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Norwalk-like Virus Sequences Detected by Reverse Transcription-Polymerase Chain Reaction in Mineral Waters Imported into or Bottled in Switzerland

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ABSTRACT

Norwalk-like viruses (NLVs) is a genus belonging to the *Caliciviridae.* NLVs are transmitted by the fecal-oral and the aerosol route and are the most common cause of outbreaks of nonbacterial gastroenteritis. NLVs are responsible for an estimated 67% of all illnesses caused by known foodborne pathogens and for 96% of nonbacterial gastroenteritis in the United States. Many outbreaks could be associated with the consumption of primarily or secondarily contaminated foods. To our knowledge, no epidemic arising from contaminated mineral water has been reported. We investigated the presence of NLV sequences in 63 mineral waters of 29 different brands that were imported into or bottled in Switzerland. NLV sequences were detected in 21 mineral waters by reverse transcription-seminested polymerase chain reaction. Specimens of two NLV genogroups (gg), gg I and gg II, were randomly present in the contaminated samples. The presence of NLV sequences could not be correlated either with bottle characteristics or with chemical properties like mineralization, pH, or the presence of carbonic acid. Nucleotide sequence analysis of 12 NLV-positive samples revealed several point mutations. All isolated NLV gg I strains have a similarity of 70 to 87% with the common Desert Shield virus (UO4469), and all isolated NLV gg II strains have a similarity of 89 to 93% with the Camberwell virus (U46500). Possible reasons for the presence of NLV sequences in mineral waters are discussed.

Norwalk-like viruses (NLVs) are single-stranded RNA viruses of the family *Caliciviridae (15).* This family includes four genera designated *Lagovirus, Vesivirus,* NLVs, and Sapporo-like viruses. The genus NLV is divided into two distinct genogroups (gg), gg I and gg II, each of which can be further divided into several clusters *(13).* At present, partial sequence data are available for more than 100 NLVs. Molecular analysis of NLVs associated with outbreaks has demonstrated both a great diversity of strains in circulation *(27)* and the presence of distinct predominant strains during several months *(4, 28, 35).*

The importance of the epidemiology of NLVs in relation to outbreaks of nonbacterial gastroenteritis has become evident with the replacement of microscopic or serological diagnostic tools by reverse transcription-polymerase chain reaction (RT-PCR). Between 1982 and 1985, serological tests suggested that NLVs could be responsible for 45 to 50% of all nonbacterial gastroenteritis outbreaks in the United States *(17, 18, 22).* Recent studies *(4, 14–16)* showed that NLVs may be responsible for 96% of all nonbacterial epidemic gastroenteritis in the United States. These results are supported by the conclusions of Vinje and Koopmans *(34),* who attributed 91% of all nonbacterial gastroenteritis outbreaks in the Netherlands to NLVs as determined by RT-PCR analysis. Surveillance data of NLV laboratory reports to the Public Health Laboratory Service *(30)* show an increase from 319 cases in 1986 to 2,049 cases in 1997. Wheeler et al. *(35)* estimated for NLVs in England and Wales a ratio of cases in the community to cases reaching national surveillance of 1,562:1, with yearly 9.4 million infectious intestinal diseases. Mead et al. *(24)* estimated a ratio of 460:1 for foodborne transmitted and for all NLV illnesses in the United States. This ratio for NLV infections reflects, on the one hand, an illness compared to bacterial infections like *Campylobacter* (7.6:1 *(35)*) or *Salmonella* (3.2:1 *(35)*), and on the other hand minimizes the fact that yearly approximately 6.9 *(24)* to 11% *(25)* of all food-related deaths are caused by NLVs *(24).* Mead et al. *(24)* estimate between 1983 and 1992 a foodborne transmission of 40% causing yearly 23 million NLV illnesses in the United States. Transmission of the NLVs by contaminated food has been documented for oysters *(2, 8, 21, 26, 29, 33),* bivalve mollusks *(13, 14),* water *(1, 9, 19, 23),* and berries *(5).* The remaining 60% of NLV illnesses seem to be transmitted mainly by person-to-person contact *(3, 11, 20).*

At present, NLVs as well as other viral contaminants are not routinely tested for in mineral waters. Because water is known to be an important transmission factor for NLVs, the presence of NLV sequences in mineral waters should be assessed. Neither national (e.g., Switzerland, EC) nor international legal limits are defined, and the possible contamination of mineral waters by these agents is unknown to everybody, including consumers, dealers, and gourmets. The aim of this study was to investigate the distribution of NLV sequences in commercially available mineral waters

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in Switzerland. We examined 63 mineral waters of 29 different brands that were imported into or bottled in Switzerland for the presence of NLVs gg I and II. For each mineral water, viral RNA was concentrated and RT-PCR performed. Selected NLV strains were sequenced for further molecular comparison.

MATERIALS AND METHODS

Mineral waters. We examined 63 mineral waters of 29 (labeled A, B, C, . . . Z, AZ-CZ) different brands, whereby 17 mineral waters were bottled in Switzerland and 46 were imported by different Swiss traders. Food inspectors of the Official Food Control Authority of the Canton of Solothurn collected 46 mineral waters; the remaining 17 bottles were collected by customs officers and sent to this Authority, where all 63 samples were examined. Different concentrations of carbonic acid were present in 10 mineral waters. Mineral water bottles were stored in the dark at room temperature before analyzing. Bacteriological analyses according the Swiss Food Manual, chapter 56, for *Enterococcus faecalis, Escherichia coli,* and *Pseudomonas aeruginosa* were performed on each mineral water and revealed no positive results (bacteriological data not shown).

Control samples and detection limits. We used two external positive control samples and four negative control samples to evaluate the RT-PCR results:

First we included an RT-PCR for polio virus strains of a known concentration to estimate the efficiency of the NLV-detection method (RT-PCR described by Gilgen et al. *(6)*). This poliomyelitis vaccine, Poloral Berna (Serum Institute, Berne, Switzerland), consists of three poliovirus strains, Sabine I (2×10^6 $TCID_{50}$ [one 50% tissue culture infective dose corresponds to 10 to 100 viral particles]), Sabine II (2×10^5 $TCID_{50}$/ml), and Sabine III (6×10^6 $TCID_{50}$/ml). We established a detection limit for our RT-PCR method of a 10^9-fold dilution, which means theoretical 3×10^{-4} $TCID_{50}$ or 0.003 to 0.03 viral particles. Nonreactive viral particles in the poliomyelitis vaccine seem to be responsible for these low viral particale numbers, because they are not considered for $TCID_{50}$ determinations.

The second and major positive control sample consists of NLV RNA of gg I and II. Because NLVs still cannot be cultivated, NLV-positive control samples were found among stool samples of patients suffering from gastroenteritis. The NLV gg I-positive control sample contained Southampton-related viruses (L07418), and the NLV gg II-positive control sample contained Lordsdale-related viruses (X86557). Detection limits for seeded NLVs in 1-liter mineral water samples were established repeatedly, and dilutions of 10^{-5} remained positive (results not shown).

Negative control samples. We include four negative control samples at different levels of the method to detect possible cross contaminations. Filtration of 1 liter of distilled water was used as an overall negative control sample and three distilled water samples for the reverse transcription and both PCRs (seminested PCR).

Virus isolation and concentration from 1 liter of mineral water. One liter samples of each mineral water and all positive and negative control samples were analyzed with the three-step isolation method published by Gilgen et al. *(6).* As an external NLV-positive control, 1 liter of distilled water was seeded with 100 μl of NLV gg I- and gg II-infected stool samples. As a secondary external control, another liter of distilled water was seeded with 100 μl of a 10^6-fold dilution of poliomyelitis vaccine. The three-step isolation procedure *(6)* used for all analyses includes two subsequent concentrations, followed by virus lysis and RNA isolation. For the first concentration step, samples were vigorously shaken and filtered through a positively charged 0.45-μm pore-size membrane (Zetapor filter membrane; CUNO Inc., Meriden, Conn.). After filtration, the Zetapor membrane was transferred to a 50-ml centrifuge tube containing 4 ml of 50 mM glycine NaOH, pH 9.5, containing 1% beef extract (Sigma Chemical Co., St. Louis, Mo.); beef extract is reported to improve the recovery rate at least 10 times *(6)* during the concentration steps but could also have inhibitory effects on RT-PCR in high concentrations *(31).* After shaking at 500 rpm and room temperature for 20 min in order to elute the viruses, the virus-containing buffer was adjusted to pH 8 with 20 μl of 1 M HCl. All 4 ml of this buffer were concentrated to 100 μl by use of a microconcentrator (Ultrafree Biomax 100K NMWL membrane 4 ml volume; Millipore Corporation, Bedford, Mass.) at $1{,}000 \times g$ and room temperature according to the manufacturer's protocol. The retentate was adjusted to 140 μl with 1× phosphate-buffered saline that was used for RNA isolation using the QIAmp HCV minikit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol. Ten microliters of the obtained 60-μl RNA solution were used for each reverse transcription.

Reverse transcription. Ten microliters of RNA were reverse transcribed by using the Sensiscript kit (Sensiscript RT Kit; QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol. Thereby, the RNAs were reverse transcribed by incubation for 60 min at 37°C followed by 5 min at 95°C to inactivate the reverse transcriptase. In a final volume of 20 μl the reaction conditions were 1× RT-Sensiscript buffer, 0.5 mM each dNTP, 1 μM primer (Microsynth GmbH, Balgach, Switzerland), 10 units RNase inhibitor (Rnasin Ribonuclease Inhibitor; Promega, Madison, Wis.), 1 μl RT-Sensiscript, and 10 μl template RNA.

Oligonucleotides. The seminested RT-PCR systems for the detection of NLVs gg I and gg II are based on primers described by Häfliger et al. *(10),* developed by alignments of all the NLV sequences available on the EMBL Genbank at this time. Primer pairs are located in highly conserved regions of the capsid gene for NLV gg I and of the RNA polymerase for NLV gg II. Table 1 shows the sequences and the localizations of all oligos. These degenerated primers allow the simultaneous detection of various NLVs (gg I: NV/8FiiA/68/US Norwalk virus [M87661], KY-89/Japan [L23828], Southampton/91/UK [L07418], DSV395/90/Saudi Arabia Desert Shield virus [U04469], and BS5/Germany [AF093797]; gg II: TV/91/Canaca Toronto virus [U02030] and Hawaii/71/US [U07611]). The seminested RT-PCR system for the detection of enterovirus (polio) is based on primers described by Gilgen et al. *(7).* All primers had been synthesized by Microsynth (Balgach, Switzerland) and stored freeze-dried at −20°C.

PCR. The seminested PCR systems for NLV gg I, NLV gg II, and poliovirus are divided into the following two PCRs: (i) PCR1: Ten microliters of the completed reverse transcription was mixed with 40 μl PCR mixture (final concentration: 1× PCR buffer for Taq DNA polymerase recombinant (GIBCO BRL by Life Technologies Inc., Paisley, Scotland), 2 μg/ml bovine serum albumin (Fluka, Switzerland), 0.2 mM dNTPs, 0.25 μM of the first primer pair, $MgCl_2$ concentration as indicated in Table 2, and 1 U of Taq polymerase (Life Technologies). Thermal cycling was performed on a Progene thermal block (Techne, Princeton, N.J.). The PCR program consists of denaturation for 180 s at 94°C for 25 cycles with 36 s at 94°C, 72 s at 55°C, and 84 s at 72°C. Finally, an end-extension for 180 s at 72°C was performed. (ii)

TABLE 1. *Seminested RT-PCR oligonucleotides for NLV gg I, gg II, and polio virus (enteroviruses); regions, polarities, and localizations are indicated*[a]

Virus and oligonucleotide	Region	Sequence 5′–3′	Polarity	Localization[a]
NLVs ggI				
SRI-1 (RT-PCR)	Capsid	CCA ACC CAR[b] CCA TTR TAC AT	Antisense	5,659–5,640
SRI-2 (PCR)		AAA TGA TGA TGG CGT CTA AG	Sense	5,344–5,361
SRI-3 (PCR)		AAA AYR TCA CCG GGK GTA T	Antisense	5,584–5,566
NLV gg II				
SRII-1 (RT-PCR)	RNA pol	CGC CAT CTT CAT TCA CAA A	Antisense	5,357–5,339
SRII-2 (PCR)		TWC TCY TTY TAT GGT GAT GAT GA	Sense	4,844–4,866
SRII-3 (PCR)		TTW CCA AAC CAA CCW GCT G	Antisense	5,046–5,028
Enterovirus				
EV03 (RT-PCR)	5′ UTR	ATT GTC ACC ATA AGC AGC CA	Antisense	601–582
EV05 (PCR)		CAC GGA CAC CCA AAG TAG T	Antisense	563–184
EV06 (PCR)		CAA GCA CTT CTG TTT CCC	Sense	448–467

[a] Localizations are in reference to Norwalk virus (M87661) and poliovirus type Sabine 3 (X00596).
[b] Mixed bases in degenerated primers: W = A or T, Y = C or T, K = G or T, and R = A or G.

PCR2: Two microliters of the first PCR reaction was used as a template for the second reaction and mixed with 48 μl PCR mixture (final concentration: same as indicated for the first PCR). Cycling was also conducted on a Progene thermal block. The PCR program consisted of denaturation for 180 s at 94°C for 40 cycles with 30 s at 94°C, 60 s at 50°C, and 60 s at 72°C and a final extension for 180 s at 72°C.

Analysis of PCR products. Ten microliters of the second (seminested) PCR reaction was mixed with 10 μl loading buffer and analyzed on 2.7% agarose gels. Electrophoresis was done at 70 mV/cm, and ethidium bromide staining and UV transillumination were done to visualize the amplicons. Fragments were compared in size with a commercially available size standard (100-bp DNA ladder, Promega). To determine the viral origin of our amplicons, we sequenced a representative number of amplicons.

DNA sequencing and sequence analysis. PCR products were directly sequenced by cycle sequencing (ABI PRISM 377 DNA sequencer, Perkin Elmer; carried out by Microsynth, Balgach, Switzerland). Nucleotide sequences of 241 and 203 bp for NLV gg I and gg II were compared with GenEMBL data bank entries using the Fasta program of the GCG software (Wisconsin Package Version 9.1, Madison, Wis.).

RESULTS

NLV sequence-positive mineral waters. We investigated the presence of NLV gg I and gg II sequences in 63 mineral waters of 29 different brands available in Switzerland by seminested RT-PCR. Overall, 33% (21 of 63) of all mineral water samples of 11 different brands were positive for NLV sequences. NLV sequences of gg I were detected in 16 samples of 10 different brands. NLV gg II sequences were detected in five samples of five different brands. NLV sequences of both gg I and II were found in four different brands but never in the same sample. Figure 1 shows ethidium bromide-stained amplicons (241 bp) of NLV gg I sequence-positive mineral water samples on a 2.7% agarose gel. Three of the eight tested samples on this agarose gel show an amplified fragment of an NLV gg I genome. Figure 2 shows ethidium bromide-stained amplicons (201 bp) of NLV gg II sequence-positive mineral water samples on a 2.7% agarose gel. Amplicons were found in four of the eight mineral water samples on this agarose gel. Table 3 summarizes the NLV gg I- and gg II-positive results listing brand designations, NLV sequence positivity, and product properties. Twelve of 21 NLV sequence-positive samples were imported into Switzerland from Italy, five from France, and four mineral waters were bottled in Switzerland.

Sequence analysis. Amplified viral sequences of 12 mineral water samples were sequenced and comparisons

TABLE 2. *PCR properties for used primer pairs, annealing temperatures $MgCl_2$ concentrations, and length of amplicons*

Virus	Primer pair	Annealing temperature (°C)	$MgCl_2$ concentration (mM)	Amplicon (bp)
Enterovirus	EV03-EV06	55	3.5	438
	EV05-EV06	55	3.0	400
NVL gg I	SRI-1-SRI-2	50	3.5	316
	SRI-3-SRI-2	50	2.0	241
NLV gg II	SRII-1-SRII-2	55	3.5	514
	SRII-3-SRII-2	50	1.5	203

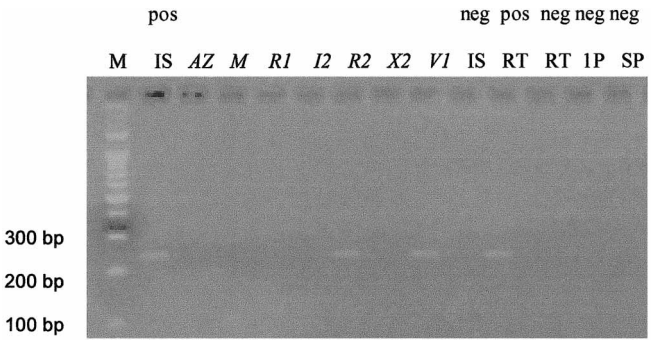

FIGURE 1. *Results of ethidium bromide staining of NLV sequences of gg I (241 bp) in selected mineral water samples. Lanes 3 to 9 (AZ to V1) are mineral water samples: NLV gg I sequence-positive samples are R2 and V1. Control samples are shown in lanes 1 and 10 to 14. As positive control sample we used a stool specimen containing Southampton-related viruses (L07418). As a negative control sample we always used distilled water. Control sample designations: IS, isolation and concentration; RE, RNA extraction; 1P, first PCR; SP, seminested PCR.*

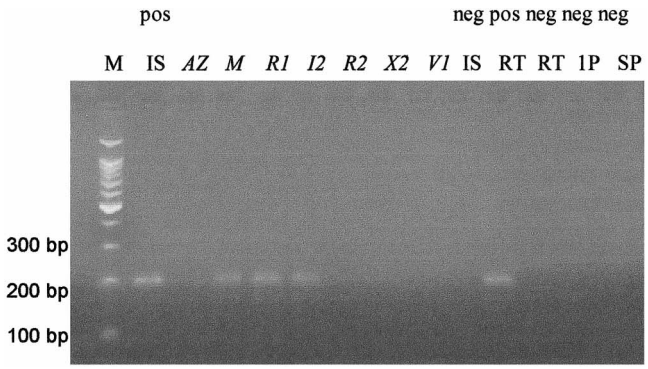

FIGURE 2. *Results of ethidium bromide staining of NLV sequences of gg II (203 bp) in selected mineral water samples. Lanes 3 through 9 (AZ to V1) are mineral water samples. NLV gg II sequence-positive samples are M, R1, and I2. Control samples are shown in lanes 1 and 10 to 14. As a positive control sample we used a stool specimen containing Lordsdale-related viruses (X86557). As a negative control sample we always used distilled water. Control sample designations: IS, isolation and concentration; RE, RNA extraction; 1P, first PCR; SP, seminested PCR.*

with the sequence database of the National Center for Biotechnology Information were done. Table 4 shows the results of the sequence alignments: Eight of 12 sequence products revealed a similarity of 26 to 81% with Norwalk virus (NV/8Fii/68/US; accession number M87661) and a similarity of 70 to 87% with Desert Shield virus (DSV395/90/ Saudi Arabia; accession number U04469). The remaining four NLV gg II-positive samples shared 89 to 93% sequence identity with Camberwell virus (Hu/NLV/Camberwell/101922/94/ Aus; accession number AF145896). There was no 100% similarity between two different samples, and all sequences were different from NLV gg I- and gg II-positive control samples used as controls.

DISCUSSION

We examined the presence of NLV gg I and II sequences in 63 mineral water samples of 29 different brands available in Switzerland by RT-PCR and could determine a contamination rate of 33%. Sequence analysis of amplified NLV gg I and II strains revealed the presence of closely related strains among all mineral water samples. These results of related strains in different European countries support a study published by Noel et al. *(28),* where a distinct strain of NLVs with a global distribution was identified. This described a 95-96-US-subset, as well as the NLV gg II-positive strains we sequenced (see Table 4) that are closely related to the Camberwell virus (U46500). Because the amplified NLV sequence regions in this study are different from the one amplified by Noel et al. *(28)* (similarity only in $<$50 bp), comparisons could not be done, although the partially corresponding regions revealed a similarity of 100% with the 95-96-US-subset. The NLV gg I sequence-positive samples we sequenced showed similarities (see Table 4) to each other (with a deviation of a few nucleotides) and with a Desert Shield virus (U04469) (84 to 88%). The modes of transmission that allow an NLV sequence cluster to spread rapidly to geographically distinct locations and the reasons for their sudden predominance and their gradual substitution *(28)* are partially unknown and raise many important public health questions. For example, import of contaminated food that is destined to be consumed uncooked (vegetables, fruits, sea food, mineral waters) and infections among international travelers such as air or cruiseship passengers could be responsible for a worldwide dissemination of distinct infectious agents like NLVs. To highlight the importance of NLVs worldwide and to demonstrate the merging or global distribution of distinct strains, the implementation of an international surveillance network with laboratories providing sequence data from a common genetic region has been suggested *(28).* The obtained data would permit the identification of international outbreaks and enable the elucidation of their modes of transmission to consider prevention. Because there are no data available for Europe, the relevance of our findings might not be assessed.

Previously to this study, different stool samples of Swiss people suffering from intestinal pain were analyzed for the presence of NLV sequences. After sequence analysis of an NLV-positive sample from a home for the elderly, we found a similarity of 100% to a previously analyzed stool sample and the NLV gg II sequence amplified from the mineral water sample of brand I.

Critical points to evaluate the relevance of NLV outbreaks caused by consuming contaminated food are the sensitivity of the detection method, the infectivity of detected agents, the quantification by PCR, and the routes of transmission.

Detection method. At present, RT-PCR is the most effective method for the detection of foodborne pathogens that cannot be cultivated. Therefore one has to keep in mind that the better the methodology, the more likely it will be that cases are identified. The detection of NLV sequences by RT-PCR results raises many difficulties. The most critical factor for NLV detection by RT-PCR is the selection of optimized primer oligonucleotides. Because the NLV ge-

TABLE 3. *NLV sequence-positive mineral waters with anonymous brand designations A through Z and AZ*[a]

Brand	PH as labeled	Bottle material	Origin	Bottle color	Expire date	Investigation date	Shelf life (days)	NLV gg I	NLV gg II
A	/	PE	Swiss	Colorless	23.12.99	20.05.99	213	Pos	Neg
B	/	PE rec	Swiss	Colorless	0.10.99	19.05.99	131	Pos	Neg
I1	7.2	PE	France	Colorless	19.10.00	20.05.99	149	Pos	Neg
I2	7.2	PE	France	Colorless	08.03.01	30.04.99	308	Neg	Pos
I3	7.2	PE	France	Colorless	19.04.01	12.05.99	337	Pos	Neg
I4	7.2	PE	France	Colorless	09.02.01	12.05.99	237	Pos	Neg
K*	7.7	PE	Italy	Colorless	0.08.00	21.05.99	399	Pos	Neg
L	7.8	PE	Italy	Blue	7.12.00	22.06.99	525	Pos	Neg
M	8.0	Glass	Italy	Green	06.04.01	30.04.99	336	Neg	Pos
N1	/	PE	Swiss	Colorless	0.04.00	20.05.99	250	Pos	Neg
N2	/	PE	Swiss	Colorless	0.04.00	19.05.99	—	Pos	Neg
R1	7.8	PE	Italy	Green	06.10.00	30.04.99	156	Neg	Pos
R2	7.8	PE	Italy	Green	05.10.00	30.04.99	155	Pos	Neg
R5	7.8	PE	Italy	Green	18.10.00	12.05.99	156	Pos	Neg
V1	7.99	PE	Italy	Colorless	—	30.04.99	—	Pos	Neg
V2	7.99	PE	Italy	Colorless	10.01.00	12.05.99	238	Neg	Pos
V3	7.99	PE	Italy	Colorless	05.01.00	19.05.99	227	Pos	Neg
V4	7.99	PE	Italy	Colorless	0.02.01	21.05.99	249	Pos	Neg
X2	7.3	PE	Italy	Colorless	0.09.00	20.05.99	460	Pos	Neg
X4	7.3	PE	Italy	Colorless	0.11.00	31.05.99	510	Neg	Pos
AZ	/	PE rec	France	White	0.10.00	19.05.99	491	Pos	Neg

[a] The abbreviation rec for brand B means a recycled bottle, and the bottle material is mostly PE (polyethylene glycol). Brand K* was the only mineral water sample (1 of 10) containing carbonic acid (concentration not indicated) to be NLV sequence positive.

TABLE 4. *Results of the amplicon comparisons with Genbank sequences of the National Center for Biotechnology Information*[a]

	NLV ggI			NLV gg II	
	% identical with Desert shield 395	% identical with Norwalk virus (capsid)		% identical with Camberwell	% identical with Hawaii
Brands	U04469	AF145709	Brands	AF145896	U07611
K	77	73	I2	89	83
N1*	87	81	M	92	39
N2*	83	80	R1*	91	57
R2*	70	66	V2*	93	59
V1*	76	72			
V4*	76	73			
X2	87	72			
AZ	83	26			

[a] Identities of sequenced NLV (gg I and gg II)-positive RT-PCR products are indicated in percent compared to known NLV strains. Asterisks denote brands with two or three different NLV sequences.

nus contains over 100 species in three genogroups that can be divided into thousands of subspecies, the main difficulty is to find sensitive oligonucleotides that allow the detection of the widest range of NLVs. Before starting our investigations, we compared different published primer oligonucleotides for NLVs by sequence alignments (not shown). We decided to use oligonucleotides designed by Häfliger et al. *(10)* that made the simultaneous detection of seven distinct NLVs (within the optimal annealing temperature for PCR) by RT-PCR possible. These primer pairs have been previously developed by sequence alignments of all NLV sequences available in the EMBL Genbank and checked for specificity with the GCG software *(10).*

Infectivity. Because the detection method (RT-PCR) used is not able to distinguish between infective and inactivated NLVs (all reverse transcribed NLV gene fragments corresponding to the applied PCR primers are amplified), the infectivity could not be assessed. Referring to the physiochemical properties of NLVs elaborated by Irving et al. *(12),* the probability of some infectivity surviving in mineral water seems to be possible.

Quantification. Quantification by RT-PCR would only be possible by using a primer-specific internal standard that is amplified by nearly the same efficiency as the amplicon. An internal standard specific for the oligonucleotides that we use will be available soon (Biosmart, Berne, Switzerland). We are now developing a quantification method for NLVs by real-time RT-PCR using a Lightcycler.

Contamination. The question of the origin of NLV-sequences in available mineral waters seems to be the critical point among producers and distributors. We consider three main contamination possibilities: first the contamination of the corresponding sources, second the contamination during the manufacturing, and/or third the contamination of the packing material (polyethylene glycol or glass bottles). Correlations made no relations possible between the presence of NLVs and the bottle properties, the pH, the mineralization, or the concentration of carbonic acid in the mineral waters, but not enough samples of one brand were analyzed to make definitive conclusions. Our results (including repetitive analysis) show a relation between the presence of NLV sequences and the lot number. The problem is that this conclusion supports all three contamination hypotheses. We are also not able to reject the hypothesis of a periodic NLV sequence contamination of mineral water sources. A possible reason could be a periodic dispersion of virus sequences from the upper layer of sediments into the source, due to the sedimentation of viruses that are adsorbed on particles and are deposited in sediment *(32).* If we consider that after serial passages of the virus sequence changes are accumulated, high sequence identity as we could observe among all mineral water brands of different countries could signify that these viruses have circulated for a long period in the environment, for instance in sediment and source water, without reproducing. To find out the real reasons for the presence of NLV sequences in available mineral waters and to assess the hypothesis of a periodic source contamination, further investigations should be done soon.

The next step will be the elaboration of a reliable quantification method for NLV sequences in food. As long as NLVs cannot be cultivated, the quantification by real-time PCR by using an appropriate primer-specific internal standard will be the next challenge. An other necessity would be the performance of an epidemiological study (case control or cohort study) to evaluate public health risks of NLV sequences in marketable mineral waters.

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