ROTAVIRUS WHOLE GENOME SEQUENCING WITH

NEXT-GENERATION SEQUENCING

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Rotavirus on maailmanlaajuisesti yksi yleisimmistä alle viisivuotiaiden lasten kuolinsyistä. Viruksen aiheuttama gastroenteriitti aiheuttaa oksentelua ja kuumeen, sekä vesiripulin, joka voi johtaa dehydraatioon ja kuolemaan. Taudin ehkäisemiseksi rotavirusrokotteet ovat osana kansallista rokotusohjelmaa 81 maassa ja saatavilla lisäksi yli sadassa muussa maassa.

Nykyään yleisin viruksen tunnistusmenetelmä on RT-PCR, ja viruksesta tutkitaan yleensä vain VP7-, VP4- ja VP6-proteiineja. Rotaviruksen genomi on rakenteeltaan varsin vakaa, mutta tutkimuksissa on havaittu yksittäisten geenien mutaatioita sekä ns. tuplareassortanttiviruksia, joissa on geenejä sekä rokote- että villityypin viruksista. Rotaviruksen koko genomin sekvensoimisen merkitys korostuu, kun halutaan selvittää viruksen osien alkuperä ja rokotteiden mahdollisia vaikutuksia rotaviruksen genomiin.

Seuraavan sukupolven sekvensointi (NGS) on huomattavasti Sanger-sekvensointia nopeampi ja tehokkaampi, mutta toisaalta monimutkainen ja kallis menetelmä. Rotaviruksen genomi on pieni, n. 30kbp, mutta jotkin geenit joudutaan sekvensoimaan Sanger-menetelmällä pienemmissä osissa. Koko genomin sekvensoinnissa NGS-menetelmä voi osoittautua aikaa säästäväksi ja kustannustehokkaaksi. Tässä tutkimuksessa kuvataan NGS-menetelmän pystytys ja käyttö rotaviruksen koko genomin sekvensoinnissa ensimmäistä kertaa Suomessa.

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1. INTRODUCTION

Rotavirus (RV) is one of the leading causes of death in children aged under five years causing 146 000 deaths in 2015 (1). The symptoms of rotaviral acute gastroenteritis are vomiting and fever, followed by watery diarrhea leading to dehydration and potentially death. Oral or intravenous rehydration was the best mortality-reducing treatment before the introduction of rotavirus vaccines (2). Before vaccinations, RV was estimated to cause 527 000 deaths annually among children under 5 years of age (3).

Currently, there are two major RV vaccines available globally. A monovalent vaccine Rotarix™ (GlaxoSmithKline) was licenced in Mexico in 2004. Two years later, a pentavalent human-bovine reassortant vaccine, RotaTeq® (Sanofi Pasteur-MSD), was launched. By 2016, 81 countries have introduced rotavirus vaccines into their national immunization programs (NIP), and RV vaccines are also available in more than 100 countries (4). In Finland, the first vaccinations were introduced with a partial coverage in 2006 with Rotarix™ and in the beginning of September 2009, RotaTeq® was added to the NIP and is given in a three dose schedule at the age of 2, 3 and 5 months (5).

Globally, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] are the most common genotypes. In Finland, before the universal mass vaccination, the most predominant rotavirus genotypes were G1P[8], G9P[8] and G4P[8] (5). After the introduction of RV vaccines, several changes in RV genotype distribution have been described, most significantly the decrease of G1P[8] and the rise of previously uncommon G9 and G12 genotypes (6).

RV vaccines have decreased the number of RV infections significantly, not only in vaccinated, but also in unvaccinated children, and are known to affect the onset and length of the RV season. Also, changes in natural seasonality have been described in the USA. (7,8) So far studies have not shown evidence if the vaccines cause antigenic shift or changes in the antigenicity of circulating rotavirus genotypes (9-11).

The complete sequence of group A rotavirus was first sequenced by Ito et al. in 2001 (12). In addition to continuous genomic drift by point mutations (13,14), vaccine-derived rotaviruses have been observed to form double reassortants with wildtype rotaviruses (15). The usual protocol of sequencing only genes encoding structural proteins VP7, VP6 and VP4 might therefore be insufficient to detect possible mutations, which may only be present in the rest of the genes. This creates pressure on sequencing the entire rotavirus genome in order to detect such changes.

Although the genome of RV is small, only 30 kbp, the length of certain genes would mean combining several shorter sequences while using the Sanger sequencing method and therefore the adaptation of a new next-generation sequencing (NGS) method would be timesaving and cost-effective. In this study, we set up and describe an NGS protocol for sequencing the whole genome of RV for the first time in Finland.

2. REVIEW OF THE LITERATURE

2.1. Rotavirus structure

Rotavirus is a non-enveloped double-stranded RNA virus. The 30 kbp-sized genome is segmented into 11 genes. Six of the genes encode structural proteins (VP1-4, VP6-7) forming the virion and five of the genes encode non-structural proteins (NSP1-6) (16). The virion consists of three layers. The outermost layer consists of VP7 and VP4, which define the G- and the P-type of the virus, respectively. VP7 protein forms the shell of the virion and VP4 protein projects outward from the shell helping the virus to attach. Both of these proteins induce neutralizing antibodies. VP4 cleaves into proteins VP5 and VP8*, which contain neutralizing epitopes. (17,18) The middle layer of the capsid is formed by VP6, which is the most immunogenic protein of rotavirus. The polymeric IgAs against VP6 inhibit viral replication by structural changes during the mRNA extrusion (19). VP2 protein forms the innermost core of the virion to which VP1 and VP3 are attached (20). VP1 acts as an RNA polymerase enzyme and VP3 as an RNA capping enzyme (21,22).

Rotavirus NSPs coordinate various stages of replication and viral assembly by adapting and modifying the cellular machinery, which leads to release of mature particles through cell lysis (23). NSP1 primarily functions as an antagonist of the host interferon response (24), and NSP2, together with NSP5, form the viroplasm (25). NSP3 suppresses host protein synthesis, and NSP4 is essential for rotavirus replication, transcription, and morphogenesis as well as an enterotoxin (26). NSP6 is known to interact with NSP5 but the precise role in replication remains to be characterized. It is not encoded by all rotavirus strains. (23).

2.2. Classification

Rotaviruses belong to family *Reoviridae* and are further divided into serogroups that include viruses that share cross-reacting antigens detectable by a number of serologic methods. Eight serogroups,

named A-H and based on the amino sequences of the VP6 proteins, have been recognized to infect various species. So far, group A, B and C viruses are found both in humans and animals and D-G only in animals. (26) Serotype A accounts of over 90% of rotavirus gastroenteritis in humans (27).

Rotaviruses are classified by their outer capsid proteins VP7 and VP4 into G-types and P-types. Currently, 27 G-types and 35 P-types have been identified. When classifying by the whole genome, the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes of RV strains are described using the abbreviations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x = Arabic numbers starting from 1), respectively. (28)

2.3. Overview of the detection methods over time

In the year 1973, Bishop et al (29) were the first to detect RV when they found it in the epithelial cells of duodenal biopsy by electron microscope (EM). Shortly after this, Flewett et al. detected rotavirus in the feces by EM (30). EM has a relatively low detection limit (10^5 to 10^6 viral particles per gram of feces), though, the quantity shed to feces during the clinical phase of the disease is much higher (31).

In the following years, many techniques were investigated to replace EM because of the costs and inefficacy when examining a large number of samples. These methods included immune electron microscopy and a fluorescent virus precipitation test (Peterson 1976), counter-immunoelectrophoresis (Middleton 1976; Tufvesson and Johnsson, 1976; Birch 1977), indirect immunofluorescence (Bryden 1976), free viral immunofluorescence (Yolken 1977), a viral RNA detection technique (Espejo 1977), radioimmunoassay (Kalica 1977), complement fixation (Zissis 1978), and enzyme-linked immunosorbent assay (ELISA) (Yolken 1977). However, some of these tests did not show the necessary sensitivity and reliability required for the detection of rotavirus. (32) In the early 80's latex agglutination methods were found to be competent (33) followed by immunochromatography (34). Later, direct detection of the viral genome was possible with guanidinium isothiocyanate combined with different purification methods (35-37).

At the moment, the most used techniques for rotavirus detection are ELISA and reverse transcription polymerase chain reaction (RT-PCR). ELISA is applied most frequently in the routine diagnostic laboratory due to the ease of use and speed of obtaining a result (16). However, RT-PCR, introduced to the research of dsRNA by Gouvea V. et al. in 1990, is highly sensitive and specific

(38) and also suitable for genotyping (39). Sequence analysis is the most definitive method for confirmation of PCR results.

The determination of the virus G-type has been one of the priorities over time. Historically, the most used characterization method has been to start with an ELISA, using serotype-specific monoclonal antibodies (Mabs) (40,41). Approximately 70%−85% of samples are typeable using one to several Mabs specific for each serotype. The method depends, however, on the presence of triple-layered particles, which are not always found due to digestion or degradation (39).

Two common sets of consensus primers, designated Beg9/End9 combined with type-specific primers (G1, G2, G3, G4, G8 and G9) introduced by Gouvea et al. in 1990 or 9con1/9con2 combined with type specific primers (G1, G2, G3, G4 and G9) developed by Das et al. in 1994, are currently used to target the VP7 gene. These consensus primers can also be used for RT-PCR amplification and nested multiplex PCR (39). Under ideal conditions, PCR can be extremely sensitive, recognizing one copy of a genome. However, the threshold of rotavirus detection is as low as 1000 virus particles per mL de facto (38).

2.4 Rotavirus epidemiology

Before vaccinations, rotavirus was the leading cause of life-threatening diarrhea causing approximately 138 million episodes of gastroenteritis, 2 million hospitalizations, and 611,000 childhood deaths each year in children under 5 years of age. The majority of rotavirus related deaths occurred in the developing countries where access to health care was limited. (42,43)

Around the world, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] are the most common genotypes. G1 strains comprised the most frequently detected serotype in each of the continents. (44) In Finland, before the universal mass vaccination, the most predominant rotavirus genotype was G1P[8] (62%), whereas other genotypes were significantly less common (G9P[8] (12%), G4P[8] (9.5%), G2P[4] (7.8%), and G3P[8] (3.6%)) (5).

As of May 1, 2016, 81 countries have introduced rotavirus vaccines in their national immunization programs. Other countries, such as Canada, India, Italy, the Philippines, Sweden, and Thailand, have introduced rotavirus vaccines in phased or regional introductions. Rotavirus vaccines are also available in more than 100 countries through the private market and they have been introduced by Global Alliance for Vaccines and Immunization in 38 countries, mostly in Africa. (4)

The implementation of RV vaccination has been shown to affect the natural seasonality, onset, and length of the RV season as well as reducing the number of cases dramatically (7). The studies also show that vaccination has significantly reduced RVGE cases in unvaccinated children (8). Studies have been carried out to determine the changes in circulating rotavirus types. In addition to changes in the prevalence of common genotypes, also new genotypes, such as G9 and G12, have emerged and spread worldwide in a very short time span (6). Point mutations and gene rearrangements among others create diversity of rotaviruses leading to a possible discovery of new strains and further reduction in vaccine effectiveness (45). However, studies conducted in Finland and Brazil have shown that there is no evidence yet supporting the hypothesis that RV vaccination leads to an increase of antigenic drift of circulating RV strains; it is more likely that the differences follow normal evolutionary trends (9-11).

Shedding of vaccine viruses has been evaluated in several studies and the results show that after immunization with RotaTeq®, 94% of children shed the virus some point during the first 28 days after the first dose of vaccination. 67% and 62% shed the virus after second and third dose, respectively. (46) The results are similar with Rotarix®: 94% and 53% shed the virus after first and second dosage, respectively. (46) Later, a Finnish study concluded that asymptomatic long-time shedding of viruses (up to 3 months after last vaccination) is not uncommon (47).

Formation and shedding of vaccine-derived double reassortant rotaviruses have been depicted (48,49), which led to propositions of potential genetic drift and generation of antigenically new assortments, thus decreasing the vaccine efficacy. It is plausible, that double reassortants could remain in circulation even longer than 1 transmission cycle leading to an increased probability of adverse events and possibility of infecting contacts especially in immunocompromised children (50). In addition to vaccine-vaccine reassortants, vaccine-derived rotaviruses have been observed to form double reassortants with wildtype rotavirus (15) increasing the potential number of VP4 and VP7 combinations. Further studies are needed to show the virulence of these strains. The nature of the shedding of vaccine strain viruses is both positive and negative. It might enforce vaccine coverage due to naïve exposure, but on the other hand it may predispose immunocompromised individuals to RVGE.

2.5 Rotavirus vaccine development

RV vaccine development started in the mid-1970's. The first vaccines were based on a Jennerian approach, when observations of a major common antigen between human and animal rotaviruses

were made (51). Next, the clinical studies with bovine rotavirus vaccine strains 4237 (G6P6[1]) and WC3 (G6P7[5]) were made (52,53), showing partial protection against the disease.

Clinical trials with animal derived rhesus rotavirus vaccines (RRV) were made in the late 1980's, which also showed only weak protection (54). A decade later, another rotavirus vaccine developed straight from an animal (lamb) rotavirus strain was developed and is still widely used in China, but shows only 44-51% effectiveness (55).

From early on, studies show that the predominating rotavirus strain may vary from year to year, which lead to a hypothesis that a polyvalent preparation might be necessary to provide effective vaccination against rotaviruses (54). Both WC3 and RRV vaccines were combined with human gene segments ("Modified Jennerian Approach") resulting in the development of RotaTeq® and RotaShield® vaccines. RRV-TV was an oral live tetravalent rhesus-human reassortant vaccine comprising three reassortant-viruses with the native strain (56). RotaShield® became the first licenced rotavirus vaccine in 1998 and showed a 90% reduction of severe rotavirus gastroenteritis and a 60% reduction of severe gastroenteritis of all causes (57). A year later, it was withdrawn from the market because of potential association between the vaccine and intussusception (58). However, a later study found no evidence of increased infant intussusception admissions during the period of RotaShield® availability (59).

Further development of reassortants based on WC3 strain was carried on by Merck (60). Several human-bovine reassortants were studied first with bivalent and tetravalent combinations (61,62) finally leading to the release of a pentavalent reassortant vaccine, RotaTeq®, in 2006. The viruses express combinations of G1P[5], G2P[5], G3P[5], G4P[5] and G6P[8]. Four viruses express the outer capsid protein from human parent strain (G1, G2, G3, and G4) and the VP4 from bovine antigen, whereas the fifth virus expresses the VP4 protein from human origin combined with bovine origin VP7 (63). The efficacy and safety trial showed that the vaccine protected 100% against severe rotavirus gastroenteritis (RVGE) and 73% against RVGE of any severity. No increased risk of intussusception was detected. (64) A follow-up study conducted in Finland showed that the vaccine shows significant protection also against G9 associated RVGEs despite not being one of the reassortants (65).

The other main vaccine used today, Rotarix™, was developed from a circulating human strain that was shown to produce broadly cross-reactive neutralising antibodies (66,67). The protection levels of the vaccine have been found to be high (over 84.7%) against severe RVGE and 58-90% against

RVGE of any severity independent of the genotype (68,69). In addition, no association with increased risk of intussusception was found (68).

The need for new RV vaccines is caused by the difference in RV epidemiology between developed and developing countries as it has been shown that the two main vaccines are not as efficient against RV disease in developing countries (70,71). Several other RV vaccines have been developed and licensed for national markets of developing countries, containing different RV genotypes than the main vaccines. Also, several vaccines are currently under development in Brazil, China, Finland, India, Indonesia, and the United States (72).

3. NEXT-GENERATION SEQUENCING

The need for new sequencing methods originated from the interest to sequence the human genome, which consumed too much time and effort with the old Sanger method. The term next-generation sequencing refers to a number of different techniques developed in the last 15-20 years. These include e.g. sequencing-by-synthesis, sequencing-by-ligation and ion semiconductor sequencing. While a Sanger reaction returns a single DNA sequence, a typical NGS run can yield up to a billion unique reads (73), which is made possible by running several samples parallel. Also, transcripts can be identified without prior knowledge of a particular gene and taking alternative splicing and sequence variation into account at the same time (74). Still the basic mechanism behind the technology is similar with the Sanger method: using fluorescently labelled bases to determine the order of the bases. Two most common products are the Sequencing series by Illumina and Ion Torrent by Thermo Fisher Scientific.

For further on, we'll discuss the basic ideology of sequencing-by-synthesis approach employed by Illumina devices. The procedure consists of three different steps: template preparation, sequencing and imaging, and genome alignment and assembly methods.

3.1 Template preparation

The method involves randomly breaking or precisely cutting genomic DNA into smaller sizes by physical fragmentation (i.e. Covaris), hydrodynamic shearing or enzymatic methods. The ends of the fragments are then repaired by phosphorylation and adenosine is added to 3' ends of the blunt DNA fragments to prevent formation of adapter dimers and concatemers. Adapters are then ligated

enzymatically. A PCR is performed to amplify the amount of DNA. From the fragments, either fragment templates or mate-pair templates are created. This is enabled by adding Illumina adapters, P5 and P7 tails, which contain a flowcell-binding site, a sequence primer site and an index sequence. The quality of the new library can be then controlled by fluorometric methods (e.g. Qubit).

High-density forward and reverse primers are covalently attached to flowcell (a glass slide), and the ratio of the primers to the template on the support defines the surface density of the amplified clusters. The amplification is composed of two basic steps: initial priming and extending of the single-stranded, single-molecule template, and bridge amplification of the immobilized template with immediately adjacent primers to form clusters. After bridge amplification, reverse strands are cleaved and washed off, leaving only the forward strands. The 3' prime ends are blocked to prevent unwanted priming. As a result, this solid-phase amplification can produce 100–200 million spatially separated template clusters, providing free ends to which a universal sequencing primer can be hybridized to initiate the NGS reaction. (73)

3.2 Sequencing and imaging

In NGS, all available free bases are modified. When polymerase incorporates a modified base into the copied strand, the clusters are excited by a light source and a characteristic fluorescent signal is emitted. The terminator and dye are cleaved from the attached nucleotide triphosphate to enable the next base to attach. The number of these cycles determines the length of the read. The complete formation of the pair strand is followed by a cleavage step, which removes the first sequencing product and index 1 primer is hybridized and introduced to the template. After completion of the index run, the 3' end of the template is deprotected to enable the template to fold and bind the second oligo on the flow cell. Index 2 is read in the same manner followed by copying of the template to form a double stranded bridge. The DNA is then linearized and the 3' prime ends blocked again. The original forward strand is then cleaved and washed away and sequencing for the reverse template is performed in the same manner as before. NGS uses positional separation. As the strands are fixed to the glass, a microscope is used to capture an image and the positions are taken into account as well as the color and the intensity of the fluorescence. At the end of the run the fluorescence color at each template position are mapped to a base.

3.3 Genome alignment and assembly methods

After NGS reads have been generated, they are separated by the index run results, and forward and reverse reads are paired to create continuous sequences. These are then aligned to a known reference sequence or assembled *de novo*. The number of reads that align at a given position of the genome is called the *depth* and the percentage of the targeted regions that are covered with at least one read is called the *coverage*. These values can be calculated beforehand to estimate and determine the number of runs and the performing method and kit needed in order to reach the desired results.

4. MATERIAL AND METHODS

4.1 RNA extraction

Viral RNA was extracted from stool samples using a Qiagen QIAamp viral RNA mini kit (Hilden, Germany) according to the manufacturer's instructions. Briefly, 10% stool suspensions were made in phosphate-buffered saline (PBS) and mixed with Buffer AVL-Carrier RNA and incubated at room temperature for 10 min. After spinning, the sample was purified by extraction with 99.5% ethanol twice. The filtrate was washed in two buffer mixes (Buffer AW1, Buffer AW2, 6000 x g (8000 rpm) for 1 min) before incubation with Buffer AVE and RNA dividing. A total of 60 μl of purified viral RNA was obtained and stored at -70°C until used in RT-PCR.

4.2 RT-PCRs

4.2.1 VP7

RT-PCR was the primary detection method for rotavirus in the study. RNA was first amplified by RT-PCR to produce a full-length copy of gene 9, encoding for VP7 glycoprotein, and further amplified in nested PCR with genotype-specific primers for variable regions of VP7, detecting genotypes G1, G2, G3, G4, G8, G9, and G12 (5,75).

Five microliters of extracted RNA with a 2 μl pool of primers Beg 9 fwd* (forward primer): 5'GGCTTTAAAAGAGAGAATTTCCGTCTGG3' (nucleotides 1-28) and End 9 rev* (reverse primer): 5'GGTCACATCATACAATTCTAATCTAAG3' (nucleotides 1062-1036) was denatured for 2 min at 94°C. Thereafter, 8μl of RT reaction mixture containing 1.8 μl Nuclease free water (Ambion), 1.2 μl of 25mM MgCl2 (Promega), 1.0 μl of 2.5 mM dNTP mix (containing 2.5 mM

each of dATP, dCTP, dGTP, and dTTP) (Promega), 3.0 μl 5XGreen GoTaq Flexi Buffer (Promega) 0.5 μl AMV RT-enzyme (Promega) and 0.5 μl RNasin (Promega) for each sample, was added to the sample-primer mixture and incubated for 60 min at 42°C. The first PCR mixture containing 20.6 μl Aqua sterilisata H_2O (Fresenius Kabi), 10.0 μl 5x Green GoTaq Flexi Buffer, 2.0 μl of 25mM MgCl2, 2.0 μl of 2.5mM dNTP mix, and 0.4 μl GoTaq DNA polymerase (Promega) for each sample was added into the RT-reaction. The first PCR mixture was denatured at 94°C for 3 min and run for 35 cycles of 20 sec at 94°C, 1 min at 56°C, 2 min at 72°C and a final extension of 5 min in 72°C. For the second PCR reaction, 48 μl of both H pool mix and C pool mix were added into 2 μl of the first PCR product and denatured for 3 min at 94°C, followed by 25 cycles of 15 sec at 94°C, 40 sec at 53°C, 1 min 10 sec at 72°C and a final extension of 5 min at 72°C. For one sample, both mixes contained 24.8 μl of Aqua sterilisata, 10.0 μl 5X Green GoTaq Flexi Buffer, 3.0 μl of 25 mM MgCl2, 4.0 μl of 2.5 mM dNTP mix and 0.2 μl of GoTaq DNA polymerase. For H pool mix, 6.0 μl of H pool primer mix was added into the second PCR mix, whereas in the C pool mix it was replaced by 6.0 μl of C pool primer mix. The PCR products from first PCR reaction and H pool and C pool reactions were run in a 2% agarose gel for 105 min at 100 V. Gel electrophoresis was used to recognize G-types different in size. Sizes of the amplicons are presented in Table 1.

Table 1. Lengths of H pool and C pool products of different rotavirus genotypes

	H pool product size (bp)	C pool product size (bp)
G1	618	298
G2	521	244
G3	682	672
G4	452	403
G8	754	161
G9	179	110
G12	387	529

4.2.2 VP4

For the determination of rotavirus P-types, an RT-PCR assay to amplify the VP4 glycoprotein was performed, detecting P-genotypes P[4], P[6] and P[8] (14,76). For RT-reaction and first amplification, 5 μl of extracted RNA with a 2 μl mixture of both primers VP4 fwd* (forward primer): 5'TATGCTCCAGTNAATTGG3' (nucleotides 132-149) and VP4 rev* (reverse primer):

5'ATTGCATTTCTTTCCATAATG3' (nucleotides 795-775) was incubated for 2 min at 94°C. Eight microliters of RT-PCR mix containing 1.6 µl of Nuclease free water, 1.5 µl of 10X PCR buffer II (Applied Biosystems), 1.2 µl of 25mM MgCl2 (Applied Biosystems), 1.2 µl of 2.5mM dNTP mix, 2.0 µl of AMV RT-enzyme 10 U/µl, and 0.5 µl of RNasin 40 U/µl was added into the primer-sample mixture and incubated for 45 min at 45°C followed by 2 min at 94°C with a hold at 8°C. For the first PCR reaction, a mixture containing 24.25 µl of Aqua sterilisata H20, 3.5 µl of 5X Green GoTaq Flexi Buffer, 4.2 µl of 25 mM MgCl2 (Promega), 2.8 µl of 2.5 mM dNTP mix and 0.25 µl of GoTaq DNA polymerase 5 U/µl was added into the RT-reaction mixture, denatured at 94°C for 3 min and run for 30 cycles of 20 sec at 94°C, 1 min at 50°C, and 1 min at 72°C following a 5 min hold at 72°C before cooling into 8°C. For the second PCR reaction, 2 µl from the first PCR product was mixed with 48 µl of second PCR mixture (28.6 µl of Aqua sterilisata, 10 µl of 5X Green GoTaq Flexi Buffer, 3 µl of 25 mM MgCl2 (Promega), 4 µl of 2.5 mM dNTP mix, 0.4 µl of GoTaq DNA polymerase 5 U/µl and 2 µl of P pool primer mixture) and denatured at 95°C for 2 min followed by 25 cycles of 35 sec at 94°C, 30 sec at 45°C, and 1 min 10 sec at 72°C, with a 5 min extension at 72°C. The PCR products from the first PCR and the second PCR reactions were then run in 1.5% agarose gel for 90 min at 100 V. The total length of the first PCR product encoding for VP4 is 664 bp. The sizes of the 2nd PCR amplicons are presented in Table 2.

Table 2. Lengths of 2nd PCR products of different rotavirus genotypes.

	2nd PCR product size (bp)
P[4]	289
P[6]	381
P[8]	151

4.3 Whole genome RT-PCR

Samples were denaturated by combining 5.0µl of sample RNA with 5.0 µl of Molecular Biograded Water (Sigma Aldrich). The PCR tube including the mixture was then heated for 2 minutes at 95°C in thermal cycler. For each sample, 40 µl of RT-PCR-mix was prepared on ice by combining 20.0µl Molecular Biograded H₂O, 10.0 µl of 5 x OneStep RT-PCR buffer (Qiagen), 2.0 µl of 10mM dNTP (Qiagen), 3.0 µl Rota FG (gene) fwd primer, 3.0 µl Rota FG (gene) rev primer and 2.0 µl OneStep RT-PCR enzyme mix (Qiagen). When using multiple primer pairs, the quantity of Molecular Biograded Water was reduced by the volume of the added

primers. The primers used are presented in Table 3 and were combined as follows; VP2, VP1+VP3, NSP1+NSP3+NSP4, VP6+NSP2+NSP5.

Table 3. Oligonucleotide primers for Rotavirus FG RT-PCR.

Name	sequence (5´-3´)	product size (approx.)
Rota FG VP1 fwd	GGCTATTAAAGCT**R**TACAATGGGGAAG	
Rota FG VP1 rev	GGTCACATCTAAGCG**Y**TCTAATCTTG	3302
Rota FG VP2 fwd	GGCTATTAAAGG**Y**TCAATGGCGTACAG	
Rota FG VP2 rev	GTCATATCTCCACA**R**TGGGGTTGG	2685
Rota FG VP3 fwd	GGCT**W**TTAAAGCA**R**TATTAGTAGTG	
Rota FG VP3 rev	GGTCACATCATGACTAGTGTG	2591
Rota FG VP6 fwd	GGCTTT**W**AAACGAAGTCTTC	
Rota FG VP6 rev	GGTCACATCCTCTCACT	1356
Rota FG NSP1 fwd	GGCTTTTTTTTATGAAAAGTCTTG	
Rota FG NSP1 rev	GGTCACATTTTATGCTGCC	1566
Rota FG NSP2 fwd	GGCTTTTAAAGCGTCTCAG	
Rota FG NSP2 rev	GGTCACATAAGCGCTTTC	1059
Rota FG NSP3 fwd	GGCTTTTAATGCTTTTCAGTG	
Rota FG NSP3 rev	ACATAACGCCCCTATAGC	1072
Rota FG NSP4 fwd	GGCTTTTAAAAGTTCTGTTCC	
Rota FG NSP4 rev	GG**WY**AC**RY**TAAGACC**R**TTCC	742
Rota FG NSP5 fwd	GGCTTTTAAAGCGCTACAG	
Rota FG NSP5 rev	GGTCACAAAACGGGAGT	667

RT-PCR mix was then pipetted to each tube containing 10µl of denaturated sample RNA, and a following PCR program was run: denaturation for 30 min at 45°C and 15 min at 95°C before being

run for 40 cycles of 45 sec at 94°C, 45 sec at 45°C, and 6 min at 68°C, with a final extension for 7 min at 72°C.

A 1.5% agarose gel was prepared with 1 x TAE buffer containing 0.08 µg/ml of ethidium bromide. 10 µl of the RT product was then combined with 2 µl of loading buffer and all 12 µl pipetted to the gel. The gel was run for 105 min at 100 V. The results were visualized under UV illumination. Positive samples were stored at -20^{0}C.

4.4 Gel purification

40 µl of the RT-PCR-product was combined with 8 µl of loading buffer. A fresh 2 % agarose gel with 1 x TAE buffer containing 0.08 µg/ml of ethidium bromide was prepared and each sample loaded into two wells. Agarose gel was then run for 90-120 min at 100V.

The cDNA fragments were visualized under UV illumination and excised from the gel with a sharp scalpel. The gel slices were weighed and 3 volumes of Buffer QC (QIAquick® Gel Extraction Kit, Qiagen) was added to 1 volume of gel and the mixture then incubated at 50^{0}C water bath until the gel slice had completely dissolved. If the fragments were under 500 bp, 1 volume of isopropanol was added to the sample. The samples were then applied to QIAquick spin columns with 2-ml collection tubes and centrifuged for 1 min at 12000 rpm. After the flow-through had been discarded, 0.5 ml of Buffer QC was added to the spin column and centrifuged for 1 min at 12000 rpm. Then, 0.75 ml of Buffer PE was added and let to stand in the column for 5 min before centrifuging for 1 min at 12000 rpm. After discarding the flow-through, the columns were centrifuged for an additional 1 min at 13200 rpm. cDNA was eluted into 30 µl of fresh Milli-Q H$_2$O by centrifuging for 1 min at 13200 rpm. The product was then stored at -20^{0}C.

4.5 Determination of cDNA concentration and sample pooling

The concentration of cDNA was determinated for every RV gene by using Qubit 2.0 Fluorometer (Life Technologies) and Qubit dsDNA HS Assay Kit as follows: Qubit Working Solution (WS) was prepared by diluting the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer. Two standard samples (#1 and #2) were made by combining 190 µl of WS and 10 µl of Qubit dsDNA HS Standard #1 and #2, respectively. For the samples, 2 µl of sample solution was added to 198 µl of WS and the mixture then incubated at RT for 2 min. The samples were run with Qubit 2.0

Fluorometer. The concentration was calculated by multiplying the numerical value given by the fluorometer with the ratio of total sample volume and the volume of sample added to the assay tube.

After determining the concentration of cDNA of each RV gene, samples were normalized to contain approximately the same amount of cDNA of each gene. Then each sample was diluted to same concentration of approximately 2.3 ng/µl.

4.6 Preparation of the NGS library

DNA was fragmented to 200 bp using Covaris S220 Focused-ultrasonicator according to manufacturer's protocol with the following settings: peak incident power of 175 W, duty factor 10%, 200 cycles per burst, treatment time of 120 seconds.

10 µl of sample was dispensed into each PCR tube. Template Preparation Master Mix was prepared by combining 2.0 µl of Template Preparation Buffer with 1.0 µl of Template Preparation Enzyme per sample. 3.0 µl of Master Mix was pipetted to each sample tube and mixed thoroughly. Tubes were then placed in a thermal cycler and the Template Preparation Reaction, 25 min at 22°C followed by 20 min at 55°C, was performed.

Library Synthesis Master Mix was prepared by combining 1.0 µl of Library Synthesis Buffer with 1.0 µl of Library Synthesis Enzyme per sample. These mixtures were kept on ice until used. 2.0 µl of the mixture was pipetted into each sample tube and mixed thoroughly. The tubes were then returned to the thermal cycler for Library Synthesis Reaction: 40 min at 22°C.

Library Amplification Mix was prepared by combining 25.0 µl of Library Amplification Buffer with 1.0 µl of Library Amplification Enzyme and 4.0 µl of Nuclease-Free H2O per sample. 30 µl of the mixture was added to each sample tube. Also 5.0 µl of Indexing Reagent was added to each sample tube and mixed thoroughly. The following PCR reaction is described in Table 4.

Table 4. Library Amplification Reaction PCR protocol.

Library Amplification Reaction			
	Temperature	Time	Number of Cycles
Extension & Cleavage	72°C	3 min	1
	85°C	2 min	1
Denaturation	98°C	2 min	1
Addition of Indexes	98°C	20 s	
	67°C	20 s	4
	72°C	40 s	
Library Amplification	98°C	20 s	
	72°C	50 s	6
	4°C	Hold	

4.7 Library processing for NGS

100 μl of AMPure XP reagent was mixed with a 100 μl aliquot of the pooled library and mixed thoroughly before incubating in RT for 5 min. The tubes were then pulse-spun, placed in a magnetic stand and let to stand for 2 min. The supernatant was discarded. 300 μl of 80% ethanol was added to the pellet. Each tube was then rotated 90 degrees and let to stand until all the beads came to halt and repeated 3 times. The supernatant was discarded and 300 μl of ethanol added. The rotations were done again and the supernatant removed. The tube was then pulse-spun and placed into magnetic stand and any residual ethanol removed after 2 min. The tubes were then incubated in a heat block at 37°C for 2-3 carefully not to over dry the pellets. The DNA was then eluted by re-suspending the beads with 50 μl of 1 x TE buffer, pH 8.0. The tubes were again pulse-spun, attached to the magnetic stand and let to stand for 2 min. The supernatant was carefully removed and transferred into a new tube.

The quality of the library was controlled with Fragment Analyzer (Advanced Analytical Technologies) according to the manufacturer's protocol using Standard Sensitivity NGS Fragment Analysis Kit.

For the next phase, molecular biology-grade 1.0 N NaOH and Tris-Cl 10mM, pH 8.5 with 0.1% Tween 20 and 0.2 N NaOH were prepared. The library was then diluted to loading concentration by combining 10.0 μl of pooled library with 590.0 μl of prechilled HT1. The mixture was then centrifuged at 280 x g for 1 min before incubating at 98°C for 5 min.

PhiX Control was first diluted to 4 nM by combining 10 nM PhiX library with 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20 and then denaturated by combining 5 µl of 4 nM PhiX with 5 µl of 0.2 N NaOH. The product was centrifuged at 280 x g for 1 min before incubating in RT for 5 min. Then 10 µl of denaturated library was combined with 990 µl of prechilled HT1, resulting in 1 ml of 20 pM PhiX library. This was again diluted to 12.5 pM by adding 225 µl of prechilled HT1 to 375 µl of 20 pM denaturated PhiX library.

As our library was of low diversity, the PhiX control spike-in was increased to 10%. For that, 540 µl of denaturated library was combined with 60 µl of denaturated PhiX.

The reagent cartridge was placed in a RT water bath until the reagents were completely thawed. The block was then prepared according to the manufacturer's instructions. 600 µl of prepared libraries were pipetted into the cartridge. The cartridge was then inserted into MiSeq, which was run according to manufacturer's instructions.

4.8 Nucleotide sequencing and sequence data analysis

A 260-cycle paired-end read sequencing run was carried out on a MiSeq desktop sequencer (Illumina, San Diego, California, USA) using the MiSeq Nano Reagent Kit v2 (500 cycles). Following preliminary analysis, the MiSeq reporter program was used to generate FASTQ formatted sequence data for each sample. Sequence data was analyzed using VirusTAP (77). Contigs were assembled from obtained sequence reads by *de novo* assembly. Assembled contig sequences were compared to previously published sequences from the nucleotide database in GenBank, using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

5. RESULTS

The samples used in the study were picked arbitrarily. We qualified six samples that were previously detected as double-reassortants or deviated from the commonly seen strains or were totally new strains in Finland. One of the six samples was a double-assortant G1P[8] from an epidemiological study conducted in 2009 – 2011 at the Tampere University Hospital (48). The other 5 samples were collected in a national rotavirus surveillance study conducted in 2012 – 2015 (47). Three of these samples had a genotype of G8P[8], one of G9P[6] and one of G5P[6]. All of the samples were collected from children aged between two months to seven years. Three of these

children were known to be vaccinated against rotavirus and one had not been vaccinated. The vaccination status of the remaining two is unclear.

The genomes of all of the six samples were successfully sequenced. The sequencing results are shown in Table 5.

Table 5. The Whole Genome Classification of the Rotavirus Strains Characterized in this Study

Study strains	Genome constellations										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
#1	RotaTeqG1	RotaTeqP[8]	I2	R2	C2	M2	A3	N2	T6	E2	H3
#2	G8	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
#3	G8	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
#4	G8	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
#5	G9	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
#6	G5	P[6]	I5	R1	C1	M1	A8	N1	T7	E1	H1

Compared with previously known constellations, the RotaTeq G1P[8] strain (sample 1) shows a bovine backbone combined with RotaTeq vaccine-originated VP7 and VP4 (78). Earlier studies have shown a similar G8P[8] constellation (samples 2-4) in Malawi and the Democratic Republic of Congo (79,80). Also a very similar constellation of G5P[6] (sample 6) has been found in Japan (81), the only difference is located in NSP3 gene.

Literature only describes G9P[6] viruses with a pure DS-1-like backbone or a backbone reassorted with Wa-like genome segments (82,83). To our knowing the genome constellation of G9P[6] found in our study (sample 5) is described for the first time.

6. DISCUSSION

In this study, we set up an NGS protocol for sequencing of the whole genome of rotavirus for the first time in Finland. As the set up was successful, we also managed to get the whole genome sequences of the six rotavirus samples.

NGS offers an almost unlimited opportunity to examine samples but on the contrary also produces massive quantities of data, which makes data-analysis procedures compulsory. The mechanism of NGS is rather complicated and elusive, which sets a challenge for inexperienced researchers. Also, for the reasons listed, we decided to control the data outcome in this study beforehand by choosing a limited number of samples. By this mean, we managed to keep the amount of output data manageable.

As a conclusion, NGS is considerably faster compared to the Sanger method but also very difficult, because of the processing of each gene individually. Because of this, it is good to keep looking for alternative ways to clarify, simplify and fasten the process.

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