruses, such as hepatitis A virus, and to compare cell culture and RT-PCR analysis. To our knowledge, this is the first study that used the cultivable canine calicivirus as a surrogate for human norovirus detection in food analysis.

This study was done as part of an European project to identify common sources of foodborne virus outbreaks in Europe. It illustrates that it might be possible to develop sensitive methods for virus detection in food other than shellfish. Analysis of food samples in an outbreak investigation will help to evaluate the true role of food in the transmission of epidemic viral gastroenteritis. To determine whether viral analysis of food samples is of practical value in confirmation of foodborne outbreaks will require further study. Method validation must be performed on foods implicated in foodborne outbreaks by epidemiological surveillance, which is the future focus of our work. The adaptation of protocols for different food matrices (e.g., fruits, liquids, meat products) will be needed before widespread use. When these aspects have been addressed, analysis of food samples for viral pathogens might become more feasible and provide valuable information.

ACKNOWLEDGMENTS

This research was supported by European Union grant no. QLK1-CT-1999-00594, Foodborne Viruses in Europe. The authors thank Christine Moe for helpful advice on the manuscript.

REFERENCES

1. Afzal, M. A., and P. D. Minor. 1994. Instant RNA isolation from virus-infected tissue culture fluid for the polymerase chain reaction. *Vaccine* 10:976–977.
2. Atmar, R. L., and M. K. Estes. 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. *Clin. Microbiol. Rev.* 14:15–37.

3. Bidawid, S., J. M. Farber, and S. A. Sattar. 2000. Contamination of foods by food handlers: experiments on hepatitis A virus transfer to food and its interruption. *Appl. Environ. Microbiol.* 66:2759–2763.
4. Bidawid, S., J. M. Farber, and S. A. Sattar. 2001. Survival of hepatitis A virus on modified atmosphere–packaged (MAP) lettuce. *Food Microbiol.* 18:95–102.
5. Bosch, A., G. Sanchez, F. Le Guyader, H. Vanaclocha, L. Haugarreau, and R. M. Pinto. 2001. Human enteric viruses in coquina clams associated with a large hepatitis A outbreak. *Water Sci. Technol.* 43: 61–65.
6. Bresee, J. S., M. A. Widdowson, S. S. Monroe, and R. I. Glass. 2002. Foodborne viral gastroenteritis: challenges and opportunities. *Clin. Infect. Dis.* 35:748–753.
7. Croci, L., D. De Medici, C. Scalfaro, A. Fiore, and L. Toti. 2002. The survival of hepatitis A virus in fresh produce. *Int. J. Food Microbiol.* 73:29–34.
8. de Witt, M. A. S., M. P. G. Koopmans, and Y. T. van Duynhoven. 2003. Risk factors for norovirus, sapporo-like virus, and group A rotavirus gastroenteritis. *Emerg. Infect. Dis.* 9:1563–1570.
9. Dubois, E., C. Agier, O. Traore, H. Laveran, and C. Crucière. 2002. Modified concentration method for the detection of enteric viruses on fruits and vegetables by reverse transcriptase–polymerase chain reaction or cell culture. *J. Food Prot.* 65:1962–1969.
10. Duizer, E., P. Bijkerk, B. Rockx, A. de Groot, F. Twisk, and M. P. G. Koopmans. 2004. Inactivation of caliciviruses. *Appl. Environ. Microbiol.* 70:4538–4543.
11. Duizer, E., K. J. Schwab, F. H. Neill, R. L. Atmar, M. P. G. Koopmans, and M. K. Estes. 2004. Laboratory efforts to cultivate noroviruses. *J. Gen. Virol.* 85:79–87.
12. Gaulin, C., M. Frigon, D. Poirier, and C. Fournier. 1999. Transmission of calicivirus by a foodhandler in the pre-symptomatic phase of illness. *Epidemiol. Infect.* 123:475–478.
13. Green, K. Y., R. M. Chanock, and A. Z. Kapikian. 2001. Human caliciviruses, p. 841–874. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Strauss (ed.), Fields virology, 4th ed. Raven Press, New York.
14. Gulati, B. R., P. B. Allwood, C. W. Hedberg, and S. M. Goyal. 2001. Efficacy of commonly used disinfectants for the inactivation of calicivirus on strawberry, lettuce, and a food-contact surface. *J. Food Prot.* 64:1430–1434.
15. Hernandez, F., R. Monge, C. Jimenez, and L. Taylor. 1997. Rotavirus and hepatitis A virus in market lettuce (*Latuca sativa*) in Costa Rica. *Int. J. Food Microbiol.* 37:221–223.
16. Koopmans, M., and E. Duizer. 2004. Foodborne viruses: an emerging problem. *Int. J. Food Microbiol.* 90:23–41.
17. Koopmans, M., H. Vennema, H. Heersma, E. van Strien, Y. van Duynhoven, D. Brown, M. Reacher, and B. Lopman. 2003. Early identification of common-source foordborne virus outbreaks in Europe. For the European Consortium on Foodborne Viruses *Emerg. Infect. Dis.* 9:1136–1142.
18. Koopmans, M., C.-H. von Bonsdorff, J. Vinje, D. De Medici, and S. S. Monroe. 2002. Foodborne viruses. *FEMS Microbiol. Rev.* 26: 187–205.
19. Leggit, P. R., and L.-A. Jaykus. 2000. Detection methods for human enteric viruses in representative foods. *J. Food Prot.* 63:1738–1744.
20. Le Guyader, F., M. K. Estes, M. E. Hardy, F. H. Neill, J. Green, D. W. G. Brown, and R. L. Atmar. 1996. Evaluation of a degenerate primer for the PCR detection of human caliciviruses. *Arch. Virol.* 141:2225–2235.
21. Le Guyader, F., L. Haugarreau, L. Miossec, E. Dubois, and M. Pommepuy. 2000. Three-year study to assess human enteric viruses in shellfish. *Appl. Environ. Microbiol.* 66:3241–3248.
22. Le Guyader, F., C. Mittelhozer, L. Haugarreau, K.-O. Hedlund, R. Alsterlund, M. Pommepuy, and L. Svensson. Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *Int. J. Food Prot.*, in press.
23. Le Guyader, F., F. H. Neill, E. Dubois, F. Bon, F. Loisy, E. Kohli, M. Pommepuy, and R. L. Atmar. 2003. A semiquantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak. *Int. J. Food Microbiol.* 87:107–112.
24. Nygard, K., Y. Andersson, P. Lindkvist, C. Ancker, I. Asteberg, E. Dannetun, R. Eitrem, L. Hellstrom, M. Insulander, L. Skedebrant, K. Stenqvist, and J. Giesecke. 2001. L'importation de salade 'roquette' partiellement responsable de l'augmentation de l'incidence de l'hépatite A en Suede, 2000–2001. *Eurosurveillance* 6:151–153.
25. Rabenau, H. F., M. Sturmer, S. Buxbaum, A. Walczok, W. Preiser, and H. W. Doerr. 2003. Laboratory diagnosis of norovirus: which method is the best? *Intervirology* 46:232–238.
26. Rosemblum, L. S., I. R. Mirkin, D. T. Allen, S. S. Safford, and S. C. Hadler. 1990. A multifocal outbreak of hepatitis A traced to commercially distributed lettuce. *Am. J. Public Health* 80:1075–1079.
27. Sair, A. I., D. H. D'Souza, C. L. Moe, and L. A. Jaykus. 2002. Improved detection of human enteric viruses in foods by RT-PCR. *J. Virol. Methods* 100:57–69.
28. Schwab, K., F. H. Neill, R. L. Fankhauser, N. A. Daniels, S. S. Monroe, D. A. Bergmire-Sweat, M. K. Estes, and R. L. Atmar. 2000. Development of methods to detect 'Norwalk-like viruses' (NLVs) and hepatitis A virus in delicatessen foods: application to a foodborne NLV outbreak. *Appl. Environ. Microbiol.* 66:213–218.
29. Sugieda, M., K. Nakajima, and S. Nakajima. 1996. Outbreaks of Norwalk like virus associated gastroenteritis traced to shellfish: coexistence of two genotypes in one specimen. *Epidemiol. Infect.* 116: 339–346.
30. Vinje, J., R. A. Hamidjaja, and M. D. Sobsey. 2004. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. *J. Virol. Methods* 116:109–117.
31. Vinjé, J., H. Vennema, L. Maunula, C.-H. von Bonsdorff, M. Hoehne, E. Schreier, A. Richards, J. Green, D. Brown, S. Beard., S. S. Monroe, E. De Bruin, L. Svensson, and M. P. G. Koopmans. 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. *J. Clin. Microbiol.* 41:1423–1433.