Noroviruses in shellfish: Challenges and facts

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Abstract. Noroviruses are among the most common causes of foodborne outbreaks of human infection. Study of the noroviruses has been difficult due to the lack of a cell culture system, so molecular techniques became the gold standard. Recently though, a cell culture system has been developed and will aid in the acquisition of new knowledge related to human noroviruses.

1. Introduction
Noroviruses cause large gastroenteritis outbreaks worldwide (1,2). They are highly contagious because of their low infective dose, their inducement of short-term immunity and their stability in the environment (3). The most common route of infection is person-to-person transmission through the faecal-oral route or by exposure via contaminated surfaces (4). Foodborne transmission can occur via contamination of food by infected food handlers or directly from contaminated foods. Asymptomatic norovirus infections are also common (5,6,7). Foods that are often implicated in norovirus outbreaks are leafy greens, fruits (raspberries), and shellfish (8,9,10).

2. Food safety regulation
Shellfish harvesting areas are classified under EC Regulation 854/2004. According to the degree of faecal pollution based on Escherichia coli levels, harvesting areas are categorized as A, B or C category. Shellfish from A category are sold directly on the market, whereas shellfish from category B and C undergo a purification or relaying process. Regulation 2073/2005 prescribes criteria for E. coli but not for viruses; E. coli in the sampled shellfish should not exceed 230 MPN/100 g in 4 out of 5 samples and no more than 700 MPN/100 g in one sample of shellfish flesh. Numerous studies agree on the resistance of human enteric viruses in shellfish during the depuration process, which is, on the other hand, effective for bacterial elimination (14,15,16). The combination of a relaying period with a final purification process allowed the diminution of noroviruses to low copy numbers (17). Cooking at 90 °C for 90 seconds is enough to inactivate enteric viruses (18).

3. Detection of the noroviruses
Before ISO 15216-1:2017 for determination of norovirus (and hepatitis A virus) was released, many different methods for the detection of noroviruses were used. The ISO describes methods of norovirus detection for food surfaces (swabs), soft fruit, leaf, stem and bulb vegetables, for bottled water and for shellfish (11). As noroviruses accumulate in the digestive glands of the shellfish (12), the extraction is made from the digestive glands using treatment with a proteinase K solution (11). Detection is made using real-time reverse transcriptase-PCR (RT-PCR) with different primers and probes.
Detection of noroviruses in shellfish samples is difficult because of the low contamination level, the presence of substances that inhibit molecular detection, wide genetic variability and the difficulty of efficient virus extraction (13).

3.1. Classification of the noroviruses
Noroviruses are nonenveloped positive-sense single-stranded RNA viruses, classified in the family *Caliciviridae*. Noroviruses are classified into genogroups and genotypes based on amino acid diversity in the complete VP1 protein (19). Seven genogroups have been classified, and each genogroup is further divided into genotypes. Genogroup GI, GII, and GIV are human noroviruses (19), GII is the most commonly detected in clinical cases, whereas GIV is rarely detected (20). Most of the reported quantitative norovirus RT-qPCR assays target the ORF1-ORF2 junction region, because this part of the norovirus genome is sufficiently conserved for the development of genogroup-specific oligonucleotide primers and probes.

3.2. Pandemic outbreaks
Among all genotypes, only GII.4 is associated with pandemics. Since the mid-1990s, outbreaks and sporadic cases of genogroup II genotype 4 (GII.4) have frequently been reported (6, 21) and have caused five pandemics of acute gastroenteritis (22). Variants of GII.4 have emerged every 2 to 3 years (19). The predominant GII.4 strains had a higher mutation rate and rate of evolution compared to the less frequently detected strains. The GII.4 lineage had higher rate of evolution within the capsid sequence compared to other noroviruses. The study of Bull et al., 2010 supports the hypothesis that epidemiological fitness is a consequence of the ability of the virus to generate genetic diversity, as the pandemic GII.4 strains were associated with increased replication and mutation rates. A parallel can be seen in the epidemiology between norovirus and influenza virus (22). In 2014, a new GII.17 variant known as Kawasaki 2014 emerged and caused an outbreak and sporadic cases across the world, and is replacing the previously prevalent GII.4 Sydney strain from 2012 (23,24,25). In the study of the norovirus strains isolated from sewage in Japan, a strain closely related to the GII.17 Kawasaki 2014 lineage had been observed in the study area a year before its appearance in the clinical cases. A similar pattern was also observed for GI.3 (26).

In a meta-analysis of norovirus global seasonality, winter peaks of noroviruses and positive association with average rainfall in the wettest months were shown (27). The number of gastroenteritis cases increased in the winter months, from November to February, and decreased in the summer months, from June to August. These trends were similarly observed in each norovirus season (26). In Slovenia, a study of norovirus strains isolated from mussels harvested at three harvesting areas (Seča, Strunjan and Debeli rtič) has been conducted. Sequence similarity among strains detected in mussels, strains isolated from sources of drinking and surface water and strains from human clinical samples was 95% at the nucleotide level and 100% at the amino acid level. A sequence similarity study showed up to 100% match at the nucleotide level with other human strains isolated worldwide (Japan, China, Korea, India). Higher levels of contamination at the Debeli rtič harvesting area (25.9% of mussels contain norovirus), the most northern point where the main Adriatic Sea current flows, can be attributed to intensive shipping in this area neighbouring the port of Koper, discharges of wastewaters, the river estuary and the main sea current that flows north (28).

4. Cultivation of noroviruses
Until recently, there were no available cultivation system for human noroviruses, so the discovery of Ettayebi and collaborators was revolutionary (29). Stem-cell-derived intestinal enteroids from duodenum, jejunum, or ileum were used as *in vitro* culture systems for human noroviruses. These cells are susceptible to human norovirus infection and exhibit cytopathic effects. Special protein staining revealed that specifically enterocytes were infected. It was shown that human norovirus infection is dependent on bile acids in enteroid cultures (29). Recent studies furthermore indicate that infection by noroviruses is also influenced by the commensal microbiota (30,31). B cells have been identified as a cellular target of noroviruses, whereas enteric bacteria could serve as a stimulator for the infection. Human norovirus infection of B cells required
the presence of free HBGA or HBGA-expressing bacteria, for example Enterobacter cloacae. When the intestinal microbiota was depleted by oral antibiotic administration, norovirus replication was reduced in vivo (30). On the other hand another bacterium, Lactobacillus, helped the recovery of human intestinal microbiota after norovirus infection (32).

5. Conclusion
There has been great progress in the field of detection and cultivation of the noroviruses in recent years. With the development of the cell culture system and the discovery of some cofactors critical for the replication of noroviruses there are some new answers, but new questions are arising. Noroviruses are a heterogeneous group of viruses, with a variety of strains and new variants. The gold standard for the detection is RT-qPCR, but new technologies, next generation sequencing and whole genome sequencing are promising tools for a broader spectrum of information about the whole norovirus genome.

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