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REVIEW PAPER

Molecular Detection and Genotyping of Noroviruses

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Abstract Noroviruses (NoVs) are a major cause of gastroenteritis worldwide in humans and animals and are known as very infectious viral agents. They are spread through feces and vomit via several transmission routes involving person-to-person contact, food, and water. Investigation of these transmission routes requires sensitive methods for detection of NoVs. As NoVs cannot be cultivated to date, detection of these viruses relies on the use of molecular methods such as (real-time) reverse transcriptase polymerase chain reaction (RT-PCR). Regardless of the matrix, detection of NoVs generally requires three subsequent steps: a virus extraction step, RNA purification, and molecular detection of the purified RNA, occasionally followed by molecular genotyping. The current review mainly focused on the molecular detection and genotyping of NoVs. The most conserved region in the genome of human infective NoVs is the ORF1/ORF2 junction and has been used as a preferred target region for molecular detection of NoVs by methods such as (real-time) RT-PCR, NASBA, and LAMP. In case of animal NoVs, broad range molecular assays have most frequently been applied for molecular detection. Regarding genotyping of NoVs, five regions situated in the polymerase and capsid genes have been used for conventional RT-PCR amplification and sequencing. As the expected levels of NoVs on food and in water are very low and inhibition of molecular methods can occur in these matrices, quality control including adequate positive and negative controls is an essential part of NoV detection. Although the development of molecular methods for NoV detection has certainly aided in the understanding of NoV transmission, it has also led to new problems such as the question whether low levels of human NoV detected on fresh produce and shellfish could pose a threat to public health.

Keywords Norovirus · Human · Animal · Molecular detection · Molecular genotyping · Real-time RT-PCR · RT-qPCR

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Introduction

The norovirus (NoV) genus belongs to the *Caliciviridae* family (along with genera *Lagovirus*, *Nebovirus*, *Vesivirus*, and *Sapovirus*) and is divided into five genogroups

(GI–GV). Human infective noroviruses (NoVs) belong to GI, GII, and GIV whereas GIII and GV enclose bovine NoVs and murine NoVs, respectively. Porcine NoVs are genetically similar to human NoV and belong to genogroup GII. Recently, NoV sequences that cluster within GIV have been detected in diarrheic feces coming from a lion cub and from dogs (Martella et al. 2008, 2009; Mesquita and Nascimento 2009). Furthermore, recently identified NoVs in sheep were shown to cluster within GIII (Wolf et al. 2009). Human infective NoVs are a major cause of gastroenteritis in adults worldwide (Koopmans and Duizer 2004) and are spread by feces and vomit through various transmission routes. These transmission routes involve contact with infected individual or fecally contaminated vehicles such as food, water, or surfaces (Berger et al. 2010; Hall 2012; Moe 2008; Zainazor et al. 2010). Although human infective NoVs have been detected in swine, dog, and cattle feces (Mattison et al. 2007; Summa et al. 2012), the risk of NoV zoonotic transmission has been considered very low (Bank-Wolf et al. 2010; Palmer et al. 2005).

To clarify these NoV transmission routes, e.g., by investigating NoV food and water-borne outbreaks, by examining food, water, and clinical samples for human and/or animal NoV presence, adequate and sensitive NoV detection and genotyping methods are needed. Detection and genotyping of animal and human infective NoVs have relied on the use of molecular and immunological methods due to the lack of an in vitro cultivation method (except for the murine NoV) (Asanaka et al. 2005; Duizer et al. 2004b; Wobus et al. 2006). The NoV genome contains three open reading frames (ORFs) encoding, respectively, a polyprotein for non-structural proteins, the major capsid protein (VP1) and the minor capsid protein (VP2). Additionally, the murine norovirus genome comprises a fourth ORF encoding a virulence factor VF1 (McFadden et al. 2011). The current review aimed to summarize methods for molecular detection and genotyping of NoVs in clinical, food, water, and environmental samples.

NoV Detection Strategy

In general, detection of NoVs—regardless of the matrix—requires three subsequent steps (Fig. 1) (Stals et al. 2012).

Virus extraction is the first step and is needed (1) for NoV extraction and concentration from any matrix and (2) for removal of inhibitors from the sample matrix. Concentration of NoV particles is particularly needed if food, environmental, and water samples are analyzed due to the low NoV levels that might occur and as NoV contamination of the latter sample categories may not be uniformly spread (Baert et al. 2011; Boxman et al. 2011; Duizer et al. 2007; Loisy et al. 2005; Stals et al. 2011a; Yilmaz et al. 2011). However, it has been estimated that 18 infectious NoV particles could be sufficient to initiate a NoV infection and thus even low levels of infectious NoV particles in foods and water could be a threat for public health (Le Guyader et al. 2003; Le Guyader et al. 2006; Teunis et al. 2008). As food products, water, and environmental samples contain inhibitors of molecular

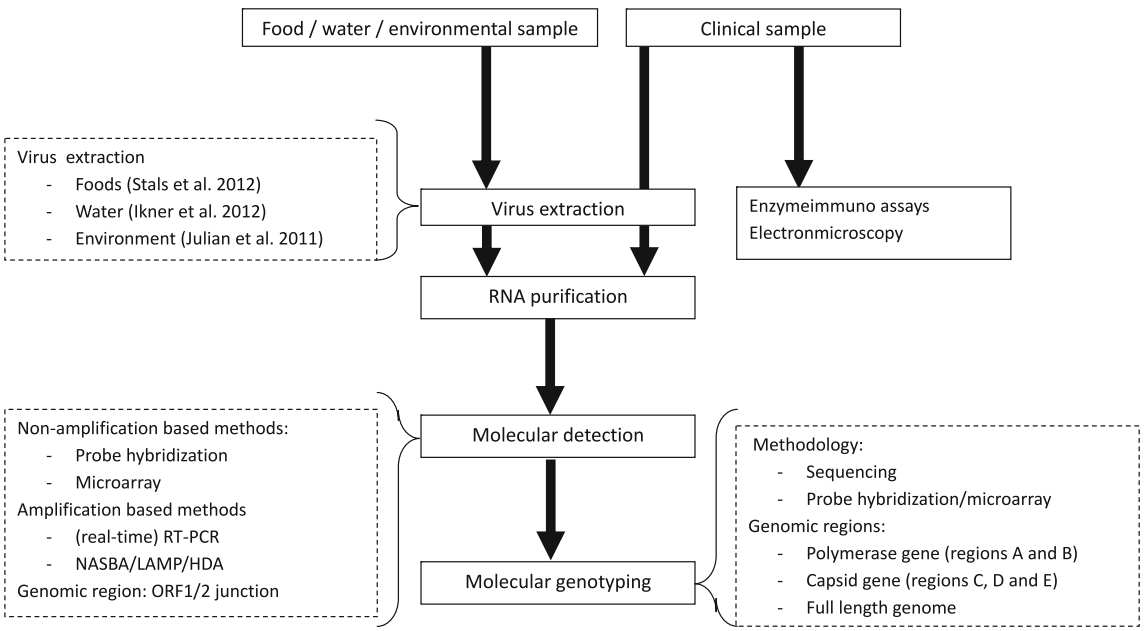

Fig. 1 Overview of strategies for detection of human and animal NoVs in clinical, food, water, and environmental samples

detection methods, removal of these inhibitory compounds is needed for a correct analysis of NoV presence in these samples (Cocolin et al. 2011; Demeke and Jenkins 2010; Escobar-Herrera et al. 2006; Rijpens and Herman 2002; Schwab and McDevitt 2003). Extraction of viruses from foods, water, and surfaces has, respectively, been reviewed by Stals et al. (2012), Ikner et al. (2012), and Julian et al. (2011).

RNA purification is the second step in the NoV detection strategy and is similar in most studies (Fig. 1). In general, the protocols to purify RNA from the virus extract involve (1) a lysis step to release the nucleic acids trapped in the viral capsid and (2) a subsequent RNA isolation step. Viral RNA purification has been reviewed recently by Stals et al. (2012) and several automated systems have been compared of late (Verheyen et al. 2012).

The third step during the NoV detection strategy is the molecular detection, in some cases followed by molecular genotyping. Important aspects regarding the molecular detection of NoV include the selection of adequate genomic target regions, selection of an adequate molecular detection method and the inclusion of the necessary quality controls, especially when investigating NoV presence in complex matrices. As reverse transcriptase-polymerase chain reaction (RT-PCR)—whether or not in real-time format—is currently considered as the golden standard for detection of animal and human NoVs (de Bruin et al. 2006; Rolfe et al. 2007; Scipioni et al. 2008a; Stals et al. 2009a), the current review did go more into detail on this method.

Molecular Detection and Genotyping of NoV

Genomic Regions for Detection and Genotyping of NoV

The genomic regions used for detection of NoVs have different properties compared to the regions used for NoV genotyping, although some genomic regions have been used for both purposes. Shorter genomic regions with a higher conservation grade are used for detection of NoVs, while larger genomic regions with a lower conservation grade discriminating between NoV genotypes are used for genotyping (Kageyama et al. 2003). After sequencing of the NoV genome by Xi et al. (1990), random target sequences were initially chosen for human NoV detection (DeLeon et al. 1992; Jiang et al. 1992). However, the great genomic diversity of NoVs both between and within genogroups made the development of sensitive and specific assays for molecular detection of a broad range of NoVs not straightforward. Primer pairs targeting highly conserved amino acid motifs, such as the GLPSG and YGDD amino acid motifs in the RNA-dependent RNA polymerase gene shared by all genera of caliciviruses, have been privileged for simultaneous detection of human and animal NoVs (Jiang et al. 1999; Vennema et al. 2002; Vinje and Koopmans 1996; Wolf et al. 2012). Even though primers targeting these regions were able to detect human infective NoVs, bovine NoVs, and porcine NoVs, they failed to detect other animal NoVs (Scipioni et al. 2008b). In addition, this genomic region may lack specificity and may give rise to cross-genera reactions (Vinje et al. 2000; Vinje et al. 2003). Despite efforts, no sensitive and specific method detecting all human and animal NoVs in one single amplification reaction could yet be developed even when highly conserved regions of the NoV genome were targeted (Jiang et al. 1999; L'Homme et al. 2009; Scipioni et al. 2008a, b; Wolf et al. 2010). Specific detection of human infective NoVs was considerably aided by the use of highly conserved genomic patterns at the ORF1/ORF2 junction, shown in Fig. 2 (Jothikumar et al. 2005; Kageyama et al. 2003; Wolf et al. 2007). A recent evaluation of different RT-PCR NoV detection assays targeting the ORF1/ORF2 junction using a variety of human infective NoV strains circulating between 2007 and 2009 in Canada confirmed that this region still allowed amplification of a broad range of human infective NoV genotypes (Mattison et al. 2010a). The European Committee for Standardization/Technical Committee 275/Working Group 6/Task Group 4 on virus detection in foods (CEN/TC275/WG6/TAG4 working group) has also selected this ORF1/ORF2 genomic junction region as target for RT-PCR detection of NoV and other viral food borne pathogens in foods (Fig. 2) (Le Guyader et al. 2009; Lees 2010). Conversely, detection of NoVs in different animal species required the use of broadly reactive primers or different sets of primers making it difficult to set up this kind of analysis in routine (Scipioni et al. 2008b). Although some assays for detection of human NoVs are capable of detecting porcine (Sugieda et al. 1998) and bovine NoVs (Dastjerdi et al. 1999; Liu et al. 1999; van der Poel et al. 2000), these assays are in general less sensitive than animal NoV-specific assays and most studies still use broad range primers to detect animal NoVs (Scipioni et al. 2008b).

Regarding genotyping of NoVs, five genomic regions have frequently been used for this purpose (Fig. 3) (Mattison et al. 2009). In detail, the polymerase gene (regions A and B) and the gene encoding the major capsid protein VP1 (regions C, D, and E) (Anderson et al. 2003; Ando et al. 2000; Gonin and Couillard 2000; Kojima et al. 2002; Noel et al. 1997; Vennema et al. 2002; Vinje et al. 2004) have been used to genotype NoV strains (Mattison et al. 2009). A schematic overview of these regions is shown in Fig. 3. The most widespread and most used NoV genotyping region is region A (Vinje et al. 2003). While region D has been proposed as complementary to region A,

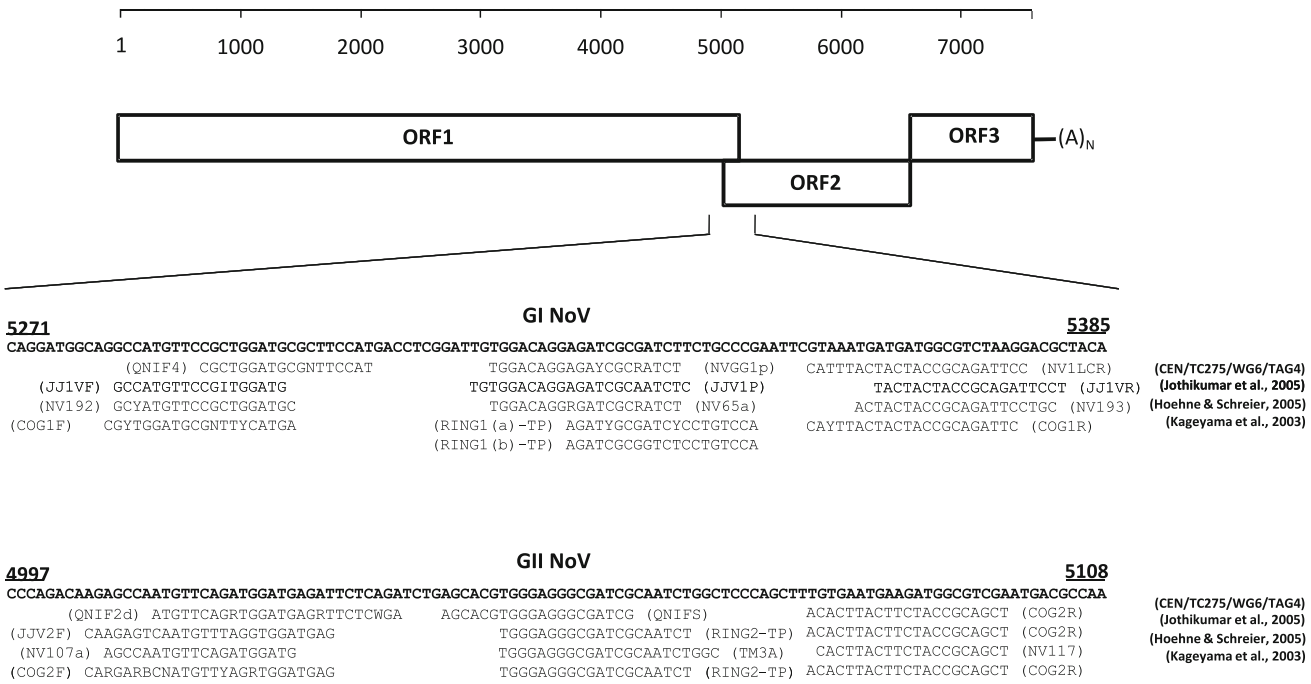

Fig. 2 Overview of various real-time PCR primers and hydrolysis probes targeting the ORF1–ORF2 junction of the NoV genome. NoV GI and GII sequences shown are deduced from EMBL/Genebank accession numbers M87661 and X86557, respectively. Names of primers and hydrolysis probes are given between *brackets* in *front* or *behind* the primer or probe sequence. Reverse primers are shown in reverse complement sequence

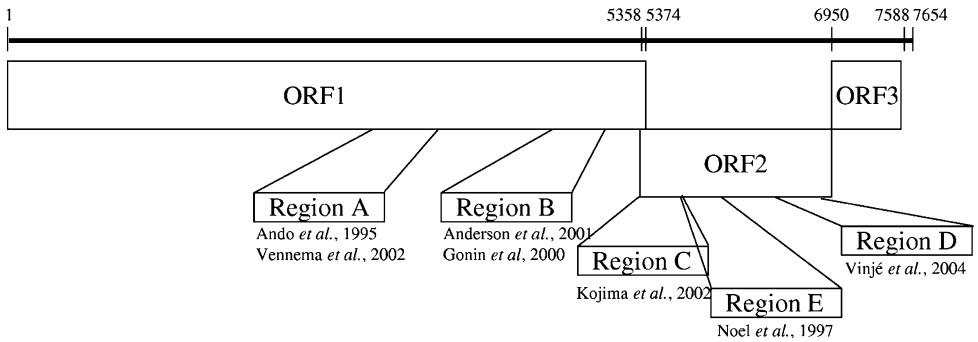

Fig. 3 Schematic representation of the NoV genome and the positions of several representative regions (regions *A–E*) that have been used for genotyping. *Numbers* refer to positions in the Norwalk GI.1 virus genome (GenBank accession number M87661). *Source* Mattison et al. (2010a, b)

a recent comparison of regions C and D demonstrated that region C was a better genotyping region. Nevertheless, both regions have been used for NoV genotyping (Mattison et al. 2009; Vinje et al. 2004).

Primer pairs amplifying the whole capsid gene have been designed for human NoVs, in particular, for GII NoVs (Kamel et al. 2009; Sdiri-Loulizi et al. 2008). For animal NoVs, the use of primers targeting the polyadenylated 3′ end of the genome in association forward primers used for the detection in the polymerase region constitutes an excellent alternative for the amplification of entire capsid gene sequences (Dingle 2004).

(Real-Time) RT-PCR Detection of NoVs

Historically, detection of animal and human NoV relied upon the visualization of virus particles by electronic microscopy in stool samples from infected individuals (Appleton et al. 1977; Kapikian et al. 1972; Thornhill et al. 1975). Other methods such as the detection of viral proteins by enzyme-linked immunosorbent assay (ELISA) or the detection (and genotyping) of viral genomes by probe hybridization have been developed for NoV detection in clinical samples (Ando et al. 1995; de Bruin et al. 2006; Geginat et al. 2011; Jaykus et al. 1996). As these methods

are rather insensitive (detection limits range between 10^4 and 10^6 virus particles per gram of sample) and/or time-consuming (Rijpens and Herman 2002), they have gradually been replaced by molecular methods amplifying specific regions of the NoV genome, especially when detecting low levels of NoVs in food and environmental samples.

A variety of amplification-based methods for molecular detection of (viral) RNA have been described including nucleic acid sequence-based amplification (NASBA) (Compton 1991), self-sustained sequence replication (3SR) (Fahy et al. 1991), and transcription amplification system (TAS) (Kwoh et al. 1989). However, the most widely used technique for the detection of RNA is RT-PCR, increasingly used in real-time format. At present (real-time) RT-PCR is considered to be the gold standard for molecular detection of NoVs in clinical, food, and environmental samples (Baert et al. 2007; Jothikumar et al. 2005; Li et al. 2012; Park et al. 2008; Scipioni et al. 2008a; Stals et al. 2009a; Wolf et al. 2007). Most important reasons for this include (1) the low detection limit (Beuret 2004), (2) the absence of post-PCR processing in case of real-time RT-PCR (Espy et al. 2006; Mackay et al. 2002), (3) the possibility for multiplexing (Gunson et al. 2008; Jones et al. 2011; Rosenfield and Jaykus 1999; Stals et al. 2009a), and (4) the possibility of NoV quantification (Mackay et al. 2002; Niesters 2002). The low detection limit ($\leq$10 target copies) is necessary because of the potentially low NoV levels in environmental and food samples and because of the estimated low NoV 50 % infectious dose (ID_{50}) of 18 infectious particles (Baert et al. 2011; Teunis et al. 2008). Numerous conventional RT-PCR assays for detection of NoV have been developed and evaluated, targeting the genomic regions as described above (Ando et al. 1995; Farkas et al. 2005; Jiang et al. 1999; Rolfe et al. 2007; Sair et al. 2002; van der Poel et al. 2000; Wang et al. 2005).

Real-time RT-PCR assays based on sequence-independent chemistries (intercalating dyes) and sequence-dependent chemistries, mainly hydrolysis probes, have been described for detection of human and animal NoV (Butot et al. 2010; Hoehne and Schreier 2006; Jothikumar et al. 2005; Pang et al. 2004; Park et al. 2008; Richards et al. 2004; Scipioni et al. 2008a; c). Alternative sequence-dependent real-time RT-PCR chemistries (hybridization probes and molecular beacons) are available but have mostly been used for NoV detection in clinical samples (Svraka et al. 2007). Other real-time RT-PCR chemistries include the Sunrise system (commercial name: Amplifluor® hairpin primers) (Winn-Deen 1998), the Eclipse® system (Afonina et al. 2002), LUX® primers (Lowe et al. 2003) and the Scorpion® primers (Nazarenko et al. 2002). However, these technologies have not been used for detection of enteric viruses yet, except for the LUX® primers for NoV and rotavirus detection (Nordgren et al. 2008, 2010).

An interesting feature of (real-time) RT-PCR is multiplexing, whereby two or more different target sequences are amplified using multiple primer pairs in a single PCR reaction (Chamberlain et al. 1988). The advantages of this setup are clear as—besides the reduced handling time and cost—samples can be evaluated for multiple targets in a single reaction tube (Edwards and Gibbs 1994). Nevertheless, the development of such a multiplex RT-PCR might be quite complex and the number of targets to be detected simultaneously is restricted (Ugozzoli and Persson 2005). Multiplex conventional RT-PCR assays have been used for simultaneous detection of rotavirus (human) NoVs, sapovirus, and adenovirus in feces samples (Kittigul et al. 2009; Yan et al. 2003).

Due to the increased number of real-time PCR fluorophores (covering a broad emission spectrum), multiplex real-time RT-PCR assays based on sequence-dependent chemistries have been developed as well. Similar assays for simultaneous detection of NoV and other enteric viruses have been described in recent literature (Beuret 2004; Hoehne and Schreier 2006; Jones et al. 2011; Shigemoto et al. 2011; Stals et al. 2009a; Tolentino-Ruiz et al. 2012; van Maarseveen et al. 2010; Wolf et al. 2010).

Alternative Methods for Molecular NoV Detection

Isothermal amplification methods such as reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Notomi et al. 2000), NASBA (Compton 1991), and RT helicase-dependent amplification (RT-HDA) (Vincent et al. 2004) constitute excellent alternatives for (real-time) RT-PCR (Sidoti et al. 2012). For the detection of enteric RNA viruses, NASBA may be especially well suited because it is an RNA amplification method, allowing the use of the initial single-stranded RNA genome as the template in the reaction. Several studies have used this method for NoV detection (Houde et al. 2006; Jean et al. 2004; Moore et al. 2004). However, the low incubation temperature (around 40 °C) in the NASBA reaction may increase non-specific amplification and as RNA is the final amplification product, the possibilities for post-amplification analyses may be limited (Jean et al. 2004). Although RT-LAMP has been used for detection of human NoVs (Fukuda et al. 2007; Li et al. 2009; Yoda et al. 2007), the need for different primer sets targeting different genomic regions might hinder development of long-term use assays due to the immunogenetic drift in the NoV genome. RT-HDA seems a promising method for detection of viral RNA (Goldmeyer et al. 2007), but has thus far not been used for NoV detection.

Genotyping and Molecular Characterization

Conventional RT-PCR amplification of specific regions in the NoV genome as described above followed by

sequencing is the most commonly used method for NoV genotyping. However, the use of different genotyping regions, combined with different primer sets per region has resulted in scattered NoV genotyping data which are often difficult to compare (Vinje et al. 2003). Furthermore, NoV classification and nomenclature still remain problematic because no international consensus has been reached yet for its taxonomy (Ando et al. 2000; Katayama et al. 2002; Vinje et al. 2003). Nevertheless, a classification system based upon the phylogenetic analysis of complete capsid amino acid sequences (ORF2) has been now widely accepted (Zheng et al. 2006, 2010). Recently, a web-based open-access typing-tool for NoVs has been developed (Kroneman et al. 2011). This tool has allowed users to upload NoV nucleotide sequences ≥100 bp which are identified by comparison to known polymerase (ORF1) and capsid (ORF2) sequences.

Problems regarding the genotyping of NoV include (1) the genetic drift of the NoV genome and (2) the need for sufficient genetic material. First of all, NoVs exhibit great genetic variability and are thought to evolve rapidly due to nucleotide substitutions and recombination (Bull et al. 2007; Kroneman et al. 2008; Matthijs et al. 2010; Siebenga et al. 2007). Recombination of NoVs and the identification of various recombinant NoV strains have been described in humans, pigs, and cattle based upon phylogenetic analysis (Bull et al. 2007; Han et al. 2004; Nayak et al. 2009). Sequence similarity analyses have located the recombination hot spot at the junction between ORF1 and ORF2 of the NoV genome (Bull et al. 2005, 2006, 2012; Coyne et al. 2006; Lam et al. 2012; Matthijs et al. 2010; Ruether et al. 2012). Therefore, recombinant strains can be evidenced when partial polymerase sequences (ORF 1) cluster differently than partial capsid region sequences (ORF 2) when aligned with published NoV sequences. In order to exclude multiple infections or contaminations, a sequence covering the ORF1–ORF2 junction confirms recombination when aligned with sequences of the putative parental strains. To be accurate, epidemiological studies based upon molecular tracing should therefore be based upon sequences of both the polymerase and the capsid gene. Second, sufficient genetic material is needed for conventional RT-PCR and subsequent sequencing. As NoVs cannot be cultivated—except for the GV murine NoVs—and due to the low viral contamination of food matrices, the amplification of large NoV sequences from food and environmental samples can be problematic while this is in general not the case for clinical samples (Baert et al. 2009). Nevertheless, some studies did obtain useful genotyping sequences from food samples using conventional RT-PCR (Boxman et al. 2007; Mattison et al. 2010b; Maunula et al. 2009). In most cases however, the only genotyping data of NoV detected in food is provided by highly sensitive real-time RT-PCR methods discriminating between NoV GI and GII without further information on genotypes within these genogroups.

Quality Control During Molecular Detection of NoV

In general, quality control of methods for detection of human and animal NoVs (and other enteric viruses) in food, water, environmental, and clinical samples consists of the inclusion of positive and negative controls at different steps of the viral detection procedure that are considered critical for correct detection and/or quantification (Fig. 1). The use of appropriate positive controls throughout the different steps of the NoV detection protocols is required to avoid detection of false negative results caused by reaction inhibition due to malfunction of thermal cycler, incorrect PCR mixture, poor DNA polymerase activity, incorrect execution of the virus detection protocol, or not least the presence of inhibitory substances in the sample matrix (Hoorfar et al. 2004; Scipioni et al. 2008a). On the other hand, as false-positive results can be caused by cross-contamination and carryover contamination (Rijpens and Herman 2002), the inclusion of negative controls in the detection methodology is equally important. As the use of process controls during detection of NoV and other viral pathogens in water, food, and environmental samples has been described by Stals et al. (2012), the current review will not go into detail regarding this matter, but will focus on the quality controls associated with molecular detection of NoV.

Positive Controls

RT Controls (RTC)

The RT step is required if methods that require DNA as target material (e.g., PCR, LAMP, and HDA) are used for detection of NoV RNA genomic material. However, this reaction is prone to inhibition when detecting viral agents in shellfish (Loisy et al. 2005; Milne et al. 2007; Schwab et al. 1998), water (Laverick et al. 2004), and other foods (Love et al. 2008). Therefore, several control systems have been described whereby exogenous RNA is added to the RT reaction mixes as RTC, often referred to as internal RNA control in scientific literature (Gibson et al. 1996; Menzel et al. 2002). A first system consists of the inclusion of RNA molecules in a viral capsid and is called "armored RNA". This system ensures (long-term) stability of RNA and a number of commercial kits are available to create armored RNA (Beld et al. 2004; Hietala and Crossley 2006). Armored RNA has been used for detection of NoV in shellfish (Greening and Hewitt 2008) and clinical samples (Medici et al. 2008). Noteworthy, Armored RNA has also been used as a process control for virus detection in

clinical samples (Sábato et al. 2007; Schumacher et al. 2007).

Another RTC system consists of in vitro transcribed ssRNA fragments containing the primer-probe binding sites of a subsequently used (real-time) PCR detection assay and has been used for detection of enteric viruses in shellfish and clinical samples (Costafreda et al. 2006; Escobar-Herrera et al. 2006; Schwab et al. 1997; Trujillo et al. 2006). Finally, a third type of RTC makes use of genomic RNA extracted from cultivable surrogate viruses such as the MS2 bacteriophage or MNV-1 which allows a constant availability of fresh RNA, although handling time may be increased due to an occasional need for cultivation of these surrogate viruses (Dreier et al. 2005; Stals et al. 2011b).

Amplification Controls

Inhibition of amplification-based methods such as PCR—whether or not in real-time format—is a thoroughly described phenomenon when detecting pathogens in foods (Rijpens and Herman 2002), water (Guy et al. 2003), and shellfish (Abolmaaty et al. 2007); and many authors have suggested the use of an amplification control to detect possible false-negative results related to this inhibition (D'Agostino et al. 2011; Hoorfar et al. 2004; Reiss and Rutz 1999). In essence, an amplification control consists of the addition of exogenous DNA to the reaction mix containing the sample or to a reaction mix run in parallel with the tested sample. These two approaches have been named internal amplification control (IAC) and external amplification control (EAC), respectively. As D'Agostino et al. (2011) have extensively described the advantages and disadvantages of both the approaches, the current review will not go into detail regarding this matter. IACs can be either competitive or non-competitive, whereby, respectively, the same or different primers (and probes) are used for amplification of IAC and target (Rijpens and Herman 2002). Advantages of the competitive IAC are (1) the ease of development as no extra molecular detection assay has to be designed and (2) a more correct accurate reflection of the (inhibition of the) amplification of the target sequence. The main disadvantage is the possibility of reduced amplification efficiency of the target sequence due to a competition shift in favor of the IAC. The risk of the latter could be reduced (although not eliminated) by assuring that the amplicon size of the IAC exceeds that of the target (Rijpens and Herman 2002). This type of IAC has been used for detection of NoV in water (Parshionikar et al. 2004) and fecal samples (Scipioni et al. 2008a). A potential disadvantage of a non-competitive IAC is that it may not accurately reflect amplification of the target sequence. This disadvantage stresses the use of very similar target sequences for both the target and the IAC. Although reduced, competition for PCR components may still occur in case of a non-competitive IAC. Therefore, the concentration of the IAC (whether or not competitive) should be kept as low as possible to avoid false-negative results (Hoorfar et al. 2003; Hoorfar et al. 2004). Several authors have developed non-competitive IACs that can be included into existing NoV detection (real-time) RT-PCR assays (Deer et al. 2010; Diez-Valcarce et al. 2011; Dreier et al. 2005; Escobar-Herrera et al. 2006; Rosenstraus et al. 1998). Noteworthy, the European Standardization Committee (CEN/TC275/WG6/TAG4 working group), in collaboration with International Standard Organization (ISO), has published a general guideline for PCR testing of food-borne pathogens, including the requirement of an IAC in the reaction mixture (Anonymous 2002).

Negative Controls and Contamination Prevention

Negative controls are needed when detecting pathogens using molecular methods, as even the slightest contamination can lead to false-positive results, especially when very sensitive molecular methods are used (Borst et al. 2004; Niesters 2002). In general, very low NoV levels are expected in food and water samples, which makes the prevention of false-positive result even more important. Despite the absence of a post-PCR processing step when using real-time PCR, contamination can still occur (Stals et al. 2009b). Contamination can result from sample-to-sample contamination (cross-contamination) as well as from the carryover of DNA (carryover contamination) from a previous amplification of the same target (Rijpens and Herman 2002). A recent study investigating the presence of NoV in bottled water highlighted the importance of negative controls by showing that the majority of positive RT-PCR results were due to cross-contamination (Lamothe et al. 2003). To avoid false positive results due to PCR contamination, a constant need remains to respect dedicated environmental conditions (e.g., separate working areas and dedicated pipettes) if real-time PCR or conventional PCR is applied (Kwok and Higuchi 1989; Stals et al. 2009b). All reagents used in PCR must be prepared, divided into aliquots, and stored in an area free of PCR-amplified product, while addition of template DNA/RNA should occur in another separated area. To reduce carryover contamination, Longo et al. (1990) described the use of uracil N-glycosilase (UNG) in combination with deoxyuridine triphosphate (dUTP) rather than deoxythymidine triphosphates (dTTP). UNG catalyzes the removal of uracil from single- and double-stranded DNA that has been synthesized in the presence of dUTP. Additional contamination preventing measures include the sterilization of the working area by shortwave UV irradiation (Cimino et al. 1990, 1991) as well as the use of aerosol-tight pipettes and sterile plastic disposables and glassware (Rijpens and Herman 2002). It is also important to mention

that highly concentrated sodium hypochlorite solutions (3 % w/v concentration; ISO 22174 2005) effectively destroy both nucleic acids and viral/bacterial pathogens, while ethanol-based disinfectants—while very effective against bacterial pathogens—can have limited disinfecting effect on viruses and do not destroy nucleic acids (Duizer et al. 2004a; Park et al. 2010).

Interpretation of Positive Results

The development of sensitive and quantitative molecular methods for detection of NoV has certainly helped clarifying the transmission routes of NoVs. However, the increased sensitivity of NoV detection methods has also raised questions on how to interpret positive results. This interpretation problem results from the incapability of molecular methods such as (real-time) RT-PCR to distinguish infectious and non-infectious NoV particles (Baert et al. 2008), despite promising results obtained by binding of NoV to porcine gastric mucin (Dancho et al. 2012; Hamza et al. 2009).

In case of NoV outbreaks, low levels of NoVs detected in a food or water sample can be related to epidemiological data and to NoVs found in clinical samples (Baert et al. 2009). However, this is not the case when food and water samples are preventively screened for NoV presence. NoV presence in shellfish has been extensively documented as these food products have frequently been involved in NoV outbreaks (Alfano-Sobsey et al. 2012; David et al. 2007; Nenonen et al. 2009). A significant difference has been shown between NoV RNA levels in outbreak-related oysters and in non-outbreak-related oysters (Lowther et al. 2012). In detail, mean levels of 2,184 and 682 NoV genomic copies were detected in outbreak- and non-outbreak-related oysters, respectively. Similarly, consumption of oysters containing <200 NoV genomic copies did not result in reported illness, while oysters with >1,000 genomic copies have been significantly related to gastroenteritis (Dore et al. 2010). However, the inclusion of a critical NoV limit for oysters and other shellfish should be discussed thoroughly, as both economical and public health factors should be taken into account (Anonymous 2012).

Regarding NoV presence on fresh produce, a single study combined data of NoV presence on leafy greens, soft red fruits, and other vegetables from Belgium, France, and Canada (Baert et al. 2011; Mattison et al. 2010b; Stals et al. 2011a). Although 28.5 % of leafy greens and 11.2 % of soft red fruits tested positive for NoV by real-time RT-PCR, no illness could be associated to any of the NoV positive samples. The authors, therefore, suggested that confirmation of NoV real-time RT-PCR results by another primer(/probe) set or by sequencing of the real-time RT-PCR amplicon may be a first step to correctly assess the public health risk related of samples testing positive by sensitive molecular methods such as RT-PCR (Baert et al. 2011). Regarding the confirmation NoV positive samples, extended primers could facilitate the sequencing of short real-time RT-PCR amplicons (Williams-Woods et al. 2011).

Perspectives

Although the bottleneck of NoV detection in complex matrices such as foods and water is clearly situated at the virus extraction step (Stals et al. 2012), there are certainly perspectives for evolution of existing methods and development/application of new methodologies for molecular detection of NoV. Regarding NoV detection in clinical samples, molecular methods are not always required and in some cases rapid and inexpensive immunochromatography strip tests can be sufficient for NoV detection (Khamrin et al. 2008; Park et al. 2012; Pombubpa and Kittigul 2012). As mentioned before, however, non-amplification-based methods are not sufficiently sensitive for NoV detection and genotyping in food, water, and environmental samples.

Concerning real-time RT-PCR detection of NoV, high-resolution melting (HRM) and the "Linear After The Exponential" (LATE) technique are two approaches that could improve NoV detection. High-resolution melting is an enhancement of sequence-independent real-time PCR chemistries and makes use of (1) instruments allowing more precise temperature increase and data acquisition and (2) fluorescent dyes with improved double-stranded DNA-binding saturation properties (Gundry et al. 2003; Wittwer et al. 2003). HRM real-time RT-PCR has successfully been used to differentiate human NoV genotypes in clinical samples after amplification with broad range NoV primers (Tajiri-Utagawa et al. 2009). Future research may clarify if HRM real-time RT-PCR could be applied for detection and genotyping of NoV in complex matrices such as food and water. Another improvement of real-time RT-PCR detection of NoVs could be the "LATE" principle. LATE-PCR is an asymmetric PCR technique—a 40–100-fold concentration difference is used between both primers—whereby the primer used in the lowest concentration is slightly modified. This approach efficiently avoids out competition of formation of primer–template strand hybrids by synthesized template DNA strands, a phenomenon that can reduce amplification efficiency in symmetric PCR assays (Pierce et al. 2005; Pierce and Wangh 2007).

Noteworthy, LATE-PCR can be combined with pyrosequencing as this PCR method generates an excess of single-stranded amplicons, the substrate of pyrosequencing (Salk et al. 2006). Pyrosequencing is a "sequencing by synthesis" method based on detecting the activity of DNA

polymerase using a chemiluminescent enzyme such as luciferase. In essence, the method allows sequencing of single-stranded DNA by hybridization of a detection primer and subsequently detecting which desoxynucleotide type was added to the complementary DNA strand. During the pyrosequencing reaction, dATP, dCTP, dTTP, and dGTP are sequentially added and removed from the reaction. A light signal is produced only when the desoxynucleotide solution complements the first unpaired base of the template DNA strand (Nyrén 2007; Sanchez et al. 2004). The combination of LATE-PCR and pyrosequencing could be useful for confirmation of the specificity of the real-time PCR amplicon.

A second perspective regarding the molecular detection of NoVs is the harmonization of current methods. Due to the importance of bivalve molluscan shellfish as cause of NoV food-borne outbreaks, the European Committee for Standardization/Technical Committee 275/Working Group 6/Task Group 4 on virus detection in foods (CEN/TC275/WG6/TAG4 working group) has recently published a standardized method for detection of NoV and other viral pathogens in shellfish (Lees 2010). Virus extraction and RNA purification were based on the protocols described by Jothikumar et al. (2005) and Boom et al. (1990), respectively. The RT step (based on the use of random hexamers) and real-time PCR were combined in a one-step protocol. The real-time PCR step included the use of hydrolysis probes targeting the ORF1/2 junction of the NoV genome, the most conserved region in the NoV genome (Kageyama et al. 2003). Although the primers and hydrolysis probes for detection of human infective NoV proposed by the CEN/TC275/WG6/TAG4 working group have successfully been evaluated and applied in several studies (Dancho et al. 2012; Lowther et al. 2012; Stals et al. 2009a; Uhrbrand et al. 2010), their specificity and sensitivity for detection of emerging NoV genotypes such as the NoV GII.12 and GIIg need to be evaluated constantly (Takanashi et al. 2011). Nevertheless, the use of this proposed standard real-time PCR assay for detection of NoV would facilitate a correct comparison between different studies. Noteworthy, publication of standardized protocols for detection of NoV and other food-borne viruses in food products has been planned in 2012 and reference materials for evaluation of molecular NoV detection methods have been made available recently (Hartnell et al. 2012).

Conclusion

Overall, substantial progress has been made regarding molecular detection of animal and human infective NoV over the last decade. Although this progress has certainly aided knowledge on NoV, e.g., regarding its transmission routes, it has also led to unexpected problems such as the relation between NoV genomic copies and NoV infectious virus particles (Knight et al. 2012). Furthermore, current challenges include the harmonization of existing NoV detection protocols, facilitating the understanding of NoV transmission. While the first steps in this important harmonization have been set, continuous development and evolution of the NoV detection methodology are needed to assure a reliable detection of NoVs in a broad range of matrices.

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