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Norovirus Structure and Classification

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Abstract

Norovirus are a major cause of acute gastroenteritis worldwide. Diarrheal disease is now the fourth common cause of mortality children under the age of 5 years but remain the 2nd most cause of morbidity. NoV are associated with 18% diarrheal diseases worldwide where rotavirus vaccinations has been successfully introduced. NoV has become major cause of gastroenteritis in children. NoV belong to family caliciviridae. They are non-enveloped, single stranded positive sense RNA Viruses. The genome consists of 3 Open reading frames, ORF-1 codes for non-structural protein, ORF-2 codes for major capsid protein VP1 and ORF-3 for minor capsid protein VP2. Based on sequence difference of the capsid gene (VP1), NoV have been classified in to seven genogroup GI-GVII with over 30 genotypes. Genogroups I, II, IV are associated with human infection. Despite this extensive diversity a single genotype GII.4 has been alone to be the more prevalent. Basic epidemiological disease burden data are generated from developing countries. NoV are considered fast evolving viruses and present an extensive diversity that is driven by acquisition of point mutations and recombinations. Immunity is strain or genotype specific with little or no protection conferred across genogroups. Majority of outbreaks and sporadic norovirus cases worldwide are associated with a single genotype, GII.4 which was responsible for 62% of reported NoV outbreaks in 5 continents from 2001 to 2007. GII.4 variants have been reported as major cause of global gastroenteritis pandemics starting in 1995 frequent emergence of novel GII.4 variants is known to be due to rapid evolution and antigenic variation in response to herd immunity. Novel GII.4 variants appear almost every 2 years. Recent GII.4 variant reported include Lordsdale 1996, Farmington Hills 2002, Hunter 2004, Yerseke 2006a, Den Haag 2006b, Apeldoorn 2007, New Orleans 2009, most recently Sydney 2012. Detailed molecular epidemiologic investigation of NoV is associated for understanding the genetic diversity of NoV strain and emergence of novel NoV variants. However, reports have revealed that not all individuals develop symptoms and a significant proportion remains asymptomatic after NoV infections.

Keywords: Acute gastroenteritis, ORF, Genogroups, Immunity

1. Introduction

The acute gastroenteritis is a major health problems, one of the most common infectious diseases among humans [1, 2]. The annual incidence of diarrheal disease is estimated of annual number is over 4.5 billion cases worldwide [3]

The global estimated of annual number of mortalities with gastroenteritis vary between 3.5–5 million cases in majority of deaths occurring among people in developing countries [4]. Diarrhoea remains into 10 most common communicable diseases found in India [5] (annual mortality is 2.5 million deaths each year in children less than five years of age in developing countries) [6].

In human enteric viruses account for more than half of all cases of gastroenteritis worldwide [7, 8]. Viral causes of gastroenteritis are follow: norovirus, rotavirus, adenovirus (group F- type 40/41), astrovirus and sapovirus [9–11].

2. Norovirus

Norwalk virus is the prototype strain of Norovirus and was associated with an outbreak of gastroenteritis at an elementary school in Norwalk, Ohio, in 1968. The discovery of Norovirus as the aetiological agent of the outbreak was made by Albert Kapikian in 1972 [12].

Using immune electron microscopy (IEM), stool samples were examined from a volunteer who had been experimentally inoculated with a faecal filtrate from the original outbreak. From these studies, Kapikian proposed the name “Norwalk virus” as the causative agent of the outbreak [12]. This was the first human virus specifically associated with gastroenteritis.

2.1 Classification

Norovirus, previously known as Norwalk-like viruses, belongs to the family, *Caliciviridae* [13]. The *Caliciviridae* family is comprised of four genera, Norovirus, Sapovirus, Lagovirus and Vesivirus [14]. Norovirus and Sapovirus are found in the genera Norovirus and Sapovirus, respectively, whilst other caliciviruses of veterinary importance, rabbit hemorrhagic disease virus and feline calicivirus, are found in Lagovirus and Vesivirus, respectively. Recently, two additional genera have been proposed within the *Caliciviridae* family, provisionally named Becovirus or Nabovirus, a bovine enteric calicivirus [15–17]. All six genera infect animals, but only Norovirus and Sapovirus contain strains that infect both humans and animals.

Based on phylogenetic analysis of the full length nucleotide sequence of the capsid gene [VP1], the Norovirus genus is divided into five genogroups (GI, GII, GIII, GIV and GV). Norovirus GI, GII and GIV are associated with human gastroenteritis. Norovirus GII includes porcine, as well as human strains, GIII contains only bovine strains, and GV contains only murine strains [18].

2.2 Structure

Norovirus is a small virion of 27 to 32 nm in diameter and has a buoyant density of 1.33 to 1.41 g/cm³ in caesium chloride [19, 20]. It is a non-enveloped, single-stranded, positive-sense, RNA virus with a genome of 7.4 to 7.7 kb [21, 22]. The RNA is polyadenylated at the 3' end. All calicivirus genomes begin with a GU [nucleotide sequence] at the 5' end terminal. A 5' end sequence, of between 16 and 28 nucleotides depending on the genus is repeated internally in the genome and corresponds to the start of the subgenomic RNA [located at the start of the capsid gene, VP1]. This sequence is thought to be part of an RNA-dependent RNA polymerase [RdRp] promoter [23].

The Norovirus genome contains three ORFs: ORF1, ORF2 and ORF3.

The initial characterisation of the genome was based on the sequence homology of ORF1 in human calicivirus to characterised proteins of picornaviruses [24]. These conserved motifs included a “2C-like” helicase [a nucleoside triphosphatase,

NTPase, in Norovirus genome], a “3C-like” protease [3CLpro] and a “3D-like” RdRp (RdRp in Norovirus genome). Proteolytic mapping and enzymatic studies using site directed mutagenesis and recombinant expression systems have revealed the presence of three other non-structural proteins within the Norovirus polyprotein, including a 3A-like protein, a viral protein-genome linked (VpG) and a N-terminal protein of unknown function [25–29]. All six non-structural proteins proceed N to C terminus in the Norovirus polyprotein (**Figure 1**).

ORF1 encodes a 200 kDa polyprotein which undergoes proteolytic cleavage mediated by a virus-encoded 3CLpro, located upstream of the RdRp. Proteolytic processing is rapid, co-translational and results in the production of six non-structural proteins [30, 31]. ORF2 encodes the major structural protein, VP1 [60 kDa], which is responsible for capsid-related functions, including self-assembly and capsid formation, host interactions and immunogenicity of the virus [32–34].

The ORF3 region encodes a small basic protein of 20 to 30 kDa involved in expression and stability of the VP1 capsid protein [34]. Downstream from ORF3, a 42 to 78 nucleotide non-translated region is present and attached to a polyadenylated tail [35].

2.3 Non-structural proteins

2.3.1 N terminal protein

Expression of the Norovirus N terminal protein demonstrated that the N terminal protein was localised to the golgi apparatus and led to its disassembly into discrete aggregates [36]. In addition, the N terminal protein interacts with the vesicle-associated membrane protein–associated protein A [VAP-A], which plays a role in regulated vesicle transport [37, 38]. Therefore, the N terminal protein is predicated to interact with intracellular membranes and may act as an anchor to membrane-bound replication complexes of Norovirus [39].

2.3.2 NTPase

NTPase protein (alternatively designated p41) of the Norovirus GI strain, Southampton virus, has NTPase activity and a helicase domain. The protein

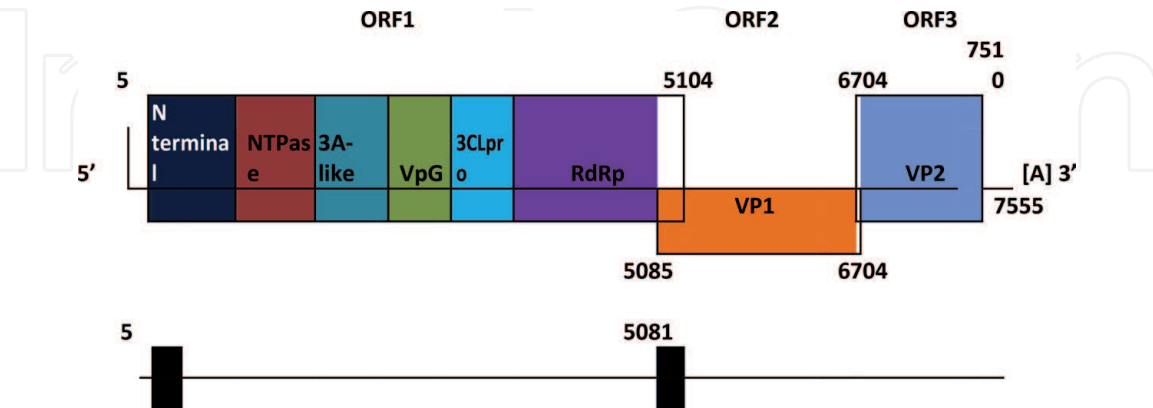


Figure 1. Genomic organisation of NoV. The genomic organisation and nucleotide positions are shown with reference to human NoV/Lordsdale virus/1993/UK, GenBank accession number X86557. The NoV genome is organised into three ORFs, with the 3' end of ORF1 overlapping the 5' end of ORF2 by 20 bp, and the 3' end of ORF2 overlaps the 5' end of ORF3 by one bp. ORF1 encodes for six non-structural proteins: N terminal protein, nucleoside triphosphate (NTPase), a 3A-like protein, viral protein-genome linked (VpG), “3C-like” protease (3CLpro) and an RdRp. ORF2 encodes for the major structural protein, VP1, which self assembles into the viral capsid. ORF3 encodes for a minor structural protein, VP2, involved in stabilisation of VP1. The polyadenylated tail at the 3' end of the genome is indicated by [a]. The two putative RdRp promoter sites are shown below the image as black boxes.

sequence of the Norovirus p41 protein showed regions of high similarity to the 2C protein of enteroviruses. Norovirus may hydrolyse NTPs for a function distinct from nucleic acid unwinding [40]. The specific role of p41 in the viral replicative cycle has not yet been determined.

2.3.3 3A-like protein

A parallel between picornaviruses and caliciviruses have been demonstrated for the 3A and 3A-like protein, respectively [41–43]. The 3A-like protein [alternatively designated p22 or p20 for Norovirus GI and GII, respectively] in the Norovirus genome occupies a position similar to that of the 3A protein in picornavirus. The specific function of the Norovirus 3A-like protein is unknown, but it has been suggested to be involved in cellular membrane trafficking and replication complexes [44].

2.3.4 VpG

VpG is essential for the production of infectious caliciviruses [45]. Human VpG has been shown to bind to translational initiation factors *in vitro* and may also be involved in the recruitment of ribosomes to viral RNA. Recently, VpG has been suggested to play a role in RNA replication [46, 47]. VpG was uridylylated *in vitro* by the RdRp, suggesting it may function as a protein-primer during RNA replication. Another study by Belliot and colleagues demonstrated that Norovirus VpG was nucleotidylated by the proteinase-polymerase form of the human Norovirus RdRp. This occurred in a template-independent manner in the presence of Mn²⁺; furthermore, the linkage between RNA to VpG was covalent. Mutational analysis identified tyrosine 27 of the Norovirus VpG as the target amino acid for this linkage, which was susceptible to phosphodiesterase treatment. Thus, the linkage of RNA to VpG via a phosphodiester bond was confirmed. In addition, there was evidence for the presence of an RNA element in the 3' end of the polyadenylated genome which enhanced nucleotidylation of the VpG by the RdRp in the presence of Mg²⁺ [48].

2.3.5 “3C-like” protease

Norovirus 3CLpro (19 kDa) is crucial to the proteolytic processing of ORF1 polyprotein into six non-structural proteins. Characterisation of Norovirus 3CLpro has revealed an active nucleophilic residue in the conserved GDCG motif, common to all chymotrypsin-like 3Cpro. The motif contains amino acid residues essential to formation of an active site. The amino acid residues exist as a catalytic triad in Norovirus, and include cysteine (Cys139), histidine (His 30), and glutamate (Glu 54), which function as a nucleophile, general base, and anion, respectively. All three amino acid residues are important to the enzymatic activity for proteolysis [49, 50].

It has also been suggested that the Norovirus 3CLpro can cleave the host encoded poly [A]-binding protein, and as a result, cellular translation is inhibited. This suggests an important mechanism of host cell modulation during viral replication [51].

2.3.6 RNA-dependent RNA polymerase

The Norovirus RdRp is a non-structural protein involved in the replication of the Norovirus genome. It has been proposed that Norovirus proteinase-polymerase precursor is a bifunctional enzyme with protease and RdRp activity both exhibited during viral replication [52].

2.4 Structural proteins

2.4.1 VP1

ORF2 encodes the major capsid protein, VP1, of the Norovirus genome. The VP1 capsid protein can be divided into three domains, the N terminal domain, shell [S] domain, which is buried inside the capsid, and a protruding [P] domain. A flexible hinge connects the S and P domains. The S domain is highly conserved and is essential for the formation of the icosahedral capsid shell. The P domain comprises of two subdomains: P1, a moderately conserved subdomain and P2, which is hyper-variable in its nucleotide sequence. The P2 subdomain of the norovirus genome is the most exposed region of the capsid structure, hence, it contains immune and cellular recognition binding sites [53–56].

2.4.2 VP2

ORF3 encodes a small minor structural protein, VP2, of the Norovirus genome. VP2 is highly variable in sequence between strains and varies in length from 208 to 268 amino acids. The function of VP2 involves the upregulation of VP1 expression and stabilisation of the VP1 in the virus structure. Furthermore, VP2 protects VP1 from disassembly and protease degradation [57]. The role of VP2 in viral replication is unknown, but it may interact with RNA, due to its highly basic, and therefore be involved in packaging of the viral genome. In addition, the VP2 protein is reported to be involved in the formation of infectious viral particles [58, 59].

2.4.3 Transmission

A highly infectious agent, Norovirus is primarily transmitted through person-to-person and commonly via the faecal oral route. Aerosolised vomitus containing Norovirus is another transmission mode by which the virus disseminates in outbreaks of gastroenteritis [60, 61]. A study by Marks et al. reported attack rates of Norovirus infections of up to 60% in individuals in close proximity (who were seated next to and on the adjacent table in a restaurant) to the index person who vomited. The attack rate of infection was directly proportional to the distance from the vomiter. Other sources of transmission include the consumption of contaminated food (oysters, vegetables, fresh and frozen produce) [62–65] or water (drinking, ice or recreational) [66–68]. In addition, fomite contamination in an outbreak setting has been demonstrated as an alternative transmission route [69, 70].

3. Clinical features and pathogenesis of norovirus

3.1 Clinical manifestation

Norovirus infection is characterised by an onset of vomiting, diarrhoea, nausea, and may also be accompanied by variable systemic symptoms including, fever, headache, chills or myalgia [71–74]. Diarrhoeal stool is non-bloody, lacks mucus and may be loose or watery. Following an incubation period of 1 to 2 days, the illness is usually mild and self-limited, which generally persists for a short duration of 1 to 3 days. A 68% sensitivity and 99% specificity was determined when the criteria was used in conjunction with laboratory detection techniques, including ELISA and nucleic acid amplification assays [75]. Norovirus infection affects all age groups and is often more severe in the elderly, the young, and in transplant and

immunocompromised patients [76–79]. Studies have shown that symptoms can persist for up to five days or longer and infection may progress to chronic disease [80]. Prolonged viral shedding can occur in the presence or absence of clinical symptoms and death may occur [81–86].

3.2 Pathogenesis

The pathogenicity of NoV was studied in human volunteers inoculated with the prototype strain, Norwalk virus [NoV GI]. Acute infection with NoV resulted in a histopathological lesion in the jejunum and correlated with a broadening and blunting of the villi and crypt cell hyperplasia of the small intestinal tract. These observations provided suggestive evidence that NoV replication is restricted to the small intestine.

Additional studies in volunteers who developed an illness or characteristic lesion, showed the levels of the small intestinal brush border enzymatic activities [alkaline phosphatase, sucrase and trehalase] were significantly reduced, resulting in transient carbohydrate malabsorption [87]. Furthermore, there was a marked delay in gastric emptying. It has been suggested that the reduced gastric motility is responsible for symptoms, specifically nausea and vomiting associated with gastroenteritis.

3.3 Immunity

The immunogenicity associated with Norovirus disease is not well defined. Early studies on host immune responses to Norovirus infection were based on human challenge studies by oral immunisation with either infectious virus or recombinant VLPs [88–93]. Challenge studies have shown that short-term immunity lasts for six to 14 weeks, and is strain specific [94]. Thus, infection is induced following challenge to a serologically distinct strain. Interestingly, individuals with high levels of pre-existing antibodies against Norwalk virus were reportedly more susceptible to infection than individuals who had a non-detectable or had low levels of serum antibodies after challenge with the same strain.

More recently, the structural recognition site of HBGAs by Norovirus has been determined by mutagenesis and crystallographic studies [95]. Based on crystallographic structures, the receptor site involved in host-cell recognition was the P domain, more specifically the outermost P2 surface on the Norovirus capsid gene [96, 97]. Such findings will provide an understanding into the complex interaction between HBGAs and Norovirus, and could lead to intervention strategies to block attachment of virus to host recognition sites. The study of the role of genetic mechanisms in Norovirus infection is a new area in Norovirus immunology, and further studies are required to understand the complex interactions between specific Norovirus genotypes (particularly, newly emergent Norovirus strains) and susceptibility to infection.

3.4 Replication

Little is known about human Norovirus biology, in particular, human Norovirus replication, immunogenicity and pathogenicity due to the lack of an in vitro cell culture and small animal model systems [98]. However, in recent times our understanding of calicivirus replication has come from other studies, including the animal calicivirus, Feline calicivirus [99], and the use of a gnotobiotic pig as an animal model for the study of human Norovirus pathogenesis [100]. However, a significant advancement in the study of Norovirus biology was the development

of the first in vitro cell culture system for the cultivation of murine norovirus 1 (MNV-1) [101]. MNV-1 was used to study immunity and pathogenesis of Norovirus in a mouse model. Subsequently, MNV-1 was successfully propagated in the murine macrophage cell line RAW 264.7 and revealed a tropism for cells of the haematopoietic lineage, specifically the macrophages and dendritic cells. It was proposed that macrophages could contribute to the spread of Norovirus through the host. Norovirus infection of dendritic cells in the lumen of the intestine also provides a point of infection for Norovirus; however, it remains unclear if human Norovirus targets such cells. Regardless, MNV share many molecular and biological properties with human Norovirus, and therefore, provides an important animal model to understand the biology and pathogenesis of human Norovirus infection. Other systems which have provided significant information regarding Norovirus replication are the replicon and reverse genetics systems.

Molecular advances have led to the development of a Norovirus replicon and a recombinant T7 vaccinia virus expressed Norovirus [102–105]. Studies have shown Norovirus RNA is infectious and capable of replication in three cell types: human hepatoma Huh7-cells, hamster BHK21 cells and human embryonic kidney [HEK] 293 T/17 cells. However, the main limitation of these systems was the inability for virions to spread to other neighbouring cells in the culture system. The inability to culture human Norovirus has been suggested to occur at the level of attachment and entry into the cells. Another in vitro cell culture system for human Norovirus was recently reported based on a rotating wall vessel bioreactor technology to engineer a 3D model of the human small intestinal epithelium (**Figure 2**).

However, the model may not provide direct evidence of in vitro propagation of human Norovirus and needs further investigation [107]. Nevertheless, the system can offer an insight into host-cell interaction in Norovirus infection.

Recently, an infectious reverse genetics system for MNV that generates infectious virus from a genomic complementary DNA [cDNA] clone under the control of an RNA polymerase II promoter was described. The principle of the Norovirus reverse genetics system was demonstrated by mutagenesis of the protease polymerase cleavage site to show that the protease-polymerase cleavage was essential for the recovery of infectious MNV [108]. Overall, the development of such systems provides an approach to perform functional analyses of the Norovirus genome, as well as the study of the molecular biology and replication of Norovirus.

3.5 RNA recombination

RNA recombination is an important mechanism in the evolution of RNA viruses. Recombination in viruses can affect phylogenetic groupings, increase the

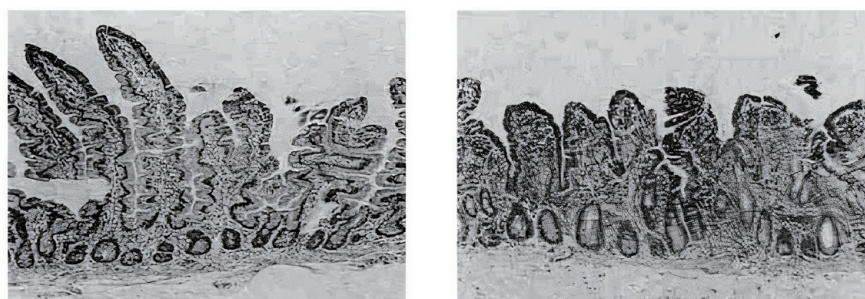


Figure 2.
Intestinal biopsy of jejunal tissue from a human volunteer infected with Norwalk virus. (A-left fig) Normal jejunal biopsy before administration of Norwalk virus. Villi and cellular morphology appear normal. (B-right fig) Jejunal biopsy after administration with the viral agent. Villi are broadened and flattened; epithelial lining cells appear disorganised. Image taken from [106].

virulence and pathogenicity of the virus, and affect anti-viral drug design. By exchange of genetic material through recombination, a new variant of the virus is produced [109]. In recent years, human Norovirus recombinants have been detected frequently in cases of gastroenteritis worldwide. This increase in prevalence of Norovirus recombinants suggests that infection with at least two virus strains is common. The proposed site of recombination in Norovirus is within the highly conserved ORF1/ORF2 overlap [110–112].

3.6 Treatment and prevention

Norovirus associated gastroenteritis is mild and self-limiting, and generally resolves without complications. However, death from Norovirus associated gastroenteritis has been previously reported [113–115]. In severe cases of Norovirus infection, hospitalisation is required and the administration of an oral fluid and electrolyte treatment is often required to replace the loss of fluids. The oral administration of bismuth subsalicylate after the onset of symptoms has been demonstrated to reduce the duration of abdominal cramps and gastrointestinal symptoms during experimentally induced Norovirus illness in adults [116]. The best control measure for the prevention of Norovirus infection is with good hygiene practices. These include, thorough and frequent hand washing, and the disposal or disinfection of contaminated materials. In addition, extra measures should be implemented in healthcare facilities to prevent large-scale outbreaks, such as restriction of staff movement between wards containing infected patients, the isolation of symptomatic patients, the exclusion of affected staff from work until 48 h after the cessation of symptoms, and the closure of affected units to limit the spread of infection. However, the impact of preventive measures in affected institutions is reduced due to the environmental stability of Norovirus outside the host. This is due to the fact that Norovirus has a non-enveloped structure, is acid stable, persists in the environment and resistant to chlorination of up to 300 ng/ml. Furthermore, quaternary ammonium disinfectants are ineffective in the disinfection of Norovirus [117, 118]. Although a combination of detergent and sodium hypochlorite solution has been reported to be effective in the decontamination of surfaces [119]. Therefore, to prevent and control the spread of Norovirus disease, strict hand hygiene and use of effective disinfectants should be enforced during outbreaks. Importantly, for the efficient implementation of precautionary measures in an outbreak setting, a rapid detection system for the diagnosis of a Norovirus infection would be ideal.

4. Laboratory diagnosis

4.1 Detection of norovirus

Detection of the aetiological agent of gastroenteritis is important as only bacterial and parasitic agents are treatable by current therapeutic agents.

Furthermore, for clinical and epidemiological studies the availability of detection methods for viral nucleic acid, viral antigen, or antibody responses is valuable. Various methods have been used for the diagnosis of Norovirus infection, including electron microscopy [EM], IEM, radioimmunoassays, ELISAs and viral RNA based nucleic acid amplification assays. Of the available detection methods, the most commonly used assays for Norovirus diagnosis include ELISAs and RT-PCR [120].

4.1.1 Electron microscopy

The detection of Norovirus has traditionally relied on EM. It enables the identification of Norovirus by their characteristic morphology. However, the sensitivity of EM detection is low, requiring at least 10^6 viral particles/g of stool for visualisation. Therefore, this technique is useful only for specimens collected immediately upon the onset of illness when substantial quantities of viral shedding occur. Furthermore, EM is a robust tool but time consuming, requires a high level of technical skill, is labour intensive and not available to all clinical laboratories. Thus, EM is not feasible for large epidemiological studies. Modifications of the EM method, such as, or solid phase IEM [121–125] have also been used to aid in virus identification. Both these methods are based on antigen–antibody reactions. However, like EM, the application of IEM is rarely applied to epidemiological investigations.

4.1.2 Elisa

An ELISA offers an efficient diagnostic method for the identification of Norovirus infection. The rapid turnover and simplicity for screening a large number of samples makes ELISAs an ideal system for use in a diagnostic laboratory. Norovirus are antigenically diverse and therefore assays may be limited in the detection of a broad range of Norovirus strains in circulation. This has probably contributed to the poor performance assessments [sensitivity and specificity] of commercially available ELISAs in different countries when compared to sensitive molecular methods, such as RT-PCR [126–128]. The potential for ELISAs to give false negatives and false-positives due to poor sensitivity and poor specificity, respectively, has limited their use for diagnosing outbreaks where large numbers of samples are being tested.

4.1.3 Reverse transcription-polymerase chain reaction

RT-PCR has remained the most reliable means of diagnosing Norovirus infection as it is the most sensitive routine method used compared to EM and ELISA [129, 130]. The availability of RT-PCR amplification has greatly facilitated sequencing and genome characterisation of Norovirus strains [131–133]. The RT-PCR assay employs primers that target conserved regions of the Norovirus genome, such as the RdRp and/or the VP1 gene. Until recently, Norovirus RT-PCR assays have used primers that targeted the RdRp [3' end of ORF1 of the Norovirus genome], which is highly conserved among Norovirus. By sequence analysis of the capsid gene [VP1] in the Norovirus genome, another conserved region located at the 5' end of the capsid gene was identified. This region offered better segregation of Norovirus genotypes by phylogenetic analysis. Moreover, analysis of both regions, RdRp and VP1 is necessary for the detection of Norovirus recombinant strains. These technical advances have improved detection and enhanced epidemiologic surveillance by molecular genotyping and sequence analysis. However, conventional RT-PCR assays have progressively been replaced by real-time RT-PCR, which is more sensitive, faster and offers quantification of RNA viruses. This technology is not only quicker but enables quantitation using the Ct of the unknown target RNA sample compared directly to the Ct of a standard curve, which contains a defined number of copies of the target virus. The Ct value is the basis for accurate and reproducible quantitation using real-time RT-PCR. The application of a standard curve in a real-time RT-PCR assay also enables the determination of viral kinetic parameters associated with Norovirus infection, such as the number of viruses excreted [that is, a measure of viral load in a sample], duration of viral excretion and the viral decay rate.

Several real-time nucleic acid amplification assays have been developed for the detection and quantitation of Norovirus RNA in clinical specimens, by the use of SYBR Green dye chemistry, and probes, including taq-man probes and hybridization probes [134, 135].

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