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Korrespondenzadresse:
Reimar.Johne@bfr.bund.de

Federal Institute for Risk Assessment, Diedersdorfer Weg 1, 12277 Berlin

Experimental accumulation and persistence of norovirus, feline calicivirus and rotavirus in blue mussels *(Mytilus edulis)*

Experimentelle Anreicherung und Persistenz von Noroviren, Felinen Caliciviren und Rotaviren in Miesmuscheln (Mytilus edulis)

Reimar Johne, Ralf-Peter Pund, Christina Schrader

Summary

Outbreaks of viral gastrointestinal diseases due to the consumption of contaminated shellfish have been repeatedly reported. Human pathogenic viruses may accumulate in shellfish after faecal contamination of the growing area and thereafter persist for several weeks. Here, blue mussels *(Mytilus edulis)* were contaminated with noroviruses and rotaviruses for 24 hours in a recirculation artificial seawater system. Noroviruses were found to be enriched 100 to 1000fold within the mussels and persisted therein for at least 4 weeks as assessed by semi-quantitative real-time RT-PCR. In contrast, rotaviruses were not efficiently enriched in shellfish and they were only detectable for approximately one week using real-time RT-PCR and cell culture. Feline calicivirus, used as surrogate for norovirus infectivity testing, was also only detectable in shellfish for approximately one week. For rotavirus, the inactivation kinetics in shellfish and contaminated seawater were largely similar. In contrast, norovirus was highly stable in shellfish for at least 4 weeks, but the amount declined in seawater and was no longer detectable therein at 3 weeks after contamination. The results indicate a unique mechanism of norovirus enrichment and persistence in shellfish. For improvement of microbial safety, surveillance may be focused on detection of noroviruses in shellfish samples.

Keywords: Shellfish, norovirus, rotavirus, feline calicivirus, accumulation, persistence

Zusammenfassung

Über Ausbrüche von Magen-Darm-Erkrankungen nach dem Verzehr von Muscheln wurde wiederholt berichtet. Humanpathogene Viren können in Muscheln nach fäkalen Kontaminations-Ereignissen in den Anbaugebieten angereichert werden und danach für mehrere Wochen in ihnen persistieren. In dieser Arbeit wurden Miesmuscheln *(Mytilus edulis)* mit Noroviren und Rotaviren für 24 Stunden in einem Zirkulationssystem mit künstlichem Meerwasser kontaminiert. Die Noroviren wurden hierbei 100- bis 1000-fach in den Muscheln angereichert und persistierten in ihnen mindestens 4 Wochen lang, wie durch semiquantitative real-time RT-PCR-Untersuchungen gezeigt wurde. Dem gegenüber wurden die Rotaviren nicht effizient in den Muscheln angereichert und waren nur etwa eine Woche lang mit real-time RT-PCR und Zellkultur nachweisbar. Das Feline Calicivirus, welches als Modellvirus für Norovirus-Infektiositätstests verwendet wurde, war auch nur etwa eine Woche lang in den Muscheln nachweisbar. Für die Rotaviren war die Inaktivierungskinetik in den Muscheln sehr ähnlich der im Meerwasser. Dem gegenüber waren Noroviren für mindestens 4 Wochen sehr stabil in den Muscheln, aber ihre Menge nahm im Meerwasser ab und sie waren hier nach 3 Wochen nicht mehr nachweisbar. Die Ergebnisse weisen auf einen besonderen Mechanismus der Anreicherung und Persistenz von Noroviren in Muscheln hin. Zur Verbesserung der mikrobiellen Sicherheit sollte die Überwachung auf den Nachweis von Noroviren in Muscheln fokussiert werden.

Schlüsselwörter: Schalentiere, Norovirus, Rotavirus, Felines Calicivirus, Anreicherung, Persistenz

Journal of Food Safety and Food Quality 62, Heft 4 (2011), Seiten 105–144

Introduction

Several disease outbreaks caused by viruses have been linked to the consumption of raw or undercooked shellfish. One of the largest reported outbreaks was recorded in 1988 in Shanghai, China, where about 300,000 people fell ill with hepatitis A after consumption of shellfish derived from a distinct contaminated growing area (Halliday et al., 1991). More recent outbreaks in Europe linked to contaminated shellfish are mostly attributed to noroviruses. For example, in 2005 a total of 200 cases of gastroenteritis in Italy and 127 cases in France could be traced to norovirus-contaminated oysters originating from a certain growing area in France (Le Guyader et al., 2006a). From January to March 2010 a total of 334 cases of norovirus-gastroenteritis due to the consumption of raw oysters were officially notified in five European countries (UK, Norway, France, Sweden and Denmark; Westrell et al., 2010). Although most of the cases are attributed to raw oysters, outbreaks involving other shellfish species and/or lightly cooked shellfish have also been described (Potasman et al., 2002).

The main shellfish-borne viral diseases are characterized by hepatitis or gastroenteritis. Among the hepatitis viruses, the hepatitis A virus is the main contaminant of shellfish, often recognized in some countries of Southern Europe (Romalde et al., 2002, Chironna et al., 2002), but relatively rare in Germany. Among the viruses causing gastroenteritis, norovirus is most frequently found in shellfish among several European countries (Prato et al., 2004; Le Guyader et al. 2010; Nenonen et al., 2009; Doré et al., 2010; Westrell et al., 2010); but there are only limited data on the occurrence of this virus in shellfish from Germany. However, infection with noroviruses is the main cause of gastroenteritis in Germany, with 171.613 officially notified cases in 2010 (Robert-Koch-Institut, 2011). Noroviruses are non-enveloped RNA viruses, which belong to the family *Caliciviridae* (Katpally & Smith, 2010). Norovirus infection causes a disease in all age classes characterized by vomiting and diarrhoea. Another important viral agent causing gastroenteritis is rotavirus. In Germany, 54.029 cases of rotavirus gastroenteritis have been officially notified in 2010 (Robert-Koch-Institut, 2011). Rotavirus disease is mostly seen in young children. Generally, rotavirus disease outbreaks due to the consumption of shellfish are very rarely described and the occurrence of rotavirus in shellfish in Germany has not been published so far.

Shellfish accumulate viruses from the environment through their filter feeding. The main source of human pathogenic viruses is faecal contamination of the shellfish growing area by sewage discharges (Lees, 2000). Filtration rates of 1.5 l/h or 6 l/h can be achieved by a blue mussel or an oyster, respectively (Riisgard, 1988), thus enabling efficient uptake of viruses. Although the human pathogenic viruses cannot replicate in shellfish, enrichment within shellfish is possible. For instance, hepatitis A virus has been shown to be 100fold enriched in shellfish after a 24h contamination period (Enriquez et al., 1992). Thereafter, viruses can persist for a long time-period, as shown for hepatitis A virus, which remains infectious for up to 4 weeks in marinated shellfish (Hewitt & Greening, 2004). For norovirus, persistence for approximately 2 months has been shown in oysters (Le Guyader et al., 2003). However, as no cell culture system exists for propagation of noroviruses, infectivity could not be assessed so far. In order to estimate persistence of infectivity of noroviruses, surrogate viruses such as feline calicivirus (FCV), which is taxonomically related to norovirus and can be efficiently propagated in cell culture, have been used in former studies with different environmental and food matrices (Bidawid et al., 2004; Duizer et al., 2004; Cannon et al., 2006; Mattison et al., 2007; Ueki et al., 2007).

Different types of viruses in combination with certain shellfish species may show different behaviour. The aim of our study was to assess the enrichment and persistence of noro- and rotaviruses, which are the most common causes of viral gastroenteritis in Germany, in the blue mussel *(Mytilus edulis),* which is the most important shellfish species commercially cultivated in Germany. Semi-quantitative real-time RT-PCR investigations and infectivity titrations in tissue culture were applied to mussels hold for up to 4 weeks in an artificial seawater system. The results should enable a direct comparison of the enrichment capacity and persistence of the different viruses and therefore give an indication for directed measures to improve microbial safety of shellfish.

Materials and Methods

Origin of mussels and animal husbandry system

The blue mussels *(Mytilus edulis)* used in the experiments were harvested from wild populations of the Baltic Sea near the shore of the Kiel Fjord (altitude 10°10' east, latitude 54°23' north) at a depth of two meters. After a 5 hour transport, the mussels were adapted to 15 °C for at least 10 hours and their surface was cleaned. The mussels were placed in a 600–800 liter recirculation artificial seawater system at 15 °C with 15–20 ‰ salinity as also found at the harvesting location (Pund, 2009). Water was prepared by dissolving an artificial salt mix (Tropic Marine salt mix, Wartenberg, Germany) with tap water. Mussel densities ranged from 300 to 500 g whole body weight per liter sea water with an average valve length of 5,4 ± 1,2 cm. Feeding was facilitated continuously using a mix of *Phaeodactylum tricornutum* and *Tetraselmis suecica* with final concentrations between 10^4–10^5/ml water. The mussels were kept for at least 4 weeks in this system before starting the contamination studies.

Cells and viruses

Bovine group A rotavirus (isolate 4630F6, kindly provided by P. Otto, Friedrich Loeffler Institute, Jena) was propagated in MA-104 cells as previously described (Elschner et al., 2005; Scherer et al., 2009). Feline calicivirus (isolate KS-20, kindly provided by U. Truyen, University of Leipzig) was propagated in CRFK cells as described (Geissler et al., 1997). A stool sample containing norovirus genogroup II.3 derived from a child suffering from enteric symptoms and diluted as 10 % suspension in PBS (Scherer et al., 2010) was used in the contamination experiments. It contained $2x10^7$ real-time RT-PCR units per ml (one RT-PCR unit is the lowest amount of norovirus RNA detectable by the applied RT-PCR protocol).

Bioaccumulation experiments

All experiments were conducted in a small-scaled recirculation seawater system under constant conditions. Two 20 l inner glass aquariums, each integrated in a 40 l outer glass aquarium serving as a thermoregulating bath (refrigerated to 15 °C) were placed under a laminar flow sterile workstation. The aquaria were autoclaved before use and covered with plexiglas plates in order to avoid aerosol

contaminations. One of the inner glass aquariums (contamination unit) was filled with 20 litre artificial sea water with a salinity of 20 ‰ and 100 mussels derived from the husbandry system were placed inside. Thereafter the virus preparation was added to the seawater and distributed by careful mixing with a circulation pump reaching a turnover rate of about 20 l/h. The dissolved oxygen concentration (DOC) never fell below 95 % saturation. After 24 h, the mussels were removed from the contamination unit and carefully scrubbed with the virucidial disinfectant Virkon® S (1 %, Noack GmbH, Warendorf, Germany) in order to remove viruses adhering to the outer surface. Thereafter, the mussels were rinsed in sterile artificial seawater and placed in the other inner glass aquarium (holding unit). This unit contained 20 l clean artificial seawater with salinity of 20 ‰. In order to mimic a natural water exchange, 10 l of the water was exchanged with fresh artificial seawater every second day. The water of the holding unit was continuously filtrated and aerated by using an air-lift filter system coupled to an aeration pump. In all experiments a natural photoperiod was chosen and the DOC was close to saturation (96–98 %). During the whole experiment mussels were fed on a commercial diet (Premium Reef Blend, MeWaStore, Darmstadt, Germany) containing a mix of the unicellular marine algae *Chlorella* sp., *Nanochloropsis* sp. and *Pheaodactylum tricornutum,* with final concentrations between 10^4–10^5/mL water.

Sampling and preparation of digestive glands

Groups of 10 mussels each were sampled shortly before and 24 h after virus contamination, which served as negative and positive controls, respectively. At 1, 3, 7, 14, 21 and 28 days after the 24-h contamination period, groups of 8–10 mussels were sampled for analysis. Water samples were collected from the contamination unit in rotavirus and norovirus experiments at the same time-points and stored at –80 °C until analysis. The mussels were carefully scrubbed with Virkon® S and rinsed in sterile artificial seawater. Thereafter, they were opened by cutting the adductor with a sterile scalpel and the digestive glands were dissected, pooled and transferred into sterile tubes. The samples were immediately frozen and stored at –80 °C until analysis.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

The digestive glands (half amount of the pooled glands of one time-point) were homogenated using a sterile razor blade. A total of 1 g of the homogenate was mixed with 1 ml proteinase K solution (30 U/mg, Boehringer Mannheim) and incubated at 37°C in a shaking incubator for 60 min followed by incubation at 60 °C for 15 min. After centrifugation at 3,000 x g for 5 min the supernatant was collected and RNA was extracted from 140 µl using the QIAamp viral RNA mini kit (Qiagen, Hilden). RNA was also isolated from 140 µl of water samples using the same kit. The extracted RNA was analysed by real-time RT-PCR using the QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany) on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, USA). The sequences of the used primers and probes are listed in Table 1. Detection of rotavirus was performed by application of a real-time RT-PCR protocol as described by Pang et al. (2004). For norovirus GGII detection, a TaqMan real-time RT-PCR assay described by Loisy et al. (2005a) was used. The same reagent concentrations and thermal profiles were also applied for detection of feline calicivirus, with the exception that other primers and probe were used (Tab. 1), which were newly designed on the basis of the published nucleotide sequence of isolate KS20 (Geissler et al., 1997). In each real-time RT-PCR assay 45 thermal cycles were performed. The ct-values of two independent duplicate tests were averaged (for mussel samples) and compared semiquantitatively to each other.

Titration of viruses in tissue culture

A 10 % suspension of digestive glands (half amount of the pooled glands of one time-point) in sterile PBS was prepared by homogenization using sterile sea sand and mortar and pestle. Debris was removed by centrifugation at 1,000 x g for 10 min, the virus-containing supernatant was sterile filtrated using disposable 0.2 µm filters (Schleicher & Schuell, Germany) and thereafter diluted in ten-fold steps in PBS to enable titration by an endpoint dilution method. Virus dilutions were inoculated on MA-104 cells or CRFK cells grown on 96-well-plates for bovine rotavirus or feline calicivirus titration, respectively. After 5 days, cytopathic changes were recorded and virus titres expressed as 50 % tissue culture infective doses ($TCID_{50}$) were calculated according to Kärber (1931). Data of two independent experiments were averaged.

Results

Rotavirus contamination

About 100 blue mussels *(Mytilus edulis)* were placed into the contamination unit containing 20 l artificial sea water and 20 ml of tissue culture supernatant containing $10^{6.5}$ $TCID_{50}$/ml of bovine rotavirus was added. After a period of 24 hours, the mussels were transferred into the holding unit

TABLE 1: *Primers and probes used for real-time PCR detection of norovirus, rotavirus and feline calicivirus.*

Assay	designation	nucleotide sequence (5'–3')	Reference
Group A rotavirus			Pang et al. (2004)
	Rota NVP3-F	ACCATCTACACATGACCCTC	
	Rota NVP3-R	GGTCACATAACGCCCC	
	Rota TM probe	FAM-ATGAGCACAATAGTTAAAAGCTAACACTGTCAA-TAMRA	
Norovirus genogroup II			Loisy et al. (2005a)
	QNIF2d	ATGTTCAGRTGGATGAGRTTCTCWGA	
	COG2R	TCGACGCCATCTTCATTCACA	
	QNIFS-probe	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	
Feline calicivirus KS20			this study
	SW-FeCV-s	GAA AGC CCA ACA AAT TGA ATT	
	SW-FeCV-as	CGTGTACCCTCTGCTCAAG	
	FCV-probe	FAM-AATGAATCTCGATCGCCAGGC-TAMRA	

Journal of Food Safety and Food Quality 62, Heft 4 (2011), Seiten 105–144

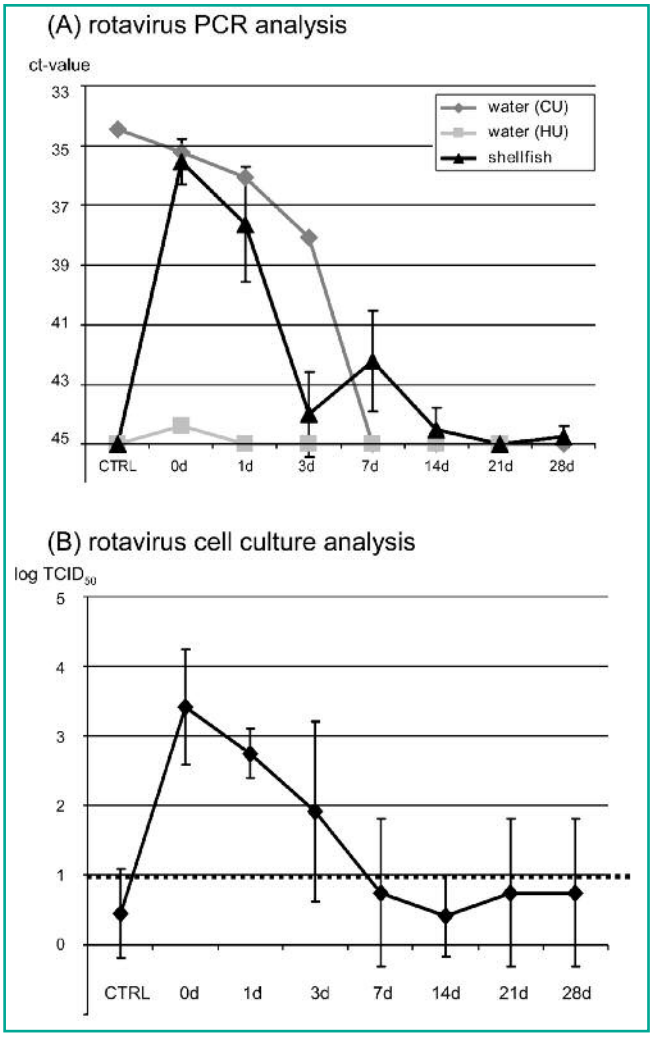

FIG. 1: *Accumulation and persistence of rotavirus in blue mussels (Mytilus edulis). (A) Real-time RT-PCR detection of the rotavirus genome in the digestive gland of shellfish (black triangles), in water of the contamination unit (CU, dark grey rhombs) or in water of the holding unit (HU, light grey squares). The ct-values derived from real-time RT-PCR are plotted against the time-points (days) after the 24-hour contamination period. CTRL: controls (non-contaminated shellfish, contaminated water for CU and non-contaminated water for HU). (B) Tissue culture titration of rotavirus present in the digestive gland of shellfish. The infectivity titres determined by cytopathic effects of digestive gland dilutions on MA-104 cell cultures and expressed in log $TCID_{50}$ are plotted against the time-points (days) after the 24-hour contamination period. CTRL: Effect of a non-contaminated shellfish sample. The dotted horizontal line indicates the titre, up to which cytopathic effects due to virus replication could be masked by toxic effects of the digestive gland homogenate.*

containing fresh artificial sea water. Samples of water and mussels were taken at different time-points and analysed by real-time RT-PCR for the presence of rotavirus RNA (Fig. 1A). In the water of the contamination unit, a moderate amount (ct=34.5) of rotavirus RNA could be detected after adding the tissue culture supernatant, which declined continuously during the next four days and rotavirus RNA was no longer detectable after 8 days after the contamination event. In the water of the holding unit, only very low amounts (ct=44.3) of rotavirus RNA were detected directly after adding the contaminated mussels, but not in later samples. Moderate amounts (ct=35.6) of rotavirus RNA were also detected in the shellfish samples after the 24 h contamination period, which declined to low amounts (ct=42.3) at day 7 after placing into the holding unit. The shellfish samples taken at days 14, 21 and 28 showed only very low ct-values between 44.5 and 45.0. Analysis of infectious rotavirus in the shellfish samples by titration in tissue culture confirmed the presence of rotavirus after the 24h contamination period (Fig. 1B). However, the infectious titre was relatively low (log 3.4 $TCID_{50}$) and declined continuously until day 3 after placing into the holding unit. The samples taken at days 7, 14, 21 and 28 showed cytopathic effects only in some of the non-diluted concentrations. However, as such cytopathic effects also occurred by using non-infected shellfish, this effect was interpreted as an unspecific toxicity of the undiluted shellfish preparation and titres below log 1 were considered as not evaluable (threshold line in Fig. 1B).

Norovirus contamination

The same experimental procedure as described for rotavirus was used for norovirus contamination. In this case, 1 ml of a stool suspension containing $2x10^7$ real-time RT-PCR Units per ml of a genogroup II norovirus was added to 20 l artificial seawater in the contamination unit. As the norovirus could not been titrated in tissue culture, only PCR analyses were performed (Fig. 2). After adding the norovirus, a moderate amount (ct=31.7) of norovirus RNA could be detected in the water of the contamination unit, which slowly declined until day 15 after the contamination event (ct=35.8). No norovirus RNA was detectable in the water samples taken from the contamination unit at 22 and 29 days after the contamination event. Low amounts (ct=39.1–42.2) of norovirus RNA were detected in the water of the holding unit until day 7 after adding the contaminated mussels, but not in samples taken at later time-points. In the shellfish samples, high amounts of norovirus RNA were detected after the 24 h contamination period until the end of the experiment. The ct-values determined for the norovirus RNA in the shellfish samples declined only slowly from 23.4 (directly after the 24 h contamination period) to 29.1 (28 days after placing into the holding unit).

Feline calicivirus (FCV) contamination

FCV was used as a surrogate of norovirus in order to analyse persistence of infectivity. The experimental procedure was identical to that described for rotavirus and norovirus, however water samples were not analyzed systematically. Contamination was performed by adding 2 ml of a tissue culture supernatant containing $10^{7.5}$ $TCID_{50}$/ml of FCV to 20 l artificial seawater in the contamination unit (resulting in FCV RNA detection at ct=33.1 in contamination water). PCR analysis of the shellfish samples (Fig. 3A) detected moderate amounts (ct=32.2) of FCV RNA after the 24 h contamination period, which declined continuously until day 7 (ct=39.7) after placing into the holding unit. In shellfish samples taken later (days 14, 21 and 28), only very low amounts (ct=43.8–43.9) of FCV RNA were detected. Analysis of infectious virus in the shellfish samples by titration in tissue culture confirmed the presence of FCV after the 24 h contamination period (Fig. 3B). However, the infectious titre was low (log 2.6 $TCID_{50}$) and declined continu-

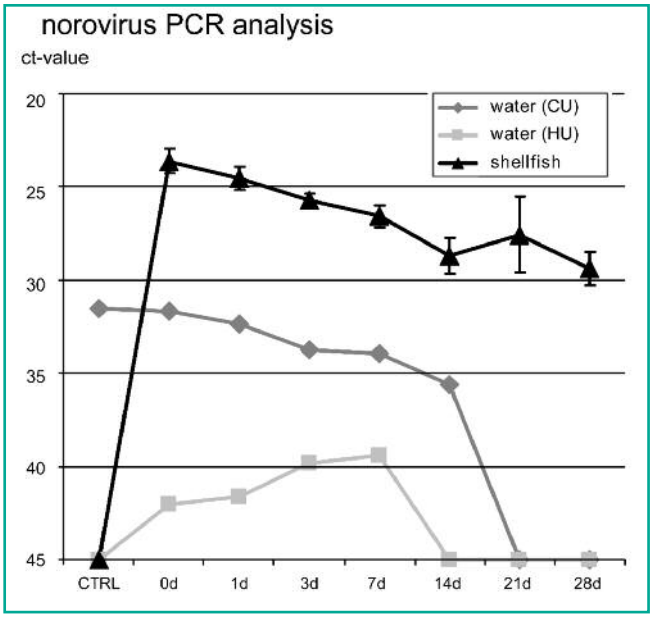

FIG. 2: *Accumulation and persistence of norovirus in blue mussels (Mytilus edulis). Real-time RT-PCR detection of the norovirus genome in the digestive gland of shellfish (black triangles), in water of the contamination unit (CU, dark grey rhombs) or in water of the holding unit (HU, light grey squares). The ct-values derived from real-time RT-PCR are plotted against the time-points (days) after the 24-hour contamination period. CTRL: controls (non-contaminated shellfish, contaminated water for CU and non-contaminated water for HU).*

ously until day 3 (log 1.6 $TCID_{50}$) after placing into the holding unit. As already described for rotavirus titration (see above), cytopathic effects occurred in some tissue cultures inoculated with undiluted shellfish samples, even by using non-infected shellfish. Therefore, titres below log 1 were considered as not evaluable (threshold line in Fig. 3B).

Discussion

It is known for many years, that enteric viruses can be accumulated by shellfish and show a long-time persistence as infectious agents (Bedford et al.,1978; Schwab et al., 1998; Hewitt & Greening, 2004; McLeod et al., 2009). Accordingly, contaminated shellfish frequently cause outbreaks of gastrointestinal diseases (Halliday et al., 1991; Le Guyader et al., 2006a; Potasman et al., 2002; Lees, 2000). In our study, we compared the two most important causative viral agents of gastroenteritis in Germany, noroviruses and rotaviruses, for their ability to be enriched and thereafter persist in shellfish. An artificial system for contamination was established using blue mussels *(Mytilus edulis),* which represent the most important shellfish species commercially cultivated in Germany.

Our artificial system for holding and experimental contamination of shellfish should enable standardized experiments under conditions resembling natural conditions as much as possible. We found that the cultivated shellfish not only survived in the recirculation artificial seawater system for several months, but also gained weight and occasionally produced gametes (data not shown). These growing conditions should therefore resemble those of the natural environment of shellfish in the growing area and enable reproduction of a realistic contamination scenario. Other researchers used a variety of different contamination systems, from simple batch systems to more sophisticated depuration systems (Schwab et al., 1998; Greening at el., 2001; Ueki et al., 2007; McLeod et al., 2009). It should be expected that the results of the studies are influenced by the distinct conditions used, including viability and nutritional stage of shellfish. In addition, the dose of virus used for contamination may influence the magnitude of its enrichment and the time of persistence. We attempted to use equal amounts of the different viruses

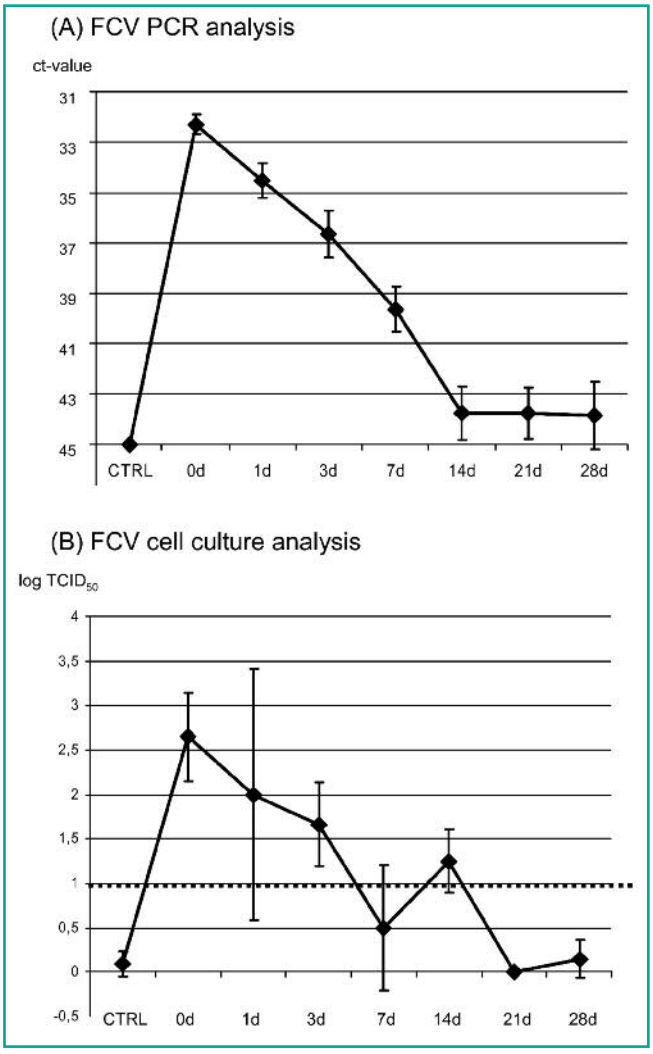

FIG. 3: *Accumulation and persistence of feline calicivirus in blue mussels (Mytilus edulis). (A) Real-time RT-PCR detection of the feline calicivirus genome in the digestive gland of shellfish. The ct-values derived from real-time RT-PCR are plotted against the time-points (days) after the 24-hour contamination period. CTRL: control (non-contaminated shellfish). (B) Tissue culture titration of feline calicivirus present in the digestive gland of shellfish. The infectivity titres determined by cytopathic effects of digestive gland dilutions on CRFK cell cultures and expressed in log $TCID_{50}$ are plotted against the time-points (days) after the 24-hour contamination period. CTRL: Effect of a non-contaminated shellfish sample. The dotted horizontal line indicates the titre, up to which cytopathic effects due to virus replication could be masked by toxic effects of the digestive gland homogenate.*

Journal of Food Safety and Food Quality 62, Heft 4 (2011), Seiten 105–144

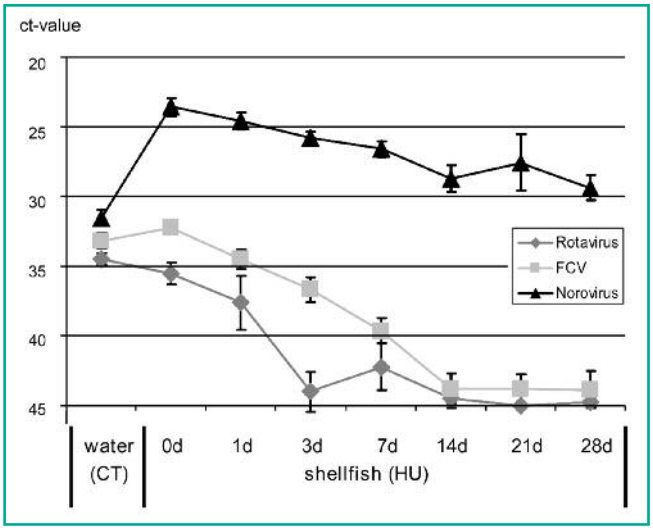

FIG. 4: *Comparative presentation of accumulation and persistence of norovirus (black triangles), feline calicivirus (light grey squares) and rotavirus (dark grey rhombs) in blue mussels (Mytilus edulis), assayed by real-time RT-PCR detection of their genomes. The ct-values are plotted against the time-points (days) after the 24-hour contamination period (shellfish, HU: holding unit). The ct-value derived for the respective virus in the water of the contamination unit (CT) immediately after contamination is presented left.*

to enable comparison and to use rather low virus doses in order to reproduce the natural situation. However, due to the low amounts of rotavirus and feline calicivirus detected after enrichment in the shellfish, infectivity titration in cell culture was difficult and prone to error. Therefore, in future experiments, the infectious dose may be increased for these viruses.

Generally, we found enrichment of all of the used viruses in shellfish followed by a period of persistence for at least 7 (PCR analysis) or 3 days (tissue culture analysis). However, marked differences between the viruses were recorded in the magnitude of enrichment and the time-period of persistence. It is evident from a direct comparison of the PCR results of the different viruses (Fig. 4), that noroviruses are enriched between 100 and 1000fold in the shellfish as compared to the contaminated water, whereas FCV and rotavirus showed only the about same amount in the shellfish as in the contaminated water. In addition, the concentration of FCV and rotavirus declined relatively fast within the shellfish as compared to norovirus. For rotavirus, the decline of the detected virus amount in shellfish was similar to that observed in the contaminated artificial seawater (Fig. 1A). This was in contrast to norovirus, which showed a higher stability in shellfish than in the water (Fig. 2). Altogether, this suggests a specific mechanism of enrichment and persistence of noroviruses in shellfish, which may be explained by the presence of specific receptor molecules for norovirus in the digestive tract of shellfish enabling a strong norovirus binding as recently described (Le Guyader et al., 2006b; Tian et al., 2007). The data are also in accordance with the number of outbreak notifications, which are often due to detection of norovirus, but only very rarely due to rotavirus detection.

Some discrepancies are obvious when our data are compared to that of other published studies. First, at least one report exists in which rotavirus-like particles have been shown to persist in oysters between 37 and 82 days (Loisy et al., 2005b). The distinct reasons for the differences to the results of our study, in which a much shorter time-period of persistence was obtained, are not known so far. However, the use of rotavirus-like particles vs. infectious rotavirus, different environmental conditions including salinity of the used water and different amounts of contaminating virus used in both studies may be considered as explanations. Second, FCV has been widely and successfully used as a surrogate for norovirus in food and environmental persistence studies (Bidawid et al. 2004; Duizer et al., 2004; Mattison et al., 2007). However, our analysis of FCV and norovirus enrichment and persistence in shellfish shows that both viruses behave largely different. Therefore, FCV may not be considered as a good surrogate for norovirus when persistence of virus infectivity in shellfish is analyzed. Indeed, other studies have also shown discrepancies between FCV and norovirus persistence (Cannon et al., 2006; Bae and Schwab, 2008). The use of murine norovirus, which is more closely related to human norovirus than FCV, has been suggested by these authors.

In conclusion, our study shows that contamination of shellfish with noroviruses possesses a significant risk for the consumer. This virus is very efficiently enriched by the mussels and thereafter shows a remarkable long persistence for more than four weeks as shown by PCR testing. These properties are different from that of some other viruses, e. g. FCV and rotavirus, and also for that of *E. coli,* which is currently used for screening the microbiological status of shellfish. As the main source of norovirus in shellfish is faecal contamination by sewage, a strict environmental hygiene regime has to be considered as the only effective way to prevent norovirus contamination of shellfish and to reduce shellfish-born human diseases. Also, direct testing of shellfish for noroviruses should be performed in monitoring in addition to testing for *E. coli.* Priority should be given to validation of the standardized detection methods for noroviruses in shellfish, which are currently developed by CEN (Lees et al., 2010) to enable routine control of noroviruses in shellfish by local control laboratories.

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Corresponding author:
Reimar Johne
Federal Institute for Risk Assessment
Diedersdorfer Weg 1
D-12277 Berlin
Germany
Reimar.Johne@bfr.bund.de