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Phylogenetic Analyses of *Norovirus* Isolates from Human Stool Samples, Mineral Waters and Oysters in Switzerland

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Introduction

Recent international studies have shown that viral infections, especially noroviruses (NV) (former name *"Norwalk-like viruses")* are the most frequent cause of gastroenteritis in the community regarding the endemic and the epidemic situation (1–6). These viruses account for an estimated 6% and 11% of all infectious intestinal diseases in England and the Netherlands (3, 5), respectively and for an estimated 23 million cases in the United States each year (7). NV are also the most common cause of outbreaks of infectious intestinal diseases in Western Europe and North America (3, 7). Two recent publications demonstrated that NV outbreaks play also in Switzerland an important role in terms of public health (8–9). Furthermore, a two-years screening for the determination of the frequency of sporadic community-acquired NV infection in the German-speaking part of Switzerland revealed that NV were detected in 18% of stool specimens from gastroenteritis cases (10). The illness is characterised by acute onset of vomiting and diarrhoea, after an average incubation period of 12–48 hours. The faecal-oral route is described to be the most common route of transmission. NV are transmitted either by contaminated fomites (such as food and water) and environment, or directly from person-to-person (11). Furthermore, outbreaks have been associated with the ingestion of contaminated drinking or recreational surface water (12). Two recent Swiss studies found RNA sequences of

NV in bottled mineral waters of various brands (12–13). In one of these investigations, samples of three European brands of mineral water without gas were monitored by RT-PCR during a period of a year. NV sequences were detected in 33 % (53 of 159) of the analysed samples (12). A further monitoring study found that 8 of 87 samples of oysters (9 %) imported into Switzerland were positive for NV. The positive-tested oysters originated from France (14). Reported here are the results of the phylogenetic analyses of Swiss human NV isolates obtained from January 2001 to July 2003 and the results of the phylogenetic comparison between these human isolates with NV sequences from the mineral waters and oysters.

Methods

NV samples

Most of the NV-positive stool samples were obtained from sporadic cases and from NV outbreak patients involved in the parallel conducted epidemiological studies between January 2001 and July 2003 (8–10, 15). Single patient stool specimens were received during epidemiological investigations undertaken by the Cantonal Laboratory Basel-Landschaft and by the Cantonal Laboratory Solothurn (data not shown). Stool samples were randomly chosen from all available patient samples. Sequence information from a monitoring of oysters (November 2001 to February 2002; used NV sequences isolated from the positively-tested oysters originating from France) (14) and from a monitoring of three European brands of mineral water (April 2000 to April 2001) (12) were included in the phylogenetic comparison.

RT-PCR methodology, sequence analyses and phylogeny

All samples were analysed with the same NV RT-PCR methodology. The method used consisted of a genogroup-specific RT-PCR system for the detection of NV GGII based on degenerate primers, located in highly conserved regions of the RNA polymerase (system B) and of a second generic RT-PCR system also based on degenerate primers (system A) (16). This combined system is a part of the detection method for NV in water samples recommended by the SFOPH in Switzerland (17). The NV RT-PCR of the human stool samples was performed at the Cantonal Laboratory Basel-Landschaft and the sequencing of the RT-PCR products was carried out by Microsynth GmbH (9436 Balgach, Switzerland). Phylogenetic analyses were performed on sequences from both NV detection systems. Alignment of the sequences was conducted by the software ClustalX 1.81. The distance-based (Kimura matrix) neighbor-joining (NJ) method in the software package PAUP 4.0b10 was the algorithm used for analysis and the phylogenetic trees were constructed with the software Treeview 1.6.6. The robustness of the NJ-trees generated were assessed by bootstrapping (1000 replications) in PAUP 4.0b10. Pairwise sequence comparisons were carried out with the software EMBOSS matcher 2.0u4s. The strains found in this study were compared with the following published refer-

ence sequences (GenBank accession numbers in parentheses): Desert Shield Virus (DSV; U04469), Bristol Virus (BV; X76716), Camberwell Virus (CAV; AF145896), Hawaii Virus (HV; U07611), Lordsdale Virus (LV; X86557), Melksham Virus (MKV; X81879), Mexico Virus (MXV; U22498).

Results

Phylogenetic comparison of Swiss human NV isolates

In total, 74 NV sequences isolated from human stool samples were phylogenetically analysed. 32 isolates were detected by the generic primer system (system A) and the other 42 isolates by the GGII – specific system (system B). The appropriate NJ-trees are plotted in the figure 1, panels A & B.

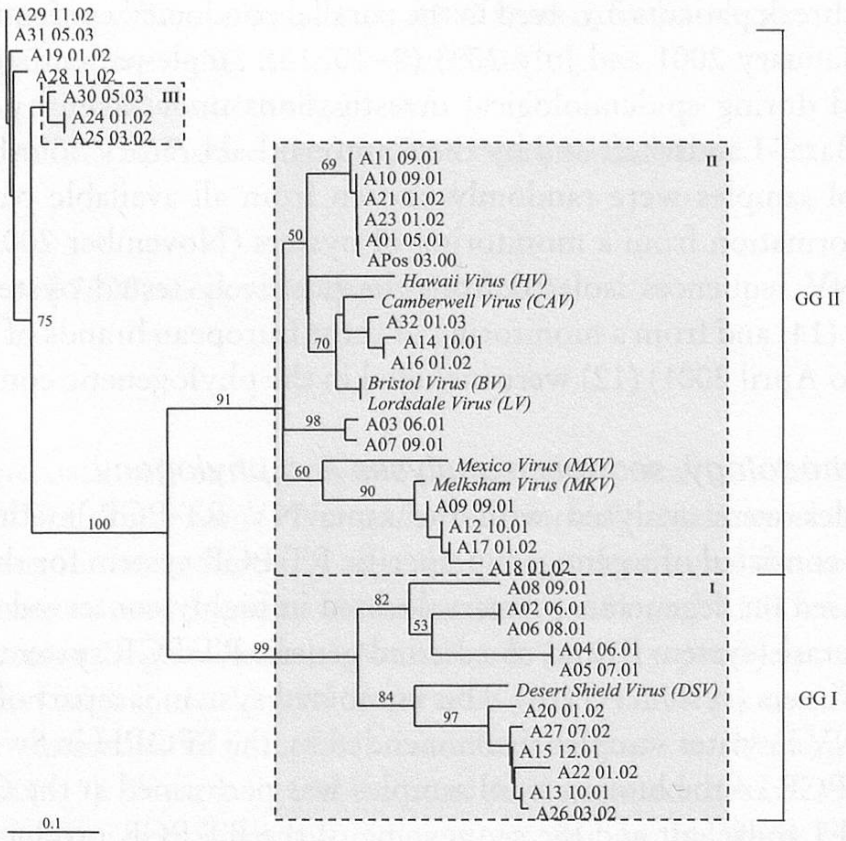

Figure 1, panel A **NJ-tree showing the phylogenetic relationship between human NV sequences from Switzerland (generated with the generic RT-PCR system A) in comparison with the following reference strains: Desert Shield Virus (DSV; U04469), Bristol Virus (BV; X76716), Camberwell Virus (CAV; AF145896), Hawaii Virus (HV; U07611), Lordsdale Virus (LV; X86557), Melksham Virus (MKV; X81879), Mexico Virus (MXV; U22498).** Bootstrapping values are indicated above the major branches. The sequence IDs are describing the used primer system (A), ID-number and date of illness (mmyy). A contamination with the used positive control ("APos") can be excluded

A region of the RNA polymerase of approximately 120 bp was amplified by RT-PCR and used for the phylogenetic analysis in the NV generic system A. Three distinct sequence clusters were detected. Cluster I revealed an overall sequence identity of 76.0 % in respect to the sequence "A08" and of 77.9 % in respect to the GGI reference strain Desert Shield Virus (DSV). Cluster II showed an overall sequence identity of 83.9 % compared to the sequence "A09". The isolates within Cluster II grouped together with the following GGII reference strains: Hawaii Virus (HV), Camberwell Virus (CAV), Bristol Virus (BV), Lordsdale Virus (LV), Mexico Virus (MXV) and Melksham Virus (MKV). The isolates from cluster II showed an overall sequence identity of 85.0 % with the Camberwell Virus. Finally, cluster III exposed a high sequence identity of 97.4 % to the sequence "A30", as seen in figure 1, panel A.

For the NV GGII – specific system B, another region of the RNA polymerase of about 120 bp was amplified by RT-PCR and used for the phylogenetic analysis. Two distinct clusters within NV GGII were found, as seen in figure 1, panel B. Cluster I

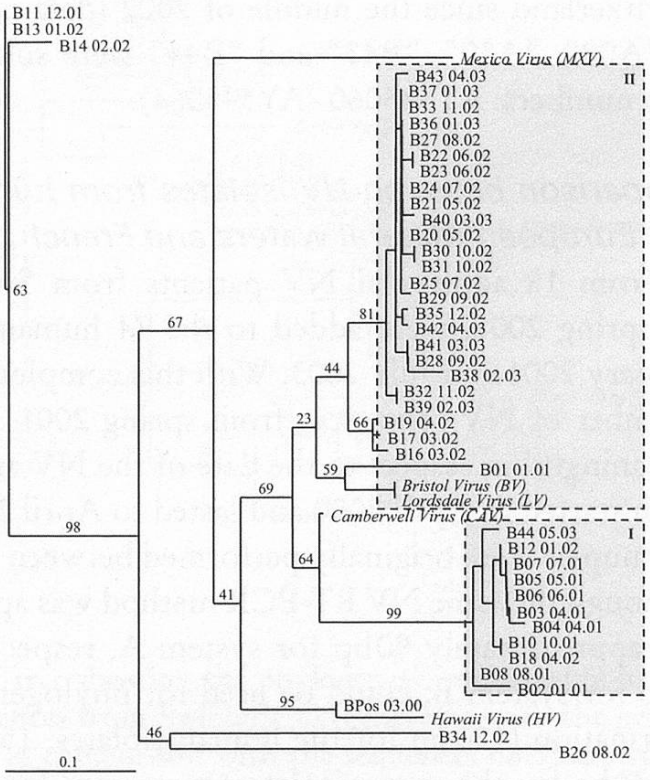

Figure 1, panel B **NJ-tree showing the phylogenetic relationship between human NV sequences from Switzerland (generated with the NV GGII – specific RT-PCR system B) in comparison with the following reference strains: Bristol Virus (BV; X76716), Camberwell Virus (CAV; AF145896), Hawaii Virus (HV; U07611), Lordsdale Virus (LV; X86557), Mexico Virus (MXV; U22498).** Bootstrapping values are indicated above the major branches. The sequence IDs are describing the primer system used (B), ID-number and date of illness (mmyy). A contamination with the used positive control ("BPos") can be excluded

showed an overall sequence identity of 96.1 % compared to the sequence "B44" and cluster II demonstrated an overall sequence identity of 97.4 % with the sequence "B43". Cluster II included the reference strain Bristol Virus (BV) and Lordsdale Virus (LV). The sequence identity between "B43" and "B44" was 80.0 %. Compared to the reference sequence of the Camberwell Virus (CAV), cluster I showed a sequence identity of 87.4 % and cluster II an identity of 91.1 %.

In total, 11 of the 74 sequenced NV isolates (15 %) belonged to the *Norovirus* GGI and 63 samples (85 %) to GGII. No local clustering of the patients where the NV sequences were derived from could be observed (data not shown). Interestingly, a temporal clustering of the NV sequences was observed: 8 of 10 NV GGII samples from the year 2001 and detected by the system B could be assigned to the found cluster I, as seen in figure 1, panel B. The cluster II on the other hand is mainly formed by sequences obtained in the year 2002 (17 of 26) and in the early months of 2003 (8 of 26). Of further interest is the fact that all samples positive for GGI were only found in the year 2001 and in the early months of 2002 by the system A, as seen in figure 1, panel A. This corresponds to the observation that GGI isolates are hardly found in Switzerland since the middle of 2002 (data not shown). The NV sequences "A08", "A09", "A30", "B43" and "B44" were submitted to GenBank (GenBank accession numbers: AY545060–AY545064).

Phylogenetic comparison between NV isolates from human stool samples from Switzerland, European mineral waters and French oysters

Sequence data from 18 additional NV patients from Switzerland, collected between 1999 and spring 2001, were added to the 74 human NV isolates of the period between January 2001 and July 2003. With this completion, the underrepresentation in the number of NV sequences from spring 2001 and before was corrected. This was meaningful in respect to the date of the NV analyses with mineral water samples which started in April 2000 and lasted to April 2001. The NV analyses with the oyster samples were originally performed between November 2001 and February 2002. Although the same NV RT-PCR method was applied on all samples, only a fragment of approximately 90 bp for system A, respectively a fragment of approximately 70 bp for system B, could be used for phylogeny. Even if the complete sequence information (120 bp for the human isolates, 150 bp for the mineral water isolates and 140 bp for the oyster isolates) were used for an improper phylogenetic comparison due to massive sequence overlaps, the main conclusions drawn on the following phylogenies remain the same (data not shown).

Figure 2, panel A, shows the results of the phylogenetic comparison based on the system A. All NV sequences obtained from European mineral waters (IDs "ABAG#") clustered entirely together in GGII and were distinct from all others except for one Swiss human NV sequence, "AAdd1", which clustered within the mineral water sequences. All mineral waters were bottled within the time period from October 1999 to August 2000 (9). The sequence "AAdd1" derived from a

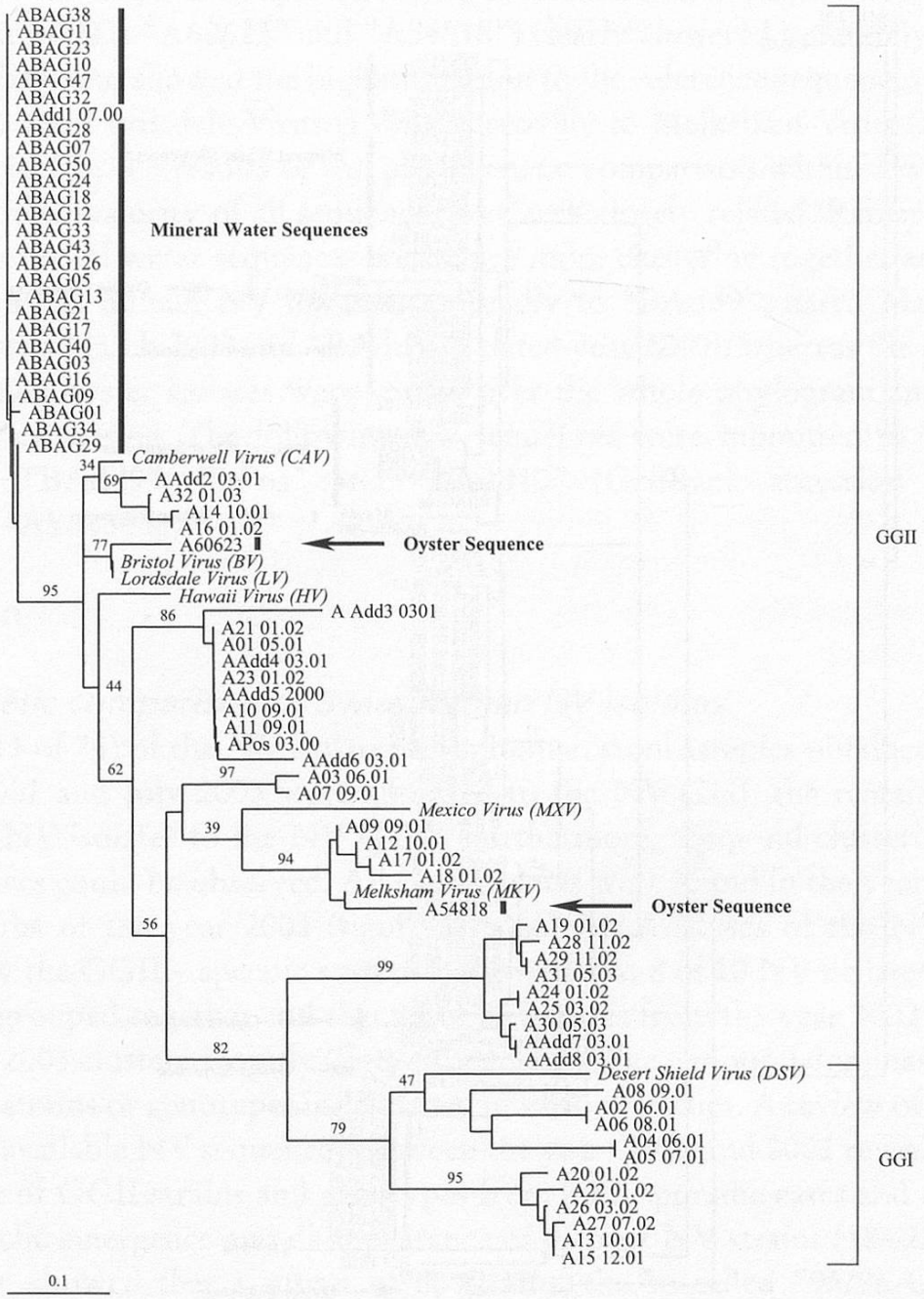

Figure 2, panel A **NJ-tree showing the phylogenetic relationship between human NV sequences from Switzerland (generated with the generic RT-PCR system A) in comparison with the sequences obtained from European mineral waters ("ABAG#"; indicated by black vertical bars) analysed between April 2000 and April 2001 and from French oyster samples (A60623 and A54818; indicated by black vertical bars) analysed between November 2001 to February 2002 and with the following reference strains: Desert Shield Virus (DSV; U04469), Bristol Virus (BV; X76716), Camberwell Virus (CAV; AF145896), Hawaii Virus (HV; U07611), Lordsdale Virus (LV; X86557), Melksham Virus (MKV; X81879) and Mexico Virus (MXV; U22498).** Bootstrapping values are indicated above the major branches. The sequence IDs of the human samples are describing the primer system used (A), ID-number and date of analysis (mmyy)

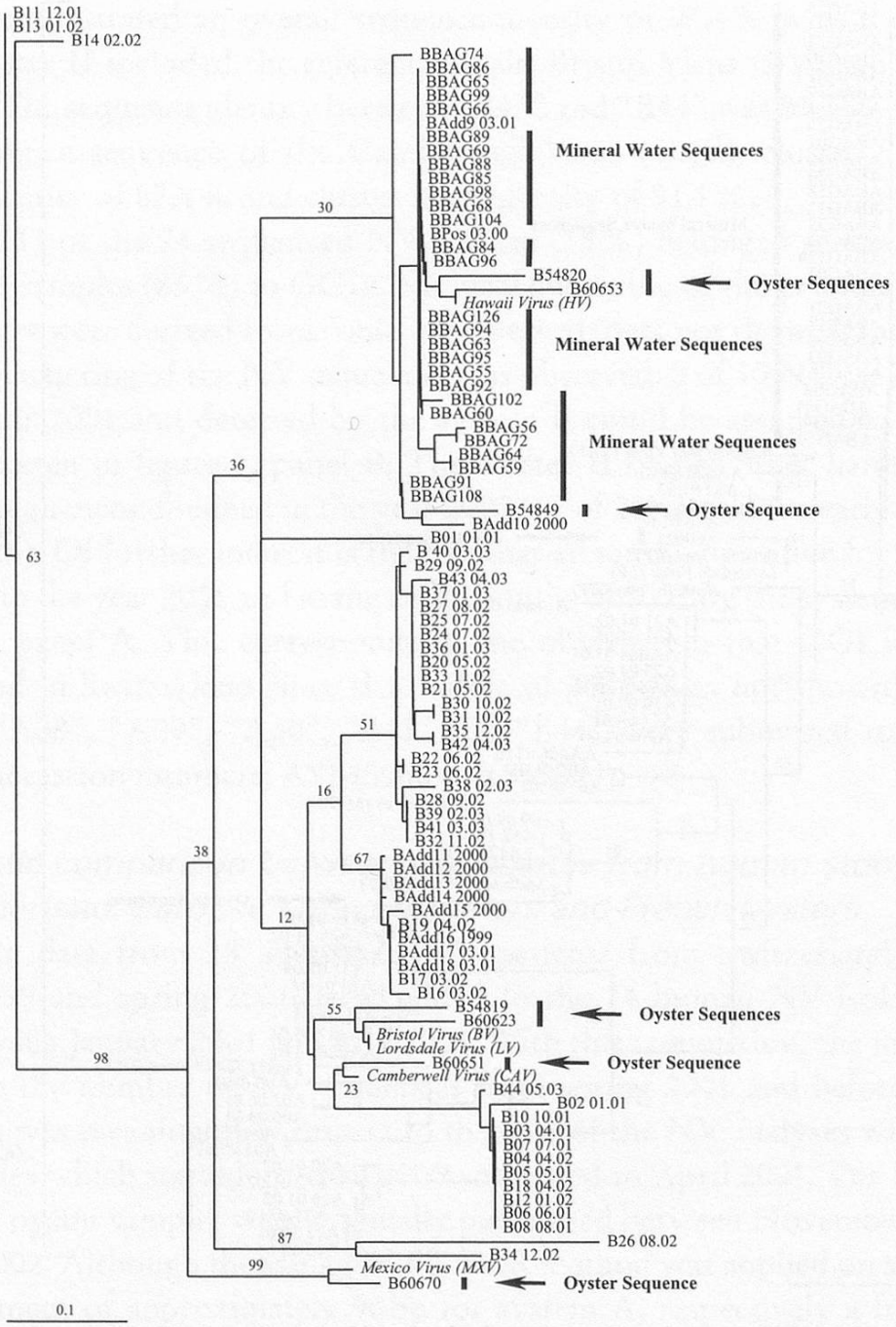

Figure 2, panel B **NJ-tree showing the phylogenetic relationship between human NV sequences from Switzerland (generated with the NV GGII – specific RT-PCR system B) in comparison with the sequences obtained from European mineral waters ("BBAG#"; indicated by black vertical bars) analysed between April 2000 and April 2001 and from French oyster samples ("B60623" and "B54818"; indicated by black vertical bars) analysed between November 2001 to February 2002 and with the following reference strains: Bristol Virus (BV; X76716), Camberwell Virus (CAV; AF145896), Hawaii Virus (HV; U07611), Lordsdale Virus (LV; X86557) and Mexico Virus (MXV; U22498).** Bootstrapping values are indicated above the major branches. The sequence IDs of the human samples are describing the primer system used (B), ID-number and date of analysis (mmyy)

NV positive tested water sample belonging to an outbreak in July 2000. The French oyster isolates (IDs "A60623" and "A54818") clearly clustered separately form the human isolates and showed the highest relation to the reference sequences of Bristol Virus (BV) and Lordsdale Virus (LV), respectively to Melksham Virus (MKV). In figure 2, panel B, the results of the phylogenetic comparison within system B are presented. The majority of all sequences was very closely related. Remarkably, the European mineral water sequences were once more clustering together and closely related to Swiss human NV isolates (especially to "BAdd9", dated March 2001; "BPos", dated March 2000 and "BAdd10", dated year 2000), whereas the sequences of the French oyster isolates were spread over the whole phylogram and showed no distinct clustering. The following NV sequences were submitted to GenBank: "AAdd1", "BAdd9", "BPos" and "BAdd10" (GenBank accession numbers: AY545065–AY545068).

Discussion

Phylogenetic comparison of Swiss human NV isolates

15 % (11 of 74) of the NV isolates from human stool samples obtained between January 2001 and July 2003 were allocated to the NV GGI, the remaining 85 % (63 of 74) NV isolates to the NV GGII. Furthermore, temporal clustering within the sequences could be observed. All GGI isolates were found in the year 2001 and early months of the year 2002 (figure 1, panel A). Analyses of the NV isolates detected by the GGII – specific system B showed that 8 of 10 NV isolates from the year 2001 grouped together and that 23 of 26 isolates from the year 2002 and early months of 2003 clustered jointly (figure 1, panel B). The temporal dominance of different NV strains or genotypes is described in various studies. A review of the internationally available NV sequences between the years 1992 and 2002 revealed a clear dominance of GGII strains and genotypes from both sporadic cases and outbreaks and the cyclic emergence and disappearance of distinct NV strains (18–25). A well cited study showed that a subset of 60 strains, the so-called "95/96-US" strain (GGII), was predominantly associated with NV outbreaks during the 1995–1996 season in the US. Furthermore, this strain showed a global distribution (19, 22). In Europe, it could be observed that NV sequences found in the years 2000 and 2001 clustered around a newly emerging variant of a GGII strain (so-called "GGIIb" strain) (23–24). Likewise, a cluster of NV isolates around a new GGII/4 strain (so-called "Farmington Hills" strain) could be detected during the year 2002 in the US (25). A striking increase in NV outbreaks occurred in 2002 in England and Wales, Germany, and in The Netherlands. This coincided with the emergence of a new predominant NV GGII/4 variant (26). In the GGII – specific system B 23 of 26 NV isolates from the year 2002 and early months of 2003 were clustering together. Within this specific cluster, Bristol virus, a GGII/4 reference strain (27) can be found (figure 1, panel B, cluster II). Most probably, these NV isolates are therefore

corresponding to the new variant found in Europe. This assumption seems to be reasonable, because of the first occurrence of these new variant in Germany (Switzerland lies next to Germany) in January 2002 (26). Furthermore, a Swiss NV screening study from July 2001 to July 2003 revealed that the detection rates of NV positives peaked between January and July 2002 (10). This circumstance may explain the grouping of certain NV GGII sequences found in this study.

Phylogenetic comparison between NV isolates from human stool samples from Switzerland, European mineral waters and French oysters

Phylogenetic analyses with both systems (NV generic system A and NV GGII – specific system B) revealed similar results with respect to the NV sequences from the European mineral water samples (figures 2, panels A & B). The majority of the mineral water sequences were highly related and clustered predominantly separate to the human NV sequences. Nevertheless, single human NV isolates from Switzerland were also found within these mineral water clusters. Furthermore, it was shown that the NV sequences found during the mineral water screening from April 2000 to April 2001 (12) were closely related to human NV isolates. The analysed European mineral waters were all bottled between October 1999 and August 2000. Interestingly, Swiss human NV isolates clustering within the European mineral water sequences ("AAdd1", "BAdd9", "BPos" and "BAdd10") came from patients of the same time period. From an epidemiological point of view, the NV positive results obtained by RT-PCR of mineral waters have to be treated with caution because the used detection method is not able to distinguish between infective and inactivated virus (12–13, 28). But it was demonstrated that the NV sequences from the analysed mineral waters were stable after 6 and 12 months of storage (12). Furthermore, a recent study could display that enteric viruses, inactivated at moderate temperatures, still have a capsid that protects the RNA from RNase (29). The origin of NV sequence contamination in bottled mineral waters also remains unclear. Water sources, packing materials or the bottling process were considered as possible origin of contamination (12). Contamination of the water source, e.g. by surface waters, may play a role. If so, could NV derive from an animal reservoir? To date, this is very unlikely because until now, no zoonotic transmission of NV was demonstrated (30). Furthermore, it is established that the NV strains in animals (calf and pig) were genetically distinct to any NV found in humans (30–32). Regarding the close relationship between NV sequences found in mineral waters and human stool samples, it can be stated that the detected NV in the mineral waters are of human origin. The sensitivity of the method used was estimated to be around 10 viral particles. This suggested a low concentration of NV sequences per litre of the tested mineral water brands (12). Additionally, a case-control study to investigate risks for NV infections did not identify mineral waters to be a measurable risk factor (15). Therefore, it can be supposed that mineral waters do not play a relevant

role in the epidemiology of NV in Switzerland. This estimate confirms a former assessment of the Swiss Federal Office of Public Health (SFOPH) (6).

The sequence analyses NV from the French oysters showed a far greater variability compared to the NV sequences generated from the mineral water samples. Oyster sequences were not found within specific clusters, but grouped together with NV sequences from mineral waters and human samples. Together with the low NV positivity rate of 9 % (8 of 87 samples) of the oyster monitoring study (14) and since the overall consumption of oysters in Switzerland is rather low (14), the direct impact on food safety and the epidemiology of the NV can be stated as rather low (33).

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Summary

Reported here are firstly the results of the phylogenetic analyses of human *Norovirus* (NV) isolates obtained from patients with sporadic infections and from outbreak cases in Switzerland between January 2001 and July 2003. Secondly, the results of the phylogenetic comparison between NV sequences from human stool specimens from Switzerland with NV sequences obtained from a Swiss recent study with European mineral waters and from an oyster screening are presented. 63 of the 74 (85 %) analysed human NV sequences belonged to NV Genogroup II and a temporal clustering was observed. The phylogenetic comparison of NV sequences from mineral waters and oysters with such of human origin revealed that the mineral water sequences were highly related and clustered predominantly separate to the human NV sequences. However, human NV sequences were also found within the mineral water cluster. Additionally, a temporal correlation between the dates of the stool specimens with the period of bottling of the mineral waters was observed. The oyster sequences displayed a far greater variability and no specific clustering with either mineral water or human NV sequences was found.

Zusammenfassung

Beschrieben sind hier einerseits die Resultate der phylogenetischen Analyse von *Norovirus*-Isolaten *(Norovirus;* NV) aus humanen Stuhlproben von Patienten mit sporadischen NV-Infektionen und von Patienten aus NV-Ausbrüchen in der Schweiz im Zeitraum von Juli 2001 bis Juli 2003. Andererseits werden die Ergebnisse der phylogenetischen Untersuchung von NV-Sequenzen aus humanen Stuhlproben mit jenen aus einer Schweizer Studie mit Europäischen Mineralwässern und einem Austern Screening aufgezeigt. 63 der 74 (85 %) analysierten humanen NV-

Sequenzen konnten der NV-Genogruppe II zugeschrieben und zeitliche Häufungen der NV-Sequenzen konnte festgestellt werden. Der phylogenetische Vergleich der NV-Sequenzen aus Humanproben mit jenen aus den Mineralwässern ergab, dass die Mineralwasser-Sequenzen einen hohen Verwandtschaftsgrad aufwiesen und sich überwiegend gesondert zu den humanen NV-Sequenzen clustern. Dennoch konnten vereinzelte humane NV-Sequenzen in den Mineralwässer-Cluster gefunden werden. Zusätzlich wurde eine temporale Korrelation zwischen den Stuhlprobeentnahmedaten mit der Abfüllperiode der Mineralwässer gefunden. Die NV-Sequenzen der Austernproben wiesen hingegen eine grosse Variabilität auf und zeigten kein Clustering; weder mit den NV-Sequenzen aus den Mineralwässern, noch mit jenen aus den Humanproben.

Résumé

Ce travail a été consacré à l'analyse phylogénétique des *Norovirus* (NV) d'origine humaine, isolés en Suisse chez des patients infectés sporadiquement et des patients présentant des symptômes épidémiques pour la période de juillet 2001 à juillet 2003. Les résultats ont ensuite été comparés à une étude effectuée en Suisse sur des eaux minérales européennes et à une étude qui avait trait aux huîtres. Sur 74 séquences de NV d'origine humaine, 63 (85 %) appartenaient au type NV-Génogroupe II. La fréquence des cas a présenté des accumulations irrégulières durant l'essai. La comparaison phylogénétique des séquences NV provenant d'échantillons humains avec les échantillons d'eaux minérales a démontré un haut degré parental chez les séquences NV des eaux minérales, celles-ci forment un cluster bien distinct des séquences NV humaines. Quelques rares séquences NV humaines ont toutefois été identifiées dans le cluster des eaux minérales. De plus, une corrélation temporelle a pu être établie entre les prélèvements de selles humaines et la mise en bouteille des eaux minérales. Les séquences NV trouvées dans les huîtres démontrent au contraire une grande hétérogénéité. Elles ne s'apparentent ni avec le cluster des séquences NV des eaux minérales, ni avec celui des échantillons humains.

Key words

Norovirus, phylogeny, clinical samples, mineral waters, oysters

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