Taking gastro-surveillance into the 21st century

Thea K. Fischer[a,b,c,*], Lasse D. Rasmussen[c], Jannik Fonager[d]

[a] *Department of Clinical Research, Nordsjaellands Hospital, Hilleroed, Denmark*
[b] *Departments of Infectious Diseases and Global Health, Clinical Institute, University of Southern Denmark, Denmark*
[c] *National Virus Surveillance and WHO Reference Laboratories, Dept of Virus and Microbiological Special Diagnostics, Statens Serum Institut, Copenhagen, Denmark*
[d] *Virus Research & Development Laboratory, Department of Microbiological Diagnostics and Virology, Statens Serum Institut, Copenhagen, Denmark*

ABSTRACT

Enteric viruses, particularly rotaviruses and noroviruses, are leading causes of gastroenteritis worldwide. Human rotaviruses are ubiquitous and globally almost every child has been infected by 3–5 years of age. Noroviruses affect people of all ages and is the leading cause of foodborne outbreaks. Rota- and noroviruses account for ˜40% and ˜17% of diarrhea-associated hospitalizations, and ˜200,000 deaths annually respectively, with most deaths occurring in developing countries. Two rotavirus vaccines have currently been implemented in ˜95 countries and several norovirus vaccine candidates are currently in development and/or clinical testing.

Surveillance of enteric viruses is an important part of outbreak investigations as well as pre- and post-vaccine impact studies but is even in developed countries often limited to investigation of sporadic cases or comprehensive outbreaks. Conventional methods for enteric virus detection and subtyping relies on standard RT-PCR methods, supplemented with Sanger-sequencing. However, for viruses with even moderate mutationrates, PCR-based-typing of only limited parts of the virus genome is challenging and requires regular update of primers. Full-genomecharacterization technologies based on sequence independent methods based on next generation sequencing (NGS), have demonstrated great potential for enteric virus detection and/or typing in both clinical and environmental samples. However, cost-benefits must balance for such methods to be widely accepted for public health purposes.

In Europe as also globally, routine use of NGS-methods for surveillance of enteric viruses is currently limited to few national public health laboratories. What important lessons can be learned from these and what is the future of NGS-based surveillance?

1. Introduction

Approximately 2.39 billion annual episodes of diarrhea (95% CI:2.3–2.5) occur globally each year. Approximately 40% (957.5 million episodes, 95% CI: 871-1.0) of these cases occur in children < 5 years of age [1]. With 114 million rotavirus cases and 684 million norovirus cases these two viruses are responsible for approximately 30% of all diarrheal cases worldwide [2] and are thus among the leading single causes for all global cases. In terms of severe diarrheal illness, rota- and noroviruses account for approximately 40% and ˜17% of diarrhea-associated hospitalizations respectively, worldwide, and each for ˜200,000 deaths annually, with the majority of deaths occurring in developing countries [1]. Fortunately, thanks to new technologies and discoveries we have improved our capability to control and prevent rotaviruses through effective vaccines. Widespread use of rotavirus vaccine [3] has more than halved the ˜450.000 rotavirus-

associated deaths in less than a decade as reported by World Health Organization in 2006. In terms of prevention and control of noroviruses, no vaccine currently exists, although several vaccines are in various stages of development [4]. Thus, the coming decade might therefore lead to similar improvements in norovirus prevention and control.

Surveillance of enteric viruses is an important part of outbreak investigations and will remain an integral part of pre- and post-vaccine impact studies. However, even in developed countries, surveillance of gastroenteric viruses, is often limited to investigation of sporadic cases or comprehensive outbreaks. Conventional methods for enteric virus detection, relies on standard polymerase chain reaction (PCR)-based methods often supplemented with Sanger-sequencing for subtyping. However, for RNA viruses with even moderate mutation-rates, PCR/sequencing-based-typing of only a limited part of the virus genome is challenging and requires regular update of primers. NGS has

Table 1
Rotavirus and Norovirus characteristics.

Virus name	Rotavirus	Norovirus
Envelope	–	–
RNA strand	Double	Single, positive-sense
Virus family	*Reoviridae*	*Caliciviridae*
Virus genus	Rotavirus	Norovirus
Type species	10 (A-J) based on sequence and VP6-inner capsid associated antigenic differences. Species A most common cause of human infections.	1 (*Norwalk virus*) [1]
Genome size (kb)	16,5	7,5
Genome arrangement	Segmented (n = 11)	Linear, consists of 3 open reading frames: ORF-1, ORF-2 and ORF-3 encoding 8 virus proteins.
Shape	Wheel (Latin rota: wheel)	Icosahedral
Structural proteins	6 (VP1-4, VP6, VP7): host specificity, cell entry, immune reactivity and various enzymatic functions involved in viral transcription	2 (VP1, VP2): regulatory, receptor binding and immune reactivity, and likely responsible for ABO histo-blood group antigen interactions [2].
Non-structural proteins	6 (NSP1-6): genome replication and antagonism of the innate immune response	6 (NS1/2-NS7) [3]: regulation of cell proliferation, replication complex assembly, inhibition of cellular protein synthesis in infected cells [2].
Nomenclature and further classification	Genotypes by a dual parameters based on species A sequence differences in glycoprotein G (VP7) and protease-cleaved protein P (VP4) encoded segments.	Genotypes by dual parameters based on the genetic lineages in the gene encoding the virus polymerase P (ORF-1) and the capsid G proteins (ORF-2) genotypes, respectively (e.g. GII.P16-GII.4 and GII.Pe-GII.4) [4].

demonstrated great potential for enteric virus characterization by whole genome sequencing, for improved molecular epidemiological investigations in both clinical and environmental samples [5–7]. However, while this technology holds great promises several technical and especially cost-related challenges remain to be solved for such methods to be widely used in routine surveillance.

A brief overview of the global disease burden and state-of-the-art detection and characterization methods of the most common gastro-enteric viruses: rotavirus and norovirus, will be provided along with perspectives on current gaps in terms of surveillance and suggestions on key elements in strengthening of relevant surveillance programs for these viruses.

2. Rotavirus

Rotavirus belongs to the *Reoviridae* family (Table 1). Rotavirus infection is clinically indistinguishable from acute gastroenteritis caused by other gastroenteritis-inducing viral agents such as norovirus, adenovirus 40 and 41, sapovirus, enterovirus and astrovirus. Rotavirus infections are often asymptomatic or mild in young infants due to maternal antibodies transferred through placenta and/or through breast milk [8]. Severe symptoms can result in rapid dehydration with electrolyte derangement, shock and death [9].

3. Rotavirus detection and characterization

A variety of rapid methods are commonly used worldwide to detect rotaviruses such as enzyme-linked immunosorbent assays (ELISA) or immunochromatography. Even though the sensitivity and specificity of such assays generally are high [10,11], the window for detection of viral shedding is typically limited to 4–7 days after symptom onset [9]. Also, samples with low virus content might be false negative using EIA [11]. The virus can be detected for longer periods by more sensitive assays such as reverse-transcriptase polymerase chain reaction (RT-PCR). RT-PCR methods based primarily on amplification of rotavirus gene segments encoding the proteins VP4, VP6 or VP7, have also been developed for rotavirus genotyping and detection [12]. Development of real-time RT-PCR for rotavirus detection and subtyping, has further facilitated assessment of viral RNA quantity in addition to detection [13].

4. Rotavirus strain classification and global distribution

To date 32 G and 47 P genotypes have been identified. However,

globally six G types (G1-G4, G9 and G12) and three P types (P[4], P[6] and P[8]) predominate, and six strains G1[P8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] account for > 90% of globally circulating species A rotavirus strains [14].

In 2014, after introduction of rotavirus vaccines in more than 60 countries, a review of global rotavirus genotype distribution based on ˜47,000 rotavirus strains from all major global regions during 2007–2012 demonstrated no major changes in the prevalence of dominating rotavirus strains as compared to preceding years [15]. Also, the continuous emergence of unusual and novel antigen combinations was documented, including some causing local outbreaks, even in vaccinated populations. In addition, vaccine strains were found in both vaccinated infants and their contacts indicating genetic interaction between vaccine and wild-type strains [15].

5. Rotavirus vaccines

Traditional hygienic measures such as clean water and improved sanitation have not substantially reduced the burden of rotavirus disease globally, and therefore vaccines are considered the best preventive measure against rotavirus disease [16,17]. Two WHO pre-qualified rotavirus vaccines are broadly used worldwide: Merck's live-attenuated pentavalent reassortant human bovine vaccine (RotaTeq) and GlaxoSmithKline's monovalent live-attenuated human virus vaccine (Rotarix). Both vaccines were widely licensed following large clinical trials in the Americas, Africa and Europe. In 2009, following efficacy studies in low-income and low-to-middle income countries in Africa and Asia, WHO recommended that all countries introduce rotavirus vaccine into their National Immunization Programs [16]. Despite the vaccines high effectiveness [18] some countries, including the United States, have reported on stagnating and even decreasing rotavirus vaccine coverage rates [19]. This recent development might indicate that surveillance of not only vaccine effectiveness but also morbidity and mortality is far from redundant despite the development of effective vaccines.

Surveillance of rotavirus is therefore still very relevant to estimate the burden of rotavirus disease and evaluate the impact of rotavirus vaccination where vaccines have been introduced.

6. Norovirus

The *Norovirus* and *Sapovirus* genera of the *Caliciviridae* family (Table 1) contain the human enteric viruses of the same names as well as a number of viruses that cause primarily enteric diseases in animals such as murine and canine norovirus. Originally norovirus was called

Norwalk-virus named after the place of its first discovery in stool specimens by use of electron microscopy during an outbreak of gastroenteritis in Norwalk, OH, in 1963, thus being the first viral agent shown to cause gastroenteritis [20]. Until 1993 detection of Norwalk virus and Norwalk-like viruses were performed using electron microscopy [21].

Norovirus is highly infectious to people of all ages, although different genotypes might prefer certain age groups [22]. Several factors enhance the transmissibility of norovirus, including the small inoculum required to produce infection (< 20 viral particles), prolonged viral shedding, and its ability to survive in the environment [23]. The main brunt of morbidity and mortality is among those most vulnerable to dehydration and electrolyte derangement such as young children, elderly and immunocompromised individuals. Norovirus is transmitted primarily from person-to-person within closed settings such as schools, cruise ships, hospitals, nursing homes etc. However, norovirus is also the most prevalent etiologic agent identified in foodborne outbreaks worldwide, and is commonly associated with foods that undergo no or little processing before consumption [21]. Norovirus outbreaks have an endemic pattern worldwide, however in regions with temperate climates, peaks are typical during the colder winter months [24]. Clinically norovirus infection has an incubation period of 24–48 hour and is normally self-limiting 1–2 days after the onset of acute nausea, vomiting, abdominal cramps, myalgias and non-bloody diarrhea. Young children < 1 year of age are more prone to diarrhea than vomiting [19]. The median duration of norovirus disease is often prolonged in immunocompromised individuals and in children [25]. Fecal excretion of norovirus infection in asymptomatic individuals is common, especially in children [27]. Outbreaks of norovirus are hard to control and can lead to considerable morbidity and mortality among the affected persons, in particular vulnerable individuals such as children, elderly and immunocompromised patients. Large outbreaks settings are often facing considerable economic losses owing to closure of hospital wards, facilities and businesses including restaurants and cruise ship industries [28,29].

Norovirus classification is based on phylogenetic distance of Sanger-sequenced partial RNA-dependent polymerase and capsid genes [30]. Today, seven genogroups GI-GVI are known, of which GI, GII and GIV can infect humans [31].Of these GII is clearly predominant being responsible of more than 92% of all cases, whereas GI is responsible for approximately 8% of the detected cases. GIV is very rare and has been observed in less than 0,05% (7/16635) of all cases reported to NoroNet over a 10 year period [37]. Genogroups are further classified into at least 30 genotypes [32]. The genetic diversity of norovirus is apparent from the observation that VP1 amino acid sequences of GII.4 strains differ by 37% to 38% from the prototypic G1.1 Norwalk virus strain and by 5%–7% within the GII genotype alone, with as much as 2.8% difference between strains of an individual virus [33]. The term 'variant' is selectively used for individual viruses within the pandemic GII.4 lineages [30]. These GII.4 variants are named after the geographical location and year of the first full-genome sequenced isolate (e.g. GII4 strains New Orleans 2009 and Sydney 2012).

Norovirus evolution is mainly driven by population immune selection pressure and new viruses evolve through both antigenic drift and shift [34,35]. Norovirus persists the acquired herd immunity through antigenic variation of epitopes targeted by neutralizing antibodies [34,35], allowing norovirus to be continuously transmitted to even previously infected individuals [36]. GII.4 Sydney 2012 has been the predominantly detected variant worldwide since its emergence in 2012, and given the replacement cycle of 2–3 years observed for previous variants [37], a new antigenic variant has been anticipated for some years. In addition to the globally prevalent GII.4 viruses, studies form Asia report on the replacement of GII.4 by a novel GII.P17-GII.17 norovirus strain (GII.17 Kawasaki 2014) in late 2014 and onwards. Still, GII.P17-GII.17 has not yet fully replaced GII.4 strains [37].

7. Norovirus detection and characterization

Several factors add to the difficulties in detection of norovirus as most other enteric viruses. The virus genome only accounts for a tiny fraction of the genetic material in the primary samples. As cultivation is not routinely available and challenging, if applied, the diagnostic analysis should therefore preferably be performed on primary samples.

In the post-electron microscopy and pre-PCR era norovirus antigen rapid kits such as enzyme-linked immunosorbent assays (EIA) and rapid immunochromatographic were main diagnostic approaches. Antigen kits are still commonly used in outbreak settings: performance of rapid assays is highly variable (sensitivity and specificity ranging from 30 to 90% and 65–100%, respectively). Newer generation EIAs based on a wider range of polyclonal or monoclonal antibodies raised against a panel of different virus-like particles (VLPs) often have relatively high specificity but are challenged by low sensitivity mainly due to their genotype-dependency [39]. Due to the low positive predictive value of a wide range of rapid antigen tests, negative samples during outbreaks requires confirmation with a second technique such as RT-PCR. [40].

Reverse-transcriptase polymerase chain reaction (RT-PCR) is the state-of-the-art norovirus detection method and numerous conventional as well as real-time RT-PCR assays are available. The first generations of PCR assays used primers targeting parts of the RdRp gene. These first assays underestimated norovirus genetic diversity and required post-amplification analyses via sequencing or hybridization probes to improve sensitivity and specificity issues [41,42] and were later improved through the introduction of more broadly reacting GI and GII primers [43]. Later, capsid gene targeting, and one-step RT-PCR improved the process [44]. With the development of real-time RT-PCR some of the challenges due to high genetic diversity were addressed, and new assays targeting a conservative region at the ORF1-ORF2 polymerase-capsid junction further improved the Norovirus detection rate and reduced the setup time. Combined RT-PCR followed by Sanger sequencing of targeted genomes have proven effective in detection of recombinant norovirus [45] and is currently the gold standard for norovirus detection and characterization [42].

A few commercial RT-PCR assays for norovirus testing are available along with several multiplex tests for diarrheal pathogens such as RT-PCR Luminex assays [46] and TaqMan arrays for detection of up to 19 enteropathogens [47,48] and a real-time nucleic acid-based loop-mediated isothermal amplification (RT-LAMP) [49]. The rapidly growing market for point-of-care testing also offers norovirus analysis for bed-side diagnostic use [50]. During even major outbreaks with widespread spatial-temporal norovirus transmission, virus mutations are common during virus passage from index patients and further on. Therefore high resolution methods with sequencing of longer fragments of the capsid gene containing the hypervariable P2 region in ORF-2 are often required to ensure sufficient discriminatory power to resolve outbreaks by extra confirmation of relatedness among norovirus outbreak patients [52,53] and detailed identification of transmission dynamics [54].

In summary, the use of conventional gene-specific primer PCR techniques combined with Sanger sequencing have solved some of the previous challenges related to relative low discrimination and detection of recombinant norovirus by increasing the genome coverage by overlapping or continuous fragments. However, norovirus genotyping is still challenged by the possible emerging possessing mutations in the primer-binding regions as well as efficient and uniform detection of multiple strains from co-infections, often seen in some foodborne outbreaks and the presence of quasi-species in clinical samples

Whole-genome sequencing (WGS) is poised to solve these remaining challenges and recent reports on the application of WGS to understand virus genetic evolution and reconstruct transmission pathways have already proven valuable and promising for surveillance and infection control purposes. Nevertheless, concerning norovirus, historical shortcomings of effective cultivation platforms, may be limiting with regards

to purity and concentration of nucleic acids used in the NGS platforms. Therefore, metagenomics detection and analysis of norovirus RNA in stool samples in which viral genomes only constitute a minor proportion of the nucleic acids presents yet another challenge. NGS approaches to achieve full norovirus genome coverage have relied on genotype-specific primers [55,56] which is both time-consuming and costly and further challenged by rapidly evolving viruses and the need for constantly updated primers. Recent developments include the use of random sequencing approaches [57,58] and application of different enrichment strategies [58–60]. In a recent study [6], the application of a poly(A)-capture technique for norovirus enrichment followed by universal random priming led to a ~40-fold enrichment of norovirus RNA in stool samples as well as reduction of non-polyadenylated bacterial RNA. This easy-to-use method was applied to samples from eight foodborne outbreaks and yielded sufficient norovirus read counts to allow the assembly of several complete or nearly complete genomes, including a new recombinant GII.P16_GII.4_Sydney, for molecular comparisons as well as identification of an additional genotype initially missed by standard PCR-based molecular characterization [6]. The method have also been applied to enrich enteroviruses [61] and might be applied to other clinically important positive-sense RNA viruses with a polyadenylated 3′ tail [61]. With future widespread access to benchtop sequencers, NGS will soon become a definitive non-discriminatory tool for virus infection control and serve to monitor both the evolution and spread of genotypes as well as enhance viral outbreak investigations.

8. Molecular epidemiology of norovirus

Worldwide GII is the most common norovirus genotype, and in a recent review GII constituted 91.7% of 16625 sequences submitted to the informal international data sharing network NoroNet during the period of 2005–2016 [37]. During this period 26 different norovirus capsid genotypes circulated and 22 different recombinant genomes were identified. GII.4 drift variants emerged with a 2 to 3 year periodicity up to 2012, from where the GII.4 Sydney capsid persisted through recombination [37].

With > 30 different human norovirus genotypes, GII.4 is still the only genotype associated with norovirus pandemics of gastroenteritis and viruses within this genetic lineage account for > 80% of all human norovirus infections at any time [62]. The most recently identified GII.4 Sydney 2012 variant is an example of a new pandemic norovirus which was identified in New Zealand in 2010 [63], two years prior to its global spread which started in 2012 and resulted in gastrointestinal illness in millions of individuals worldwide during the following years. The early identification of the pandemic variant resulted in global awareness and enabled activation of preventive strategies in institutional settings [64]. Interestingly this variant along with the New Orleans 2009 variant didn't only evolve using standard antigenic drift mechanisms from previous GII.4 viruses through mutations within the protruding domain of the capsid as earlier pandemic norovirus variants [65]. Instead, the New Orleans 2009 and Sydney 2012 also evolved through combined antigenic drift and shift as both antigenic capsid variation and intragenotype recombination at the ORF1-ORF2 overlap has taken place [45,66].

The continuous changes in global norovirus genetic diversity and the constant emergence of new pandemic norovirus strains with the potential to immune escape underscore the need for sustained global norovirus surveillance.

The historical lack of ability to propagate norovirus in cell cultures has greatly hindered the development of long-lasting effective vaccines and therapeutics. However, recently a norovirus *in vitro* infection model has successfully demonstrating that norovirus GII.P4 can be cultivated in human B-cells using commensal bacteria as a co-factor for infection [67]. With this important milestone achieved development of norovirus vaccine candidates can therefore be expected in the near future.

9. Conclusions

Despite recent decades of global improvement in hygienic measurements and vaccine development, gastrointestinal disease caused by common enteric viruses such as rotavirus and norovirus still pose a considerable burden on morbidity both within healthcare institutions and in the broader communities worldwide. The development of an animal model or cell culture system to measure neutralizing norovirus antibodies will provide an important first step in developing safe and effective vaccines against norovirus. However, the limited use of effective and available rotavirus vaccines might indicate future challenges ahead for introduction of even effective vaccines against common diseases including norovirus.

Interventive/preventive measures exist for rotavirus in the form of a vaccine, which, despite covering a broad repertoire of rotavirus strains, is not expected to provide complete protection against all strains. No vaccine or other therapeutic measures exist for norovirus and interventive measures to stop ongoing transmission is dependent on quick action to isolate persons or remove contaminated food items. The precision of such actions is significantly enhanced by fast and correct identification of transmission chains.

NGS has already proven to be a reliable and effective method providing important information in a timely manner. Further development of this method with a reduction in the costs will in the interim phase -while awaiting development and/or wide use of effective infection control and preventive measures- be the most useful tool in the ongoing monitoring of these common diseases.

In that case, surveillance of not only vaccine effectiveness but also morbidity and mortality is far from over with the development of effective vaccines.

Conflict of interest

I approved the final manuscript.

Funding

None of the authors have received funding for this study. We are all government employee.

Ethical approval

Not relevant – a review.

Randomized controlled trial

N/A.

CRediT authorship contribution statement

Thea K. Fischer: Conceptualization, Methodology, Writing - review & editing. **Lasse D. Rasmussen:** Writing - review & editing. **Jannik Fonager:** Writing - review & editing.

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