MARIA HEMMING

Rotavirus Infections in Children

Clinical features and effects of large scale prevention by rotavirus vaccination

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ACADEMIC DISSERTATION
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Abstract

Rotaviruses (RVs) are the most common causative agents of acute gastroenteritis (AGE) in young children worldwide. Before the launch of rotavirus vaccines, rotavirus infections caused an estimated 2400 hospital admissions and 3600 outpatient clinic visits among young children in Finland every year. In 2006, two live oral RV vaccines were licensed. As of 1 September 2009, bovine-human RV vaccine RotaTeq® was added to the National Immunization Programme (NIP) of Finland. A major part of the present study focuses on the impact of universal rotavirus vaccination on hospital admissions for AGE and its possible effects on circulating wild-type RV strains and on the appearance of vaccine-originated RV strains.

Rotavirus causes an intestinal infection, the symptoms of which include diarrhea, vomiting, and fever. In 2002 it was discovered that many RV AGE patients also have RV antigen in their blood. To study the association between systemic spread of the virus and clinical severity of the disease, stool, serum, and whole- blood samples from 155 children hospitalized for RV AGE during the pre-vaccination years 2006-2008 were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). RV RNAemia and antigenemia were found to be common events in RV AGE patients, occurring in 67% and 61% of the cases, respectively. Children positive for RV RNA and RV antigen in both serum and stools were more likely to have a higher level of fever and more severe vomiting than children with RV detected only in stools. These data indicate that the clinical severity of RV disease is related to extraintestinal spread of the virus.

To determine the effect of universal mass vaccination on hospital admissions and outpatient clinic visits for rotavirus gastroenteritis (RVGE), results from two similar epidemiological studies using the same methodology were analyzed. Material for the first study was collected from pre-vaccination (pre-NIP) years 2006-2008 and the other from post-vaccination (post-NIP) years 2009-2011. In both studies, children seen for AGE in the outpatient clinic or emergency department were enrolled in the study. In the pre-NIP period 809 children with available stool specimens were recruited, whereas in the post-NIP period only 330 patients were recruited from the same population (59% decrease). In the pre-NIP period, RVs accounted for 52% of all cases of AGE, which dropped to 26% in the post-NIP years, giving an 80% overall reduction in RVGE cases. Hospital admissions for RVGE were reduced by 76% and outpatient clinic visits by 81%. The large drop in RVGE cases was accompanied by a relative

increase in cases of norovirus (NoV) AGE. Although the absolute number of NoV cases remained fairly stable, the proportion of cases in which NoV was the causative agent in children hospitalized for AGE rose to 37%, and NoV became the leading cause of AGE in children seen in hospital.

Previous studies have suggested that large-scale use of monovalent G1P[8] human rotavirus vaccine may have an effect on the prevalent G1 VP7 subtype in wild-type RVs. We investigated whether the use of RotaTeq® vaccine would have any such effects on the most common circulating rotavirus genotype G1P[8]. As RotaTeq® has human-bovine reassortants containing both VP7 protein G1 and VP4 protein P[8] of human origin, we followed the changes in the sequences of those two outer layer proteins (VP7 and VP8*) at nucleotide and amino acid level over a 20-year period from 1992 to 2012. The study material included G1P[8] strains from pre-vaccination years 1992-1994 and 2002-2004, strains from 2006-2008 when rotavirus vaccine (mainly Rotarix™) was available, and the post-NIP years 2009-2012. For G1 VP7 the circulating rotaviruses were divided into two sublineages, both present in each study year. For VP8* protein of G1P[8] strains we observed periodical fluctuation of sublineages over the 20-year period, with multiple changes at both nucleotide and amino acid levels. The fluctuation of sublineages was not correlated to the use of the RotaTeq® vaccine, but did have a temporal correlation with the use of Rotarix™ in 2006-2008. These observations indicate that the universal immunization with RotaTeq® vaccine has not affected the circulating wild-type G1P[8] rotavirus strains.

Overall, the G- (and P-type) distribution of wild-type rotavirus strains has not changed much after this introduction of universal rotavirus vaccination. It is of interest, however, that the few breakthrough infections in vaccinated children that have been detected seem to be associated with RV genotypes other than G1P[8].

Virus strains from live-attenuated RV vaccine are shed in the stools. Usually shedding is symptomless, but diarrhea has been reported in 1-2% of infants receiving human-bovine reassortant vaccine. We studied the presence of RV vaccine virus in children hospitalized for AGE. Stool samples from all cases of RV positive AGE in the post-NIP years 2009-2011 and 2012-2013 were analyzed by sequencing the two outer layer proteins VP7 and VP4, as well as the middle layer protein VP6, to determine the origin of the virus. If the VP7, VP4 and/or VP6 proteins were identical to the cognate gene segments from RotaTeq® vaccine, the stool samples were tested for the presence of rotavirus antigen by ELISA, and, if positive, were propagated in MA104 cells. Final RT-PCR and sequencing were performed on the viruses extracted from the cell cultivation to confirm the stability of the new virus. In the first post-vaccination years 2009-2011 we found three recently vaccinated children with symptoms of acute GE due to a new vaccine-derived human-bovine double reassortant rotavirus. In the 2012-2013 season we detected the vaccine-derived double reassortant in an unvaccinated 7-

year-old girl with AGE. Cultivation in MA-104 cells confirmed the virus to be a new double-reassortant rotavirus G1P[8] between two vaccine strains G1P7[5] and G6P[8]. These data indicate that the vaccine-derived double-reassortant G1P[8] may be formed in RotaTeq®-vaccinated infants and may occasionally cause gastroenteritis symptoms. The virus remains stable in the environment for more than one transmission cycle, and may potentially infect unvaccinated contacts. In addition, we commonly found original human-bovine reassortants in the stools of AGE patients, but in all such cases another gastroenteritis virus (norovirus) was present at the same time. We conclude that shedding of vaccine virus is not associated with clinical symptoms.

Taken together, the vaccine-associated issues do not offset the value of the universal rotavirus vaccination program in Finland, which has greatly reduced the burden of AGE in children. Moreover, intussusception, which is a well-known albeit rare complication of rotavirus vaccination, has not occurred in connection with the rotavirus NIP in Finland.

Tiivistelmä

Rotavirukset (RV) ovat maailmanlaajuisesti yleisimpiä pienten lasten vakavan ripulitaudin (gastroenteriitti) aiheuttajia. Ennen rotavirusrokotuksia lasten rotavirusinfektiot aiheuttivat Suomessa vuosittain arviolta 2400 sairaala- ja 3600 poliklinikkakäyntiä pienillä lapsilla. Kaksi elävää suun kautta annettavaa rotavirusrokotetta sai myyntiluvan vuonna 2006. Vasikan ja ihmisen rotavirusten yhdistelmistä (reassortanteista) muodostettu rokote RotaTeq® lisättiin Suomen kansalliseen rokotusohjelmaan 1. syyskuuta 2009. Tutkimuksesta suuri osa käsittelee kansallisen rokotusohjelman vaikutuksia akuuttien gastroenteriittien (AGE) aiheuttamiin sairaalakäynteihin sekä rotavirusrokotteen mahdollisia vaikutuksia luonnossa kiertäviin villityypin rotaviruksiin sekä rokotteesta peräisin olevien virusten aiheuttamiin oireisiin.

Rotavirusten aiheuttama ripulitauti on lapsille vakava tauti, joka voi runsaan oksentelun, ripuloinnin sekä kuumeilun kautta johtaa vaikeaan kuivumaan ja hoitamattomana kuolemaan. Rotavirukset infektoivat ensisijaisesti suolistoa, mutta ne voivat edetä verenkiertoon aiheuttaen systeemisen infektion. Systeemisen infektion yhteyttä kliinisen taudinkuvan vakavuuteen tutkittiin analysoimalla uloste-, seerumi- ja kokoverinäytteet 155 rotavirusgastroenteriitin vuoksi sairaalaan joutuneelta lapselta PCR-menetelmällä rotaviruksen RNA:n ja ELISAlla rotaviruksen antigeenin osoittamiseksi. Tutkimuksessa havaittiin 67%:lla lapsista samanaikainen RV RNAemia ja 61%:lla antigenemia. Niillä lapsilla joilla RV RNA:ta ja antigeenia löytyi seerumista, oli todennäköisemmin korkeampi kuume ja voimakkaampaa oksentelua, kuin lapsilla joilla rotavirusta löytyi pelkästään ulosteista. Vaikka RV:n leviäminen suoliston ulkopuolelle näyttää liittyvän vaikeampaan taudinkuvaan, ei varmuudella tiedetä lisääntyykö RV muuallakin kuin suolistossa.

Rotavirusrokotteen vaikutusten arvioimiseksi verrattiin AGE:n vuoksi tehtyjä sairaalakäyntejä Tampereen yliopistollisessa sairaalassa ennen rokotuksia vuosina 2006–2008 aikana tehdyn tutkimuksen ja rokotusten aloittamisen jälkeen vuosina 2009–2011 tehtyyn tutkimukseen. Tutkimusten potilasvalinta ja tutkimusmenetelmät olivat identtiset. Vuosina 2006-2008 tehtyyn tutkimukseen osallistui 809 lasta kun taas rokotteen käyttöönoton jälkeiseen tutkimukseen osallistui 330 lasta. Ennen rokotuksia rotavirukset aiheuttivat 52% kaikista AGE tapauksista ja rokotusten jälkeisinä vuosina ainoastaan 26% (80% vähenemä). Rotavirusrokote vähensi rotaviruksen vuoksi tehtyjä sairaalahoitoja 76% ja poliklinikkakäyntejä 81%. Kaikkien AGE tapausten määrä väheni

Tampereen yliopistollisessa sairaalassa 59%. Rotavirustapausten määrän väheneminen johti noroviruksen nousuun yleisimmäksi taudinaiheuttajaksi pienillä lapsilla. Norovirus aiheutti 37% sairaalahoitoisista AGE tapauksista rokotusten jälkeisinä vuosina.

Laajojen rotavirusrokotusten on arveltu voivan aiheuttaa immunologista painetta luonnossa kiertäviin villityypin rotaviruksiin. Tätä tutkittiin seuraamalla yleisimmän rotavirus genotyypin G1P[8] kahden uloimman proteiinin VP7 ja VP8* nukleiinihapposekvenssejä ja aminohappojärjestystä 20-vuoden ajalta eli ennen rokotuksia ja rokotusten jälkeen. VP7 ja VP8* proteiinien tutkiminen osoitti, että näiden antigeenisilla alueilla tapahtuu kausittaista vaihtelua, johon RotaTeq® rokotteella ei kuitenkaan ole ollut todettavaa vaikutusta. RotaTeq® rokotteen käytöllä ei havaittu olevan vaikutusta G1P[8] RV genotyypin ilmiasuun ja G1P[8] säilyi vallitsevana RV genotyyppinä myös rokotusten jälkeisinä vuosina. Mielenkiintoisena havaintona kuitenkin oli että harvoissa todetuissa läpimurtoinfektioissa (AGE tapauksia) aiheuttajina olivat muut genotyypit kuin G1P[8].

Aikaisemmin on havaittu, että elävällä rotavirusrokotteella rokotetut lapset erittävät usein rokoteviruksia ulosteisiin. Tutkiessamme gastroenteriittiin sairastuneita lapsia rokotteen käyttöönoton jälkeisiltä vuosilta löysimme RT-PCR menetelmällä uuden rokotteesta peräisin olevan yhdistelmäviruksen neljältä rokotetulta ja yhdeltä rokottamattomalta lapselta. Sekvensoimalla rokoteperäinen G1P[8] rotavirus, huomattiin sen olevan uusi yhdistelmä kahdesta RotaTeq® rokotteen sisältämästä viruksesta G1P[5] ja G6P[8], jossa vasikan rotaviruksen sisäkuoren (VP6) pinnalla onkin kaksi ihmisen rotaviruksen pinta-antigeenia. Rokotteesta peräisin oleva yhdistelmävirus G1P[8] osoittautui stabiiliksi soluviljelyssä, minkä perusteella sen voi olettaa olevan stabiili myös luonnossa. Kaksoisyhdistelmävirus voi aiheuttaa gastroenteriitin oireita rokotetuilla lapsilla ja se voi myös tartuttaa rokottamattomia lapsia.

Kokonaisuutena rotavirusrokotuksen todetut haittavaikutukset ovat vähäisiä suhteutettuna rokotusten antamaan suureen hyötyyn. Rotavirusten molekyyliepidemiologiaa on kuitenkin syytä seurata jatkossa varsinkin kun tiedetään, että rokotevirus ja villityypin virus voivat vaihtaa genomijaokkeita odottamattomalla tavalla. Rotavirusrokotuksiin liittyvää harvinaista komplikaatiota, suolistoinvaginaatiota, ei ole esiintynyt Suomen nykyisen rokotusohjelman aikana, mutta tätäkin on jatkossa seurattava. Rotavirusten hyödyt näyttävät jatkuvan tämän työn seurannan jälkeenkin; rotavirusten aiheuttamat AGE:t ovat olleet harvinaisia ainakin 4 vuotta rokotusten aloittamisen jälkeen. Rotavirusrokotus ei kuitenkaan kykene juurimaan villityypin rotavirusta.

List of original publications

The thesis is based on the following publications, which are referred to in the text by their Roman numerals. The original articles are reprinted with the permission of their copyright holders.

I. Hemming M, Huhti L, Räsänen S, Salminen M, Vesikari T. Rotavirus antigenemia in children is associated with more severe clinical manifestations of acute gastroenteritis. Pediatr Infect Dis J. 2014 Apr; 33(4):366-71

II. Hemming M, Räsänen S, Huhti L, Paloniemi M, Salminen M, Vesikari T. Major reduction of rotavirus, but not norovirus, gastroenteritis in children seen in hospital after the introduction of RotaTeq vaccine into the National Immunization Programme in Finland. Eur J Pediatr. 2013 Jun; 172(6):739-46

III. Hemming M, Vesikari T. Genetic diversity of G1P[8] rotavirus VP7 and VP8* antigens in Finland over a 20-year period: No evidence for selection pressure by universal mass vaccination with RotaTeq® vaccine. Infect Genet Evol. 2013 Oct;19C:51-58

IV. Hemming M, Vesikari T. Vaccine-derived human-bovine double reassortant rotavirus in infants with acute gastroenteritis. Pediatr Infect Dis J. 2012 Sep31;(9):992-4

V. Hemming M, Vesikari T. Detection of RotaTeq® vaccine-derived double reassortant rotavirus in a 7-year-old child with acute gastroenteritis. Pediatr Infect Dis J. 2013 Dec 9.

Abbreviations

AGE	Acute gastroenteritis
Bp	Base pair
CC	Cell culture
CPE	Cytopathic effect
DLP	Double-layered particle
ED	Emergency department
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ER	Emergency room
IFN	Interferon
IS	Intussusception
MEM	Minimum essential media
NIP	National Immunization Programme
NoV	Norovirus
NSP	Non-structural protein
ORS	Oral rehydration solution
PCV	Porcine circovirus
RT	Room temperature
RT-PCR	Reverse-transcriptase polymerase chain reaction
RV	Rotavirus
RVGE	Rotavirus gastroenteritis
TLP	Triple-layered particle
UMV	Universal mass vaccination
vdG1P[8]	Vaccine-derived double-reassortant G1P[8] rotavirus
VP	Viral protein
5-HT	5-hydroxytryptamine

Contents

1 Review of the literature

1.1 Introduction

Rotaviruses (RVs) were first discovered in 1973 when Bishop et al. studied intestinal biopsies of children with acute gastroenteritis (AGE) in electron microscopy (1). Soon after, rotavirus particles were also found in stool and given the name "rotavirus" because the viral particle looked like a wheel (Latin: *rota*) when visualized in electron microscopy (2).

After the initial finding, rotaviruses were observed to be responsible for the majority of cases of acute gastroenteritis requiring hospital admission in children below the age of 5 all around the world, and to be associated with high mortality rates in developing countries (3-8). In the early 1980s, rotaviruses were estimated to cause over 870 000 deaths annually (9). Globally, almost every child is infected during the first 5 years of life (10,11). Rotavirus disease is typically associated with vomiting and fever, followed by profuse watery diarrhea, and the main cause of death is dehydration. The most important therapy is oral or intravenous rehydration. While oral rehydration therapy has reduced mortality, by the time of the first rotavirus vaccine was licensed in 2005 there still were about 450 000 deaths due to rotavirus gastroenteritis (RVGE) around the world. (12)

1.1.1 Rotavirus structure

Rotaviruses are non-enveloped double-stranded RNA viruses, with a segmented genome (13). Each of the 11 gene segments codes for a single protein except for segment 11, which codes for two different non-structural proteins (Table 1). Six of the gene segments encodes viral structural proteins (VP1-4, VP6-7) which are incorporated into the virion, and five encode non-structural proteins (NSP1-6) (gene segment 11 encodes both NSP5 and NSP6). (14)

The infectious rotavirus particle is a virion formed of three concentric layers. The outer capsid of the virus consists of two proteins, VP7 and VP4, both of which induce neutralizing antibodies (15-17). The majority of the outer capsid is formed of the VP7 proteins which form the shell around the virion (Fig. 1) (16). The VP4 proteins form protease-activated spikes which project outward from the capsid for attachment. In the

intestines, the presence of trypsin-like proteases yields to cleavage of the VP4 protein into two polypeptides VP8* and VP5*(18,19). The VP8* forms the head of the VP4 spike, whereas the VP5* forms the stalk and base of the protein (20). Both proteins contain sequential neutralizing epitopes and surface-exposed neutralizing epitopes (discussed later) (17,21-24).

The inner capsid/middle layer of the virus is comprised of VP6 protein in icosahedral symmetry. VP6 is the most conserved, abundant, and immunogenic protein of rotavirus. VP6 protein induces heterotypic cross-protective immunity by eliciting T cell (CD4+) responses and circulating IgA antibodies which neutralize the virus by intracellular action. (25-28)

The virion core consists of viral proteins VP1-VP3 and dsRNA (13,29,30). The innermost layer of the virus is formed of VP2 proteins, to which the VP1 and VP3 proteins are attached from the inferior side (Fig. 1) (13). The VP1 is a viral RNA-dependent RNA polymerase enzyme, whereas VP3 is an RNA capping enzyme (29,30).

The non-structural proteins NSP1-NSP6 are essential for rotavirus replication because they modify the cell functions to enable the release of new virions from the infected cells (31). NSP4 was the first viral enterotoxin to be described (32). In addition to its role in rotavirus morphogenesis, NSP4 has been shown to induce diarrhea by action on Cl^- and Ca^{2+} channels and to induce immune responses (32-34). NSP1 acts as an IFN antagonist whereas NSP3 shutdowns the cellular protein synthesis (35,36).

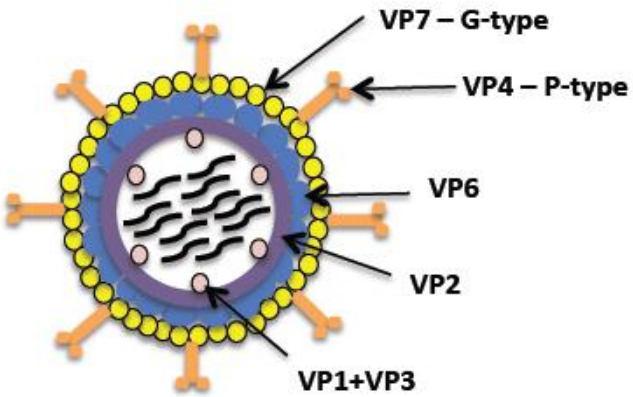

Figure 1. Rotavirus structure

RNA gene segment	Protein	Size (bp)	Molecular weight (kDA)	Copies/ particle	Location	Function
1	VP1	3302	125	<25	innermost layer/virion core	mRNA transcription (polymerase complex with VP3)
2	VP2	2690	102	120	innermost layer/virion core	activate VP1 and VP3 - sdRNA synthesis, non-neutralizing antigen
3	VP3	2591	88	<25	innermost layer/virion core	mRNA transcription (polymerase complex with VP1)
4	VP4 (VP5*+VP8*)	2362	87	120	outer layer/capsid	cell attachment, neutralizating antigen
5	NSP1	1611	59	0	nonstructural	interferon antagonist
6	VP6	1356	45	780	inner layer/capsid	non-neutralizing antigen (main target of IgA)
7	NSP3	1104	37	0	nonstructural	shutdowns cellular protein synthesis
8	NSP2	1059	35	0	nonstructural	forms viroplasm together with NSP5, non-neutralizing antigen
9	VP7	1062	37	780	outer layer/capsid	neutralizating antigen
10	NSP4	751	20	0	nonstructural	enterotoxin, outer capsid assembly, non-neutralizing antigen
11	NSP5+NSP6	667	22	0	nonstructural	forms viroplasm together with NSP2

Table 1. Rotavirus RNA gene segments and their location and function

1.1.2 Rotavirus classification and nomenclature

Groups

Rotaviruses belong to the *Reoviridae* family and are subdivided into different groups based on the amino acid sequences of their VP6 protein (14,37). So far, infectious serogroups from A to H have been recognized as infecting various species (37). However, only groups A-C and H can infect humans, group A viruses being responsible for over 90% of all infections (14). Groups D-G rotaviruses circulate in avian species (38,39).

Rotaviruses may be classified according to their VP7 and VP4 antigenic properties into G- and P-types (discussed below), and into different subgroups according to their VP6. VP6 subgroups (SG) can be referred to as SG-I, SG-II, SG-I/II, and non SG-I/II, depending on the presence or absence of subgroup-specific epitopes. (40-43)

G- and P- types

As already mentioned, rotaviruses are most commonly classified by their two outer capsid proteins VP7 and VP4 into G-types and P-types. The G- and P-genotyping system is based on reverse transcription polymerase chain reaction (RT-PCR), where different genotypes may be recognized by their length and further sequenced (44,45). At the moment, 27 G-genotypes (from G1 to G27) and 35 P-types (from P[1] to P[35]) have been described. (46)

Different G- and P-types are further subdivided into different phylogenetic sublineage groups by their aligning in phylogenetic trees after nucleotide or amino acid sequencing.

Gene Constellation – New nomenclature

Rotaviruses may also be classified by their whole genome, where genome segments for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 are represented by the acronym Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (where x= an Arabic numeral ≤1) (46). Each of the nine other internal gene segments (other than G- and P-typing gene segments) have more than 8 genotype alternatives.

Sequencing of the full genome has revealed that the internal gene segments of the most common genotypes with P[8] P-type (G1P[8], G3P[8], G4P[8], and G9P[8]) usually belong to genogroup 1, whereas the internal gene segments from G2P[4] strains belong to genogroup 2 (47-50). In addition, phylogenetic analyses have revealed that the human genogroup 1 rotaviruses have developed from the same origin as

porcine rotaviruses, whereas the genogroup 2 viruses have a link to rotavirus strains of bovine origin (50).

1.1.3 Rotavirus genotypes in Finland and around the world

Around the world, the most common circulating genotypes are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (51), which accounted for 86-99% of rotavirus infections in European countries before the launch of rotavirus vaccines. Of all rotavirus detections worldwide, the G1P[8] genotype is the most common, followed by the G4P[8]. Fluctuations in the most predominant genotype are relatively common (such as from G1P[8] to G4P[8]), but the shift commonly occurs within those common genotypes. (52-61)

In Finland, before the universal mass vaccination, the most predominant rotavirus genotypes were G1P[8] (62%) and G9P[8] (12%). Other common genotypes G4P[8], G2P[4], and G3P[8] were observed to a lesser extent (9.5%, 7.8% and 3.6%, respectively). (62) Similarly to the genotype distribution of Europe and Latin America, G1P[8] accounted for 78.5% of all rotavirus infections in the United States between 1996 and 2005, followed by G2P[4], G9P[8] and G3P[8] (9.2%, 3.6%, and 1.7%, respectively) (63,64). In developing countries, the same common genotypes account for the majority of the infections. However, more uncommon genotypes (such as G12 and G8) and different G- and P-type constellations (such as G12P[6] or G2P[8]) are detected relatively more often than in developed countries (65-69). (Fig. 2)

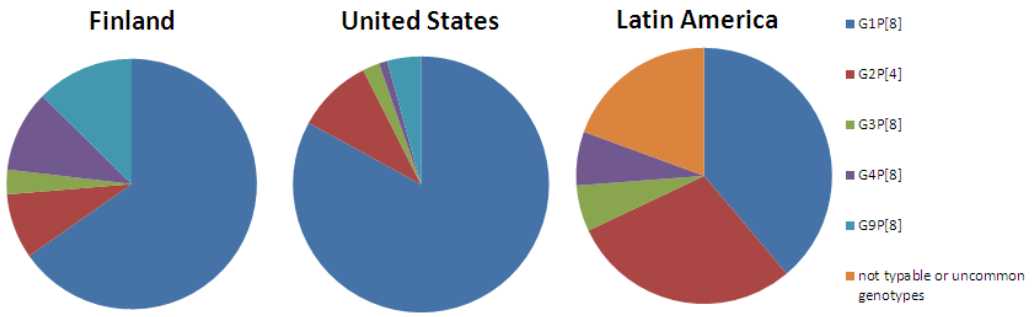

Figure 2. Rotavirus genotypes in Finland, U.S. and Latin America before the universal mass vaccination.

1.2 Rotavirus epidemiology and burden of disease

1.2.1 Epidemiology

Before rotavirus vaccinations in 2000-2004, rotavirus infections were estimated to cause over 500,000 deaths annually worldwide (70). The majority of rotavirus related deaths occur in developing countries (especially in India and Africa), where access to health care is limited (70).

Each child is normally infected at least once before the age of five, the majority of them before 2-years of age (10,11). Neonatal and adult RV infections are more uncommon and often asymptomatic (71,72).

In regions with a temperate climate, such as Europe, RV has a clear seasonal distribution, the most active months being in the winter and/or early spring (73). In subtropical and tropical climates the distribution of RV disease is not as clear as in Europe; the most active months are during the cool and dry season, but sporadic infections may be detected during the whole year (74,75).

1.2.2 Disease mechanisms and clinical picture

1.2.2.1 Transmission and Pathology

Rotaviruses are highly infectious and may be transmitted via the fecal-oral route, in respiratory droplets or via fecally contaminated water (76-78). Infected children start shedding rotaviruses in their stools before the onset of symptoms and may excrete more than 10^{10} or 10^{11} rotavirus particles per gram of feces; fewer than 100 particles are required to infect new contacts (79-82). The risk of transmitting rotavirus can be lowered by frequent hand washing and treating contamined materials in high temperatures (over +50°C). The virus can be inactivated by several disinfectants, especially 95% ethanol, which exerts its effect by removing the outer-most layer. (83,84)

Viral replication

After ingestion, rotaviruses infect the mature enterocytes at the tip of the villi of the small intestine and replicate in the cell cytoplasm (77). The triple-layered rotavirus particles (TLPs) are transcriptionally-inactive and are attached to the cell membrane by

VP8* (located in the tip of the VP4 spike) after trypsin cleavage of VP4 into VP8* and VP5*. The virion is delivered to an early endosome via endocytosis, where reduced calcium concentrations lead to uncoating of the virion from VP7 proteins and induce membrane penetration by VP5*, resulting in a transcriptionally-active double-layered rotavirus particle (DLP) in the cytosol (85-87). Immediately after the removal of the outer capsid and release to the cytosol, VP1 and VP3, polymerase complexes located on the inferior side of the innermost layer, start to transcribe mRNAs from the 11 gene segments (88-93). RNA transcription from DLPs occurs at the base of the Type I channel located in the five-fold vertices of the VP6 layer. The minus-strands of the dsRNA genome segments are used as templates for (+)RNAs, which are further used as mRNAs for viral protein synthesis by cellular ribosomes or as templates for (-)RNAs during genome replication (94). The capped mRNAs are further extruded from the DLPs via the Type I channels in the five-fold vertices and translated in large inclusions formed from NSP2 and NSP5 in the viroplasm (95). Viroplasm-associated (+)RNAs are further packaged into VP2 cores, which activate VP1 (and possibly VP3) to initiate dsRNA synthesis to form the nascent core (96). At the same time, NSP2 and NSP5 interact and regulate the assembly of different proteins to control the assembly of the structural proteins (97-101). In addition, the VP7 proteins are gathered directly to the endoplasmic reticulum to prevent too-early formation of TLPs (102).

The DLPs are formed after VP6 particles attach to the nascent core (103). For the triple-layered structure, DLPs form complexes with NSP4 and VP4 at the cytosolic site of the endoplasmic reticulum (ER) and these penetrate further through the ER membrane, which is coated with VP7 proteins (104,105). Thereafter, the ER membrane is removed and VP7 collects on the particle, creating a TLP (106). TLPs may be released from infected cells by several mechanisms at least, including direct lysis, secretion from the apical cell surface, and via lysosomes (107,108). (Fig. 3)

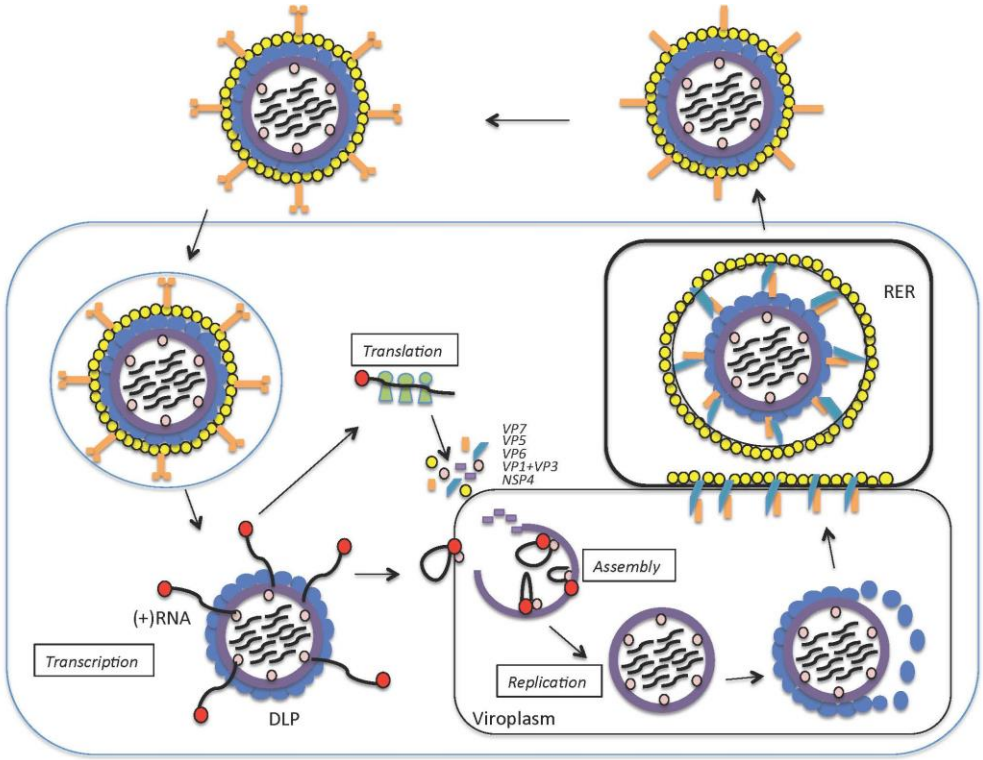

Figure 3. Rotavirus replication cycle in target cell. DLP=double-layered particle, RER= rough endoplasmic reticulum membrane.

Extraintestinal spread

Rotaviruses may be spread from the intestines into the circulation, as rotavirus RNA and RV antigen (VP6) have been detected in blood (serum) of infected children (109). Rotavirus RNAemia and antigenemia are common events, occurring in respectively 58-72% and 33-90% of RV infected children (109-114). In addition, extraintestinal spread of rotavirus into cerebrospinal fluid has been described in case reports (115,116), and RV RNA has been detected in multiple extraintestinal organs, such as heart, kidney, spleen, testes, bladder, and liver (117-120). Although the presence of RV RNA and antigen in serum implies the presence of infectious RV particles in blood, the isolation and culture of infectious RV particles from human serum has been unsuccessful, possibly because of the presence of serum inhibiting factors and too few infectious viral particles (121,122). However, in a study by Blutt and co-workers, the detection of antigenemia was found to be directly related to the presence of viral particles in serum

as the infectious rotavirus particles were detected in HT-29 cells using a modified virus isolation technique and immunofluorescence staining (123).

1.2.2.2 Symptoms

Children infected by rotavirus may shed the viruses in their stools for several days before the onset of symptoms (124). The clinical symptoms of primary rotavirus infection most commonly start after a 48-hour incubation period with forceful vomiting followed by fever and diarrhea lasting from 4 to 7 days (125). Elevated transaminase levels (126,127), complicated diseases possibly leading into seizures (128,129), and encephalitis (130,131) have been detected in rotavirus-infected children.

Diarrhea

The most typical sign of rotavirus infection, diarrhea, may be caused by several mechanisms; it may be osmotic, secretory and/or exudative (132) (133).

RV diarrhea has commonly been explained by damage to the epithelial cell line and by changes in the intracellular Ca^{2+} concentration. After infection of mature enterocytes, the intracellular Ca^{2+} concentration increases (probably with the involvement of NSP4) to enable viral replication. The increased Ca^{2+} concentration leads to inhibition of Na^{+} cotransporters, and together these reduce the absorptive capacity of the intestinal epithelium. The unabsorbed organic molecules increase the osmolality of the intestinal contents and absorb water from the epithelium, causing osmotic diarrhea. However, recent studies have shown the NSP4 protein to be functionally responsible for increasing the cytoplasmic Ca^{2+} concentration (134,135). In enterocytes NSP4 results in disruption of tight junctions while in crypt cells it stimulates secretion (136). In addition, NSP4 may stimulate the release of serotonin (5-hydroxytryptamine, 5-HT) from the enterochromaffin cells, activating the enteric nervous system (137,138). Treating rotavirus-infected children with hypotonic oral rehydration solution (ORS) has been shown in several studies to reduce the length and severity of diarrhea (139,140). Hypotonic or isotonic ORS promotes rehydration via sodium-coupled solute co-transporters as water passively follows the osmotic gradient (139,140). In addition, supplements such as *Lactobacillus* (especially strain GG) (141-143) and zinc have shown to be efficacious in reducing rotavirus diarrhea (144).

Vomiting

RV infection has been found to cause delay in gastric emptying, which changes the pressure gradients between the stomach and the duodenum (145). The pathology of gastric delay includes stimulation of vagal nerves and gastrointestinal hormones, mediated by the 5-HT_3 receptors and sodium glucose co-transporter (SGLT-1) (146-149).

During RV infection, intestinal enterochromaffin cells are thought to release 5-HT, which interacts with 5-HT_3 receptors and stimulates the vagal afferent nerve projecting to the vomiting center of the brain (137). Using the same mechanism, anti-emetic drugs (5-HT_3 receptor antagonists) are used to attenuate vomiting in children with RVGE (150,151). In addition, 5-HT_3 receptor antagonists have been shown to attenuate rotavirus-induced diarrhea, and RV has been shown to directly stimulate the vomiting center in mouse models (152)(137).

Fever

Rotavirus infection stimulates the release of several pyrogens, such as prostaglandins and interleukins, from infected cells. In addition to their temperature-moduling effect prostaglandins (PGE_2) may stimulate water secretion (153,154). Treating rotavirus-infected children with aspirin, which inhibits prostaglandins' converting enzyme cyclooxygenase (COX), may reduce the duration of rotavirus-associated diarrhea (155) (156).

Extraintestinal spread

Previous studies have found sporadic associations between extraintestinal spread and clinical manifestations of RV infection. Although the mechanism and site of replication leading to extraintestinal spread has remained unclear, RV infection may be associated with systemic symptoms, and meningitis, encephalopathy, and encephalitis have been reported in children with RV RNA detected in their cerebrospinal fluid (112,157-159). So far, in studies assessing the linkage between serum antigen levels and severity of illness, only sporadic associations have been found. In a study by Fischer et al., more severe illness was found to be associated with higher serum antigen levels, but the difference was not statistically significant (110), while in other studies the antigen level has been found to be associated to the probability of convulsions in RV infected children and to the level of interleukin 8 and 10 (160,161). Interestingly, in a study by Ray et al., the genotype G1 was found to be associated with antigenemia (113).

1.2.3 Laboratory diagnosis

As described earlier, rotavirus particles were first studied by electron microscopy in samples taken from stools (1). At the moment, rotaviruses are most commonly studied by enzyme linked immunosorbent assays (ELISAs) which recognize the RV antigen against middle-layer protein VP6, or by reverse-transcription polymerase chain reaction (RT-PCR). Although RT-PCR can be used to solve the whole 11-segment viral genome, usually only VP7 and VP4 proteins are studied as they are used for classification (44,45,162). The RT-PCR method has been shown to be more sensitive and specific than ELISA, however, both methods can be used for several types of specimen, including stool, serum and whole blood (163). Rotaviruses may still be detected by electron microscopy and also by virus isolation and polyacrylamide gel electrophoresis (164,165).

1.2.4 Immunity and protection

Generation of immune response

Rotavirus infection induces several protective mechanisms in the human host, and the virus has several ways to modulate the innate immunity. When mature epithelial cells are infected by RV, they release certain cytokines which further mediate B-cell and T-cell responses for induction of antigen-specific immunity.

The protective effect of symptomatic primary rotavirus infection against subsequent severe infections was first observed by Bishop and co-authors in a 3-year follow-up study of infants who had had RV infection neonatally (71). Neonatal RV infections were also studied in India by Bhan and co-workers, who observed that infants infected with RV as newborns (nosocomially) often had less severe or even asymptomatic RV infection later on (166). Later, in Mexico, Velazquez et al. reported that the first rotavirus infection provided 87% protection against moderate to severe diarrhea and the second infection provided 100% protection against severe diarrhea in following infections (10). Even though the protection rates against less severe diarrhea were lower after each infection, the third RV infection was observed to provide 99% protection even against mild diarrhea (10). Altogether, in several studies, the humoral immunity obtained from the primary infection was found to correlate with protection (10,167-169).

Interferons and cell-mediated response

In studies using animal models, rotavirus infection has been shown to induce type I (IFN-γ) and type III (IFN- λ) interferon (IFN) mRNA expression, which reduces viral replication (170). In addition, the capacity of RV strains to inhibit the IFN system has been shown to affect the degree of extra-intestinal spread of the virus in mouse models. Although rotaviruses may potentially inhibit all types of IFN response by inhibiting their transcription factors (IRF3, IRF5, IRF7, and NF-κB) by NSP1 or inhibiting their signal complexes (STAT1 and STAT2) (171-173), the human RVs have been shown to inhibit the IFN response less efficiently than the animal RV strains (35). In addition, rotaviruses potentially inhibit the capacity of dendritic cells to activate type 1 helper T-cells (Th1 cells) by stimulating secretion of a regulatory cytokine, TGF-β, in Caco-2 cells *in vitro* (174,175).

Although rotavirus infection has shown to elicit cellular immune response, its role for protection is unclear. Rotavirus infection has been shown to relatively poorly induce cytokine-secreting virus-specific CD8+ cells, which are present in the pherpheral blood in most adults, and T-helper Th cells are presented in the convalescent sera of rotavirus-infected children (176). Marcelin and co-authors observed that lymphocytes contributed (but were not required) to the clearance of RV antigenemia in mouse model, but that treating infected mice with convalescent-phase sera or nonneutralizing serum antibodies was efficient to delay the development of RV antigenemia (177).

Antibody responses in rotavirus-infected children

The immune response to RV infection includes systemic response and mucosal response. To initiate systemic response, viral RV antigen is presented by the antigen-presenting cells to activate Th cells to further activate B- and T-cell responses. The B-cells generated in Peyer's patches enter the blood circulation, antibody-secreting B-cells homing into the lamina propria for secretion of polymeric IgA (intestinal antibody IgA) and memory B-cells returning to the Peyer's patches. Further, the presence of viral antigen stimulates the formation of both kinds of B-cells in the spleen (antigen-presenting and memory cells). The memory B-cells circulate in the bloodstream before returning into the spleen, whereas the antigen-secreting B-cells home into bone marrow and secrete monomeric IgG and IgA (serum IgG and IgA). (178)

Although both serum IgG and IgA are developed after the RV infection, only the serum IgA levels have been shown to correlate with the level of protection in children and serum IgA is used as a marker of protection against RV disease, i.e., against moderate to severe diarrhea (179-182). While the serum IgA represents a correlate of

intestinal IgA, it has an independent role in clinical protection through intracellular neutralization of the virus (27).

The role of IgG in protection is less clear. In a Mexican study the high titers of IgG were shown to protect children against RV infection but not against the disease (183), whereas in a study conducted in Bangladesh, the IgG response was found to correlate with the protection against clinical illness (184).

Rotavirus infection induces mucosal antibodies and the presence of mucosal antibodies has some correlation with subsequent protection (185). However, the role of mucosal antibody alone may not be too significant as indicated by the inability of breast milk to prevent rotavirus infection and uptake of RV vaccine (186,187).

In a study conducted in Indonesia, RV neutralizing antibodies were detected in 56% of colostrum specimens, decreasing to 41% in transitional milk specimens (188). The role of breastfeeding in protection from RV disease is still unclear (189-191).

Antibody response to rotavirus antigens

In 1986, Chiba and co-authors observed that the protection against RV infection could be serotype specific and related to the levels of neutralizing antibodies against the specific (homotypic) virus (192). In the same study, neutralizing antibody levels ≥1/128 were shown to provide protection against subsequent RV infections. Later, children with primary RV infection were found to have both homotypic and heterotypic neutralizing antibodies indicating the presence of cross-reactive neutralizing epitopes in the virus (193). Rotavirus-specific IgA antibodies also act as neutralizing antibodies, and may react with epitopes eliciting heterotypic protection (194).

However, studies of convalescent sera after RV infection have also detected antibodies directed against non-neutralizing antibodies (VP6, VP2, NSP2 and NSP4) (195-199). These antibody responses are quantitatively much stronger than the VP7- or VP4-specific responses (196).

Neutralizing antigens

The outer layer proteins, VP4 (VP8* and VP5*) and VP7, have been shown to induce neutralizing antibodies which may directly inhibit rotavirus infection of mature enterocytes by blocking specific epitopes needed for attachment and penetration.

Glycoprotein VP7 contains two structurally defined antigenic regions (7-1 and 7-2), which both include several antigenic epitopes (16). Although the 7-1 region is immunodominant, both regions bind homotypic and heterotypic antibodies, suggesting that the capacity to bind different antibodies is not due to the location of the epitope

alone (200-202). Neutralizing antibodies interacting with VP7 most likely stabilize the trimer formation of VP7 so that uncoating the virion from VP7 to enable penetration of VP4 (VP5*) is inhibited (16).

The proteolytic cleavage products of VP4, VP8* and VP5*, contain both sequential and surface-exposed neutralizing antigenic epitopes. VP8* has four surface-exposed epitopes (8-1 to 8-4) which induce serotype-specific neutralizing antibodies and five sequential neutralizing epitopes (I-V) which induce more cross-lineage neutralizing antibodies (17,21,23). Glycoprotein VP5* has five surface-exposed antigenic epitopes (5-1 to 5-5) which show more cross-reactive neutralization among strains belonging to different VP4 serotypes. The neutralizing activity of VP4 has been proposed to act by inhibiting the attachment to the cell (203).

The neutralizing antibody responses to VP7 and VP4 after primary and secondary rotavirus infections were described by Gorrell and Bishol in 1999 (204). In the primary infection, VP7 response was shown to be serotype-specific, although it was immunodominant in the production of cross-neutralizing antibodies and neutralizing antibody titer after the subsequent infection. In the same study, VP4 response was found to be more heterotypic. However, the specific role of VP7 and VP4 (VP8* and VP5*) in the production of serotype-specific and cross-reactive neutralizing antibodies is still unclear.

In principle, VP7- and VP4-specific neutralizing antibodies act at an early stage of RV infection, and prevent infection rather than disease.

Non-neutralizing antigens

In addition to epitopes of VP7 and VP4, antibodies can be directed against immunodominant epitopes of VP6, VP2, NSP2, and NSP4, with the highest serum titers directed against VP6 (196,205). Serum IgA is mostly directed against VP6, and a high level of serum IgA correlates with protection against disease rather than infection (183). IgG and/or IgA responses have been detected for VP2, VP6, and NSP2, while fecal IgA has shown to react to NSP2 and VP6 in convalescent samples (206).

Recent studies have identified the dominant type of human humoral response to RV infection to consist of VP6-specific antibodies encoded by the V_H1-46 gene (207,208). Previous in vitro studies have shown the anti-VP6 IgA monoclonal antibody to inhibit RV replication (209). The neutralizing mechanism of VP6 was recently discovered by Aiyegbo and co-workers, who observed that polymeric IgAs may inhibit viral replication by binding near type I channels at the five-fold axis, thus blocking the extrusion of mRNA from DLPs during viral replication (210).

NSP4 was the first viral enterotoxin to be described, and has been shown to induce dose- and age-dependent diarrhea in mice (211). In addition, NSP4 was shown to have

adjuvant properties, enhancing both mucosal and systemic immune responses to model antigens (212). A recent study discovered that NSP4 triggers the secretion of proinflammatory cytokines from murine macrophages and induces secretion of interleukin-8 in vitro (213). Previous studies have detected the NSP4-specific antibody responses with varying levels of immunogenicity (205,214), however, the NSP4 response is thought to be at least partially heterotypic (215,216).

Both NSP2 and VP2 proteins have been shown to induce immune responses to RV infection, although the mechanisms by which they limit viral replication are not understood (199). In addition, VP2 DNA vaccine given intranasally to mice has been shown to increase cytokine levels (IFN-gamma and IL-4) with production of anti-VP2 IgG antibodies (217).

1.3 Rotavirus vaccines

1.3.1 History and development of rotavirus vaccines

The development of a live oral rotavirus vaccine started in the mid-1970s, when researchers found that previous infection with animal rotavirus strain protected laboratory animals from human rotavirus infection (218). Although humans can be infected by animal strains, interspecies transmission of animal rotavirus to humans is relatively uncommon (219).

The first vaccines were based on a Jennerian approach (pioneered by Jenner in 1798 for human smallpox vaccination); when antigenically related non-human rotavirus strains were given orally, they acted as immunogens for VP6, but not for VP7 or VP4, inducing a similar immune response to that caused by the natural rotavirus infection (220-222).

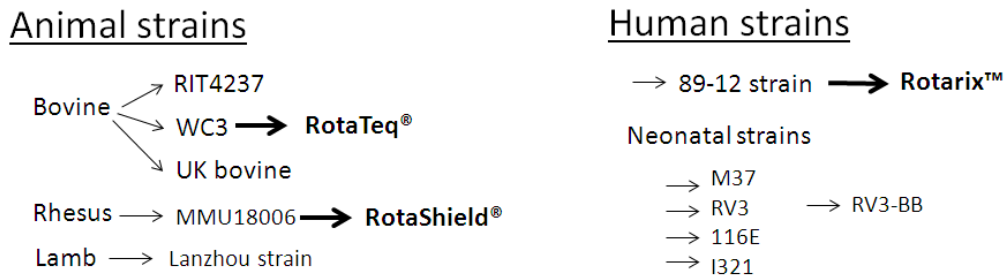

Figure 4. Development of rotavirus vaccines from animal rotavirus strains and human rotavirus strains.

1.3.1.1 Vaccines from animal rotavirus strains

Bovine rotavirus strain

The first clinical vaccine studies started with bovine rotavirus vaccine strain 4237 (G6P6[1]) derived from bovine rotavirus NCVD, which was isolated and highly attenuated in cell culture (223). The first results from efficacy trials in infants in Finland showed greater than 80% protection against rotavirus disease (223-225). Soon after, the vaccine was also tested in newborns. In contrast to previous results, however, the vaccine did not protect against rotavirus infection, but it did give gave partial protection against disease, with the clinical picture of RVGE being significantly milder in vaccine recipients than in placebo recipients (226,227). These results were similar to those seen in infants with natural neonatal RV infection (71,166).

In a study by Lanata and co-workers in Peru, the vaccine was shown to provide serotype specific protection especially against G1 serotype viruses, whereas, despite the number of immunizations, protection rates against G2 were significantly lower (228). In the same study, the vaccine was shown to provide a 40% level of protection against RVGE of any severity and between 58% and 75% protection against severe RVGE. Other trials in developing countries showed lower efficacy, and the development of the RIT4237 vaccine was discontinued in 1986 (229,230).

Soon after, the development of another bovine rotavirus vaccine started with bovine rotavirus WC3 (G6P7[5]), which was isolated from a calf and further propagated in CV1 cell line (231). The WC3 vaccine was developed at the Wistar Institute in Philadelphia and produced by Pasteur Merieux (232). In small vaccine trials, WC3 vaccine was shown to induce neutralizing antibodies in vaccinated infants, but the results in terms of RV disease prevention were inconsistent, as the results from a small efficacy trial in Pennsylvania were promising, but a subsequent trial in Cincinnati showed only low-level protection. (231-234)

Rhesus rotavirus strain

The first rhesus rotavirus vaccine (RRV) was based on simian rotavirus strain MMU18006 (G3P[3]), which shared neutralization specificity with human-origin G3 strains and grew efficiently in cell culture (235). In a study conducted in Finland, the RRV was found to be highly reactogenic, resulting in febrile reactions in 64% of vaccine recipients (236). The initial dosage was then lowered, which led to lower efficacy rates (38% against any rotavirus disease and 75% against severe disease) (237). In Sweden, the efficacy of the original high dosage was studied with a single dose in

children 4-12 months of age, but as in other studies, the protective efficacy was poor and reactogenicity was unacceptably high (238-241).

Later, both WC3 bovine vaccine and RRV vaccine, were modified to incorporate gene segments from human rotaviruses in a "Modified Jennerian approach" resulting in development of the RotaTeq® and RotaShield® reassortant vaccines (discussed later). Both reassortant vaccines were based on a presumption of serotype-specific protection, although the evidence for its specific role was not well established.

Lamb rotavirus strain

The only other rotavirus vaccine developed directly from an animal rotavirus strain was licensed and has been used in China since 2000. The Lanzhou lamb rotavirus vaccine is a monovalent G10P[12] (Group A rotavirus) live-attenuated oral vaccine, and over 30 million doses have been administered to children younger than 5 years of age. The vaccine has not shown any evidence of side effects and has been reported to give partial protection (44-53%) against RV infection. (242,243)

1.3.1.2 Human rotavirus vaccine strains

Neonatal RV infections are often asymptomatic and caused by different RV strains than those circulating in the community (71). The development of oral rotavirus vaccines from human RV strains obtained from infected neonates started after Bishop et al. found that postneonatal infections in infants that had experienced RV infection early in life (as newborns) were significantly milder and less frequent than in those infants experiencing their first RV infection (71). The first vaccine candidate of human-origin was M37, which was well tolerated and immunogenic in infants but induced only a low level of protection (244-246). Bishop et al. found a naturally attenuated G3P[6] human rotavirus strain, from an asymptomatic infant which was shown to protect against clinically severe reinfections (71). The RV3 (G3P[6]) was formulated as a low-titer vaccine, but did not elicit good immune responses (247,248). The RV3 vaccine is currently being developed as a high-titer vaccine (RV3-BB) (249). In addition, vaccines from two neonatal strains isolated in India (116E and I321) from strains G9P[11] and G10P[11] have been evaluated (250,251). Newly published results from the 116E efficacy trial in *The Lancet* show 54% protection against severe disease after three doses of vaccine given at 2, 4 and 6 months (not neonatally) (252).

Vaccine candidate strain 89-12 was isolated from a child infected with a G1P[8] rotavirus strain in Cincinnati in the 1988-1989 season (234). Strains similar to the 89-12 strain were shown to produce broadly cross-reactive neutralizing antibodies, and 89-12

was shown to provide excellent protection against reinfections (167,253). To produce a vaccine strain the 89-12 was propagated in a cell culture for 33 passages, without losing immunogenicity (254). The 89-12 strain vaccine showed 89% efficacy against any gastroenteritis and 100% efficacy against severe gastroenteritis, but was mildly reactogenic in infants 3-5 months of age (254). Thereafter, after the 89-12 strains was licensed to GlaxoSmithKline, a single virus was chosen from the end-point dilution of passage 33, followed by 12 passages in Vero cells, resulting in a fully attenuated virus and development of the Rotarix™ vaccine, designated as strain RIX4414 (discussed later) (255,256)

1.3.2 RRV-TV

RRV-TV is an oral, live, tetravalent rhesus-human reassortant vaccine based on a G3P[3] simian rotavirus strain. Before the final vaccine formulation was determined, different vaccine compositions including single G1 or G2 serotype rhesus-human reassortants were evaluated (257). A high dose of G2-reassortant vaccine was shown to provide 89% protection against severe RVGE, and 67% protection against any RVGE, whereas the protection provided by a low dose of G1 reassortant vaccine was much lower, giving only 44% protection against severe RVGE indicating low significance of VP7 serotype-specific protection (257).

The final vaccine formulation of RRV-TV comprises three human-rhesus reassortant-viruses expressing the VP7 antigen of human origin (G1, G2, or G4), while the other gene segments are from the rhesus origin G3P[3] virus, plus the native G3P[3] strain as the fourth component. After several efficacy trials conducted in the U.S, Finland, and Venezuela, the RRV-TV vaccine became the first licensed rotavirus vaccine, licensed in August 1998 by the US Food and Drug Administration as RotaShield® (Wyeth Lederle Vaccines and Pediatrics). In the pre-licensure studies, RotaShield® vaccine had a 57-66% protective effect against rotavirus disease, with an 82-91% protective effect against severe diarrhea (258-261). In the Finnish study by Joensuu et al., the efficacy against severe RVGE was 90% and the protection against severe AGE of any cause was 60% (258).

Soon after the licensure, the vaccine was recommended by the Advisory Committee on Immunization Practices and the American Academy of Pediatrics for the routine immunization of children on a 2-, 4- and 6-month schedule and, in the first year, catch-up vaccination of infants up to 9 months of age (262,263).

Between November 1998 and July 1999, an estimated 600 000 children received their first dose of RotaShield® vaccine (264). During the post-licensure surveillance, a rare association between RRV-TV vaccination and intussusception was found and

recommendation for the vaccine was withdrawn by its manufacturer within less than one year. The pathogenic mechanisms and host-dependent risk factors for intussusception have been studied since the withdrawal, and show that age at vaccination was strongly associated with risk of intussusception (265,266). At first, the risk of intussusception was estimated to be between 1 in 2500 and 1 in 5000 vaccinated infants, but later studies have shown the risk to be much lower, between 1 in 10000 and 1 in 32000 vaccinated infants (267-270). Intussusception was found to be more common in older infants who received their first dose after the age of 3 months (265).

RRV-TV efficacy studies are still continuing; in a recent study in Ghana using a two-dose schedule in neonates, the vaccine showed good results with about 60% efficacy (271).

1.3.3 Human-Bovine Reassortant vaccine WC3

After the WC3 vaccine had shown low efficacy, subsequent human-bovine reassortants based on the WC3 strain were acquired and further developed by Merck (232). The reassortants expressed the human VP7 and VP4 antigen on a bovine backbone, and several genotypes and their combinations were tested before the final composition containing 5 rotavirus reassortants ("RV5") was licensed (272,273). At first, the vaccine was tested with bivalent (G1 and G2) and tetravalent (G1-G4 and G1-G3 with P[8]) combinations in double-blind, placebo-controlled trials in the United States (274,275). The bivalent vaccine composition produced 73-87% protection against all RVGE and did not show any statistically significant differences in the prevalence of fever, diarrhea or vomiting in patients who received the vaccine within 42 days compared with the placebo group (275). The tetravalent vaccine composition including genotypes G1-G3 and P[8] had similar efficacy rates to the bivalent composition: 74% protection against all RVGE and 100% against severe RVGE (274). The proportion of children with diarrhea or vomiting was greater among patients who received the vaccine than among those who received placebo, but the difference was not statistically significant. In addition, 4.4% of vaccinated children were found to shed vaccine viruses in their stools 3-5 days after the first dose. Of these patients, five shed the original vaccine composition P[8] and two shed a recombinant rotavirus protein with both G1 and P[8] on a bovine backbone (274). In the bivalent combination study, the shedding was evaluated by plaque assay, with 3% of vaccine recipients shedding original vaccine viruses G1 or G2 in their stools after the first dose (275).

Soon after, the vaccine composition was tested with another tetravalent composition including genotypes G1-G4, before the P1A[8] genotype was added to produce broader coverage and with the hope of producing increased efficacy against all

rotavirus strains . This tetravalent composition produced a 68-69% level of protection against all RVGE and 88-100% protection against severe RVGE (261). The prevalence of fever was not higher in immunized children, although the absolute number was larger (276). Vaccination was followed by a great 97% seroresponse and 15% of children were shedding the vaccine viruses in their stools within 7 days after the first immunization (261,276).

With the success from studies using bivalent and tetravalent compositions, the RV5 rotavirus vaccine was developed to contain five human-bovine reassortant rotaviruses. All five available compositions (G1 and G2, G1-G3 with P1A[8], G1-G4, G1-G4 with P1A[8] and P1A[8]) at three dose levels were further compared in a Finnish study, which proved all of them to be efficacious against RVGE, without significant differences in fever, vomiting, or diarrhea among different vaccine composition groups (277). However, the pentavalent composition at the middle dose level was chosen as the final composition, as the addition of P1A[8] was presumed to provide wider protection. Pentavalent vaccine was highly immunogenic and protected against RVGE in the second and third post-vaccination seasons as well (277). P1A[8] reassortant alone was less efficacious than the pentavalent composition (277).

In the final pentavalent composition, four viruses express the outer capsid protein VP7 from human parent strain (G1, G2, G3, and G4 from strains WI79-9, SC2-9, WI78-8, and WI79-4, respectively) and the VP4 P7[5] from bovine antigen, whereas the fifth virus expresses the VP4 protein P1A[8] from human origin combined with bovine origin VP7 G6 (WC3) (Table 2). (277)

A large scale, multinational Rotavirus Efficacy and Safety Trial (REST) was conducted in 2001-2005 in 11 countries and nearly 70,000 infants (23,500 from Finland) were enrolled in the study, in which most of the children were followed for one full rotavirus season after vaccination. (278) The results from REST indicated that the vaccine protected against severe RVGE and RVGE of any severity (100% and 73%, respectively) as early as 14 days after the first dose, and reduced clinic visits for RVGE (of G1-G4 genotypes) by 86%. The vaccination was administered in a three-dose schedule, starting in infants 6-12 weeks of age and no increased risk of intussusception was associated with the vaccine. (278) The REST study was followed by the Finnish Extension Study (FES), in which 89% of the Finnish children (~21,000 infants) enrolled to REST continued to be followed for an average of 3.1 years after their first dose. The results from FES confirmed the impact in terms of reduction of health care visits due to RVGE or any gastroenteritis pathogen (279). The FES study confirmed significant protection against severe RVGE associated with G1, G2, G3, G4, and also G9, which is not included in the vaccine composition (279).

The pentavalent human-bovine reassortant vaccine was licensed in the U.S and Europe in 2006 and registered under the trade mark RotaTeq® (Sanofi Pasteur-MSD).

Reassortant rotavirus	Bovine parental strain	Human parental strain	Parental origin of genome segment				Min. dose levels
			VP3	*VP4*	*VP7*	*VP1,2,6 + NSP1-6*	
WI79-9 (G1)	WC3	WI79	***Human***	*Bovine P[5]*	***Human G1***	*Bovine*	2.2
SC2-9 (G2)	WC3	SC2	***Human***	*Bovine P[5]*	***Human G2***	*Bovine*	2.8
WI78-8 (G3)	WC3	WI78	*Bovine*	*Bovine P[5]*	***Human G3***	*Bovine*	2.2
BrB-9 (G4)	WC3	BrB	*Bovine*	*Bovine P[5]*	***Human G4***	*Bovine*	2.0
WI79-4 (P[8])	WC3	WI79	*Bovine*	***Human P[8]***	*Bovine G6*	*Bovine*	2.3

Table 2. Composition of RotaTeq® vaccine. Minimum dose levels in10^6 infectious units.

1.3.4 Human rotavirus vaccine RIX4414

The human rotavirus vaccine RIX4414 was developed from human parent G1P[8] strain 89-12 in cell culture. The parental strain was isolated from a rotavirus-infected child who took part in the WC3 vaccine study and received placebo. The strain was passaged 33 times in monkey kidney cells, followed by plaque purification and a further 12 passages in vero cell culture at GlaxoSmithKline (280). Compared to the parent vaccine strain 89-12, the large number of passages made the RIX4414 strain more attenuated and very mildly reactogenic (256). The vaccine's immunogenicity was evaluated in Finnish infants by measuring the rotavirus-specific IgA antibodies in the serum after two doses given at 2 and 4 months of age, and the vaccine was found to be 96% immunogenic. It was also found to be well-tolerated, with no associated febrile reactions. (256)

The first efficacy trial of the RIX4414 vaccine was conducted in Finland in 405 infants aged 2-4 months who received two doses of the vaccine with a 2-month interval between. The vaccine showed 72% efficacy against all RVGE and 100% against severe RVGE (281). Almost all infections were caused by G1 rotaviruses and the vaccine seemed also to show protection against G1 rotavirus infection, not just disease. In a study conducted in Latin America, the rotavirus infections in vaccinated children were for the first time associated with non-G1 strains. Still the protection against RVGE caused by non-G1 genotypes was 77%, while for G1 rotaviruses it was 88% (282). The immunogenicity and efficacy were studied in several vaccine trials around the world, and the encouraging results led to phase III safety and/or efficacy trials (282-284).

Like RotaTeq® in the REST trial, the safety and efficacy of the Rotarix™ vaccine were evaluated in a large, multinational, placebo-controlled trial involving 63,225 infants in Finland and 11 Latin American countries (255). The vaccine was found to be efficacious against severe RVGE (84.7-100% protection), and no association with increased risk of intussusception was found; in fact, the prevalence of intussusception cases was actually higher in placebo group (255). In the same study, the protection against RVGE was found to be best for G1 strains or strains with the equivalent P[8] genotype, whereas for a totally different genotype strain such as G2P[4], the protection level was only 41%. In a subsequent 2-year study conducted in six European countries, protection rates remained high, with 90% protection (96% in the first year and 86% during the second year) against severe RVGE (285). The overall protection against RVGE of any severity associated with different genotypes (G1-G4 and G9) ranged from 58% to 90%, G2 being the lowest. The vaccine was also shown to reduce hospital admissions for gastroenteritis of any cause by 72%. (285)

In July 2004, the RIX4414 vaccine was licensed in Mexico by GlaxoSmithKline under the trade mark Rotarix™. The Rotarix™ vaccine is currently licensed in over 100 countries for prevention of RVGE and has been available in Europe since 2006. The vaccine is administered orally in a two-dose schedule. The two-dose schedule should be completed by 24 weeks of age (in the U.S) or 16 weeks of age (in Europe); the first dose may be given from 6 weeks of age with an interval of at least 4 weeks between doses. (286)

	RotaTeq®	Rotarix™
Contents	Five human-bovine reassortant rotaviruses containing human G1, G2, G3, G4 and P1A[8]	Human G1P[8] rotavirus strain
Administration	Given orally in 3 doses starting at 6 to 12 weeks of age. Subsequent doses administered at 4- to 10-week interval. Third dose should be given before 32 weeks of age.	Given orally in 2 doses starting at 6 weeks of age. Second dose administered after an interval of at least 4 weeks and prior to 24 weeks of age.
Adverse events	Diarrhea, vomiting, irritability	Irritability and vomiting
Effectiveness	Against severe RVGE cases 100%. Against RVGE cases of any severity 73%. [278] Indicated against G1, G2, G3 and G4	Against severe RVGE cases 84.7-100% [255]. Against RVGE cases of any severity 58-90% [285] Indicated against G1, G3, G4 and G9
Intussusception risk	No increased risk observed before licensure	No increased risk observed before licensure
Shedding of vaccine viruses	After 1st dose: 0-13% of vaccinated infants	21-61% in 7 post-vaccination days after 1st dose
Transmission of vaccine virus and vdG1P[8]	Formation of vdG1P[8] observed with tetravalent composition	Transmission of vaccine viruses into unvaccinated siblings observed in prelicensure trials

Table 3. RotaTeq® and Rotarix™ vaccine. Intussusception risk, Shedding of vaccine viruses and transmission of vaccine virus and vdG1P[8] based on pre-licensure studies.

1.3.5 Effects of universal mass vaccination with RotaTeq® and Rotarix™ vaccines

1.3.5.1 Use of vaccines

Soon after licensure, in 2006 rotavirus vaccines were introduced into the NIPs of Austria and Australia, followed thereafter by several other countries such as Brazil, USA and Belgium. In Finland, RotaTeq® vaccine was added into the NIP in September 2009. However, before the implementation of RotaTeq®, Rotarix™ was used, with 22% coverage between 2006 and 2007 and 35% coverage in 2007-2008 (29% Rotarix™ and 6% RotaTeq®). (62)

As of January 2014, 53 countries around the world have introduced rotavirus vaccines in their NIPs and several countries such as Canada, Germany, Thailand and regionally in the United Arab Emirates (287). Currently, 30 of these countries have introduced RV vaccine with donor support from the Global Alliance for Vaccines and Immunization. Rotavirus vaccine is also available in the private market in more than 100 countries.

In Europe, rotavirus vaccination is in the NIPs of Finland, Belgium, Austria, Luxembourg, the United Kingdom, and five Federal states of Germany. Finland is the only European country using exclusively RotaTeq® vaccine (287).

1.3.5.2 Impact on RV burden of disease

The implementation of RV vaccination has been shown to affect the natural seasonality of RV disease. In a study performed by the US Centers for Disease Control and Prevention (CDC), the rotavirus testing data from a 10-year surveillance was analyzed (288). The onset of the RV season was delayed by 2-4 months in the first year following universal vaccination, and the season was 12 weeks shorter than during the 6 previous years, before RV vaccines became available. However, the season was longer again in the following (second post-NIP) year. (288,289) Similar results have been observed in epidemiological studies in Brazil and Belgium, where the epidemic onset has shifted by 1-2 months after implementation of RV vaccination (290-293). As this kind of shift was not seen before the vaccines were launched, it is likely that the use of RV vaccines has affected RV seasonality (294). In tropical countries such as Brazil, RV disease has occurred during the cool and dry seasons, but sporadic infections or outbreaks may occur during the whole year (74,75). A shift in the onset or shortening of RV season has not been detected in Brazil (75).

1.3.5.3 Effectiveness on RVGE cases

In several countries of Europe, the use of the two rotavirus vaccines RotaTeq® and Rotarix™ has dramatically reduced the numbers of hospital admissions and outpatient clinic visits for RVGE.

In Belgium, the use of Rotarix™ was reflected as a 61% decline in laboratory-confirmed RVGE cases in the first year (292). After two years of immunization in the NIP, the reduction in cases of RVGE in children eligible for vaccination (2-24 months of age) was 80% (295). Similarly, in Austria, RVGE-related hospital admissions were reduced by 74% in children eligible for vaccination (296). In a recent study from Brazil,

Rotarix™ vaccine effectiveness was estimated to be 85%, with a significant reduction in hospital admissions and mortality due to gastroenteritis of any cause in children less than 1 year of age (by 48% and 54%, respectively) (297). In Australia, with combined use of both RV vaccines, RVGE-related hospital admissions were reduced by 68-93% in children less than 1 year of age (298).

After the introduction of the RotaTeq® vaccine, hospital admissions for RVGE in children were reduced by 74-85% in the US (299,300). In addition, in a recent study from the United States, RotaTeq® vaccine was found to effectively reduce RVGE-related health care visits even after only the first and second immunizations (301). Health care visits due to RVGE were reduced by 88% after the first dose and 94% after the second dose, while health-care visits due to gastroenteritis of any cause were reduced by 44% after the first dose and 40% after the second dose (301).

Indirect effect in unvaccinated children

In addition to reduction of RVGE in children eligible for vaccination in the NIP, implementation of RV vaccination has also reduced RVGE cases in unvaccinated children.

In Belgium, in the second post-NIP year, the number of RVGE cases in children too old to be vaccinated in the NIP was reduced by 64% (295). In Australia, with combined use of both vaccines, hospital admissions for RVGE were reduced by over 50% in children older than 2 years of age (not eligible for RV vaccination in the NIP) (298). In the United States, the use of, mostly RotaTeq® vaccine was reflected in a 42-45% reduction in RVGE cases among children too old or too young to be included in the RV NIP (288).

1.3.5.4 Genotype distribution

To monitor the effect of RV vaccines, systematic research into the changes in circulating rotavirus genotypes has been carried out in some countries (302,303).

Especially in countries that exclusively use the Rotarix™ vaccine, such as Belgium and Brazil, an increase in G2P[4] genotype strains was detected soon after the introduction of the vaccine (293,304-307). In Aracju, in North West Brazil, the predominance of G2P[4] genotypes was associated with the disappearance of other genotypes (308). In Belgium, the G2P[4] genotype accounted for less than 5% of all RVGE cases before universal mass vaccination, but rapidly increased, accounting for 30-40% of all RVGE cases in the following three years (309). In a study by Braeckman and co-workers, the efficacy of Rotarix™ vaccine against G1P[8] rotaviruses was 95%,

while for G2P[4] strains it was 85% (310). Although the strong re-emergence of G2P[4] strains was at first supposed to be related to the mass use of vaccine, as the G2P[4] genotype is phylogenetically further than the other common human RV genotypes, the causal relationship is unclear, since the emergence of G2P[4] had already been identified before the vaccines were available (in Spain and in Portugal), and has also been found in countries without rotavirus vaccine in a NIP (Lithuania and several countries in Central and South America) (302,311-314).

Australia was one of the first countries to introduce rotavirus vaccine into its NIP. Both vaccines are licensed and in use in different Australian states. Interestingly, with an overall vaccine coverage of more than 80% of the population, during the first two years after the vaccines were introducted, in states using Rotarix™ the G2P[4] became the most predominant RV genotype, while in states using RotaTeq® G1P[8] and G3P[8] accounted for the majority of RV infections (298). However, more recent data has shown that the predominant RV genotypes in states using Rotarix™ and RotaTeq® respectively have changed places with each other (315).

In the United States, RotaTeq® and Rotarix™ were approved for immunization in 2006 and 2008, respectively (316). Before introduction of the vaccine, G1P[8] was the prevalent genotype for several years, but soon after the introduction of RotaTeq®, the proportional role of G1P[8] decreased to 30.7% while G3P[8] became the predominant genotype at 36.3% (316).

In addition to shifts in the genotype distributions, since the introduction of both RV vaccines, emergence of previously uncommon genotypes such as G9 and G12 has been observed worldwide (317). In the most recent data from the United States, a new rotavirus genotype, G14P[24], was detected from children with RVGE, possibly indicating that the proportion of unusual rotavirus genotypes to increase in future (318). However, as the introduction of the vaccines has taken place within a short time period, it is still too early to speculate as to which of the changes in genotype distribution are due to natural fluctuation of genotypes and which to the use of vaccines and to vaccine-induced selection pressure.

1.3.5.5 Effects on rotavirus genomics

It has been suggested that the large-scale use of RV vaccines has the potential to cause genetic drift of the virus genome or even new reassortments leading to antigenically new strains (319,320). As the diversity of rotaviruses is generated by several mechanisms including point mutations and gene rearrangements, surveillance of the circulating rotavirus genotypes is essential to detect possible new strains, the emergence of which may result in decreased vaccine effectiveness (319).

Antigenic pressure on VP7 and VP4 proteins

The possible antigenic pressure on outer capsid proteins VP7 and VP4 has not yet been studied to a great extent. To date, worldwide circulation of four VP4 P[8] sublineages (P[8]–I to P[8]–IV) and at least 11 VP7 G1 lineages (G1–I to G1–XI) has been described(321,322). In a recent presentation from Brazil, the P[8] sublineages circulating after the introduction of the Rotarix™ vaccine show more intragenotypic variety than strains circulating in the pre-NIP years. After mass immunizations with Rotarix™, the majority of circulating VP4 P[8] strains belonged to the P[8]–III sublineage, which is phylogenetically distant from Rotarix™ P[8], which belongs to sublineage P[8]–I (Poster presentation: Silva MFM et al. VP8 P[8] lineages of group A rotaviruses circulating over 20 years in Brazil. 11th International Symposium on dsRNA viruses, 27 Nov–1 Dec 2012, Puerto Rico). RotaTeq® VP4 P[8] belongs to the P[8]–II sublineage(323). Previous studies have indicated that circulating group A rotaviruses differ from both vaccine strains in their antigenic epitopes of VP7 and VP4 proteins (323). However, circulation of several VP7 sublineages at the same time has been reported from many countries both before and after rotavirus vaccines became available (53,60,324).

1.3.5.6 Shedding of original vaccine viruses and vaccine-derived reassortants

Shedding

The shedding of vaccine viruses has been evaluated in several pre-licensure studies, which indicated shedding of RotaTeq® viruses to be a relatively uncommon phenomenon, whereas, Rotarix™ appeared to be commonly shed after the first dose of vaccine (256). In the prelicensure studies of RotaTeq®, the shedding occurred in 0-13% of vaccinated children after the first dose, in 0-7% after the second dose and in 0-0.4% after the third dose (277,278). In both studies, the shedding of vaccine viruses was evaluated using the viral culture method with plaque assay and electropherotyping. However, more recent studies from the post-licensure period that use ELISA have shown the actual shedding rates to be much higher. In a study by Yen and co-workers, 21% of vaccinated children were found to have vaccine strain in stools collected during the first 9 days after the first immunization (325). In the same study, the viral load was assessed at between 4.5 x 10^7 and 7.0 x 10^{12} copies per gram of stool (325). Hsieh and co-authors studied the shedding after each immunization with both RotaTeq® and Rotarix™ vaccine using RT-PCR and ELISA. After immunization with RotaTeq® vaccine, 56% and 94% of children (ELISA and RT-PCR, respectively) shed the virus at

some point during the first 28 days after the first immunization. Shedding was less common after the second dose (9.3% and 67%) and after the third dose (8.1% and 62%) (326). The low level of ELISA positivity and high level of RT-PCR positivity in shedding after the second and third doses of RotaTeq® may suggest that viral multiplication is common but low-level in vaccinees in whom the first dose has already taken.

In the same study by Hsieh et al., shedding rates after immunization with Rotarix™ were similar. After the first immunization, 43% and 94% of children (ELISA and RT-PCR, respectively) shed the vaccine virus in their stools diminishing to 25% and 53% after the second dose. (326) In the pre-licensure studies, the shedding of the Rotarix™ strain was relatively common, with 21-61% of vaccinated children shedding the vaccine viruses in their stools during the first 7 days following the first immunization and 11-21% after the second dose (327-329). Unlike with the RotaTeq® vaccine, transmission of the Rotarix™ strain to an unvaccinated placebo group (often twins), and prolonged shedding in some individuals, had already been noticed before licensure (327-329). With the RotaTeq® vaccine, prolonged shedding up to 14 months of age has been observed in children with severe immunodeficiency (330).

Vaccine-originated viruses and new reassortants

Since the launch of the RotaTeq® vaccine, shedding of a novel vaccine-derived double reassortant rotavirus has been described from the United States and Australia. The first vaccine-derived human-bovine double reassortant virus was detected in January 2009 in the United States, where an unvaccinated boy was found to have vaccine-originated rotavirus in his stools after a health care visit for symptoms of gastroenteritis (331). After full or partial RT-PCR and sequencing of all 11 RV gene segments, the virus was found to be a novel vaccine-derived reassortant G1P[8] (vdG1P[8]) rotavirus derived from two original vaccine strains G1P7[5] and G6P[8]. The source of infection was thought to be his recently vaccinated sibling, who had no signs of gastroenteritis. (331) Soon after, Donato et al. published a study on children hospitalized for AGE after immunization with RotaTeq® (332). The study found that 13 of these children (21%) were shedding RotaTeq® vaccine strains in their stools, and in four of them a novel vaccine-derived double reassortant was detected. The virus was identified by genotyping and sequencing of VP3, VP4, VP6, and VP7 gene segments. The presence of vdG1P[8] was thought to be possibly associated with altered immune function, as some of the children had underlying medical conditions. (332) The same vdG1P[8] has been described by Boom and co-authors, who detected vdG1P[8] in an unvaccinated 22-month-old boy hospitalized for AGE (possible sibling transmission) and in a

recently vaccinated child hospitalized for bronchiolitis (detected concomitantly with adenovirus) (333).

Interestingly, the reassortant between P[8] VP4 and G1 VP7 proteins was described earlier in the prelicensure studies of the tetravalent vaccine composition. In a study by Clark et al., 7 of 161 children immunized with tetravalent vaccine composition shed vaccine virus in their stool 3-5 days after the first immunization. Of these seven children, vaccine strain P[8] was detected in five, while 2 were concomitantly shedding P[8] and G1 components of the vaccine on a bovine WC3 backbone. (274)

In addition to the vaccine-vaccine reassortants derived from the RotaTeq® vaccine, a new vaccine-wildtype reassortant has been observed in Nicaragua in two RotaTeq®-vaccinated children. The new reassortant has a typical genome constellation for a wild-type G1P[8] virus, but the NSP2 gene segment is identical to the cognate gene segment in RotaTeq® vaccine. (334)

In the study by Bloom et al., rotavirus VP7, VP4, and NSP2 gene segments identical to Rotarix™ vaccine strains were detected in stool from an unvaccinated 6-month-old boy. The child was hospitalized for signs of AGE and no other gastroenteritis pathogens were detected; however, the source of the Rotarix™-derived virus remained unclear. (333) Similar surprise detection of Rotarix™ in an unvaccinated child with AGE in territory using exclusively RotaTeq® has been reported in the United States (Daniel Payne (2013), Personal communication, Centers for Disease Control and Prevention, Atlanta, Georgia, USA). Previously, the horizontal transmission of Rotarix™ vaccine strain in twins has been studied by Rivera et al., the results indicated that horizontal transmission is relatively common, occurring in 15 of 80 twins; however, none of these transmissions led to symptomatic gastroenteritis in the infected twin. (335)

1.3.5.7 Vaccine-related safety issues

Porcine circovirus

In March 2010, DNA of porcine circovirus type 1 (PCV-1) was found in Rotarix™ vaccines in the United States (336). Rotarix™ vaccine was observed to contain full-length PCV-1 genomes representing infectious viruses. Although PCV-1 does not infect humans, the finding led to temporary withdrawal of Rotarix™ in the United States and some European countries (336,337).

Soon after, in May 2010, RotaTeq® vaccine was also observed to contain fragments of DNA of PCV-1 and porcine circovirus type 2 (PCV-2). The source of the PCV-1 and PCV-2 genomes was identified in a trypsin lot used in the vaccine production

(338). In a subsequent study by Esona and co-workers, the stools of RotaTeq®-vaccinated children were studied for the presence of PCV-1 or PCV-2. Of all vaccinated children enrolled in the study, 58% were found to have PCV-2 in their stools, while PCV-1 was not present in any sample. Shedding of PCV-2 DNA occurred most frequently in the 5 days following immunization, and the DNA was detected for up to 9 days. However, although the shedding rate of PCV-2 DNA was relatively high, the DNA was not associated with viable PCV particles. (339)

In Spain, both rotavirus vaccines were unavailable for a 5-month period due to contamination with PCV-1. A later analysis estimated the costs of RV disease in children who were unvaccinated in the period of withdrawal as very high (337). Rotarix™ is still not available in Spain.

Intussusception

Although no increased risk of intussusception was found in the prelicensure studies of RotaTeq® vaccine (278), a potential risk has been identified in post-licensure surveillance programs conducted in the United States and Australia (340-345). The risk of intussusception was found to be greatest after the first and second doses of vaccine; with a total risk of approximately 1 intussusception case per 65000 RotaTeq®-vaccinated infants (343). In the Australian studies, the relative risk of intussusception was from 5.3 to 9.9 (95% confidence interval, 1.1-15.4 and 3.7-26.4, respectively) during the first 7 post-vaccination days with an even smaller risk after the second dose (340,341). The relative risk of intussusception in the 1-21 days after the first dose of vaccine was 3.5 (95% CI 1.3-7.6) (340). In a recent study from the United States, immunization with RotaTeq® vaccine was shown to induce 1.5 excess cases of intussusception in 100,000 vaccinated children, with the risk limited to the first dose of the vaccine (346).

In Australia, the use of both rotavirus vaccines combined has been estimated to cause an excess of 14 intussusception cases annually (341). Furthermore, the risk of intussusception after both vaccines has been observed to cluster on post-vaccination days 3-6 (342).

Newly published results from the relative risk of intussusception after RotaTeq® and Rotarix™ in *The New England Journal of Medicine* show the relative risk for intussuception to be significant after immunization with Rotarix™ but not RotaTeq® vaccine (1.1 and 8.4 relative risk after RotaTeq® and Rotarix™ vaccine, respectively) (347).

The association between intussusception and Rotarix™ vaccine has been studied in Mexico, Brazil and Australia. In Mexico and Brazil, the risk of intussusception was estimated at between 1 in 51,000 and 1 in 68,000, respectively (348), with

intussusception cases clustering in the first 7 days after the first immunization (349). In a study conducted in Australia, the relative risk (RR) after Rotarix™ was lower than for RotaTeq® vaccine during days 1-21 after the first immunization (RR 1.5, 95% CI 0.4-3.9) (340). In Australia, the risk of intussusception in the 7 days following the first immunization was 6.8 (95% confidence interval, 2.4-19.0; P<0.001) (341), similar to Japan, where the relative risk was 3.6 (95% CI 1.3-9.9) (Poster presentation: Nakagomi and Nakagomi. Estimating the risk of intussusception during the first week after the first dose of monovalent human rotavirus vaccine to Japanese infants 6-20 weeks of age. Vaccines for Enteric Diseases 2013, 6-8 Nov 2013, Bangkok, Thailand).

In smaller efficacy and safety trials, either no intussusceptions have been observed in the study population or no associations with the vaccine has been established (350).

The mechanism of intussusception was studied after administration of RotaShield® vaccine. Previously, intussusception has been associated to the presence of several viruses (i.e. adenovirus) and to the presence of mesenteric lymphadenopathy and inflamed Peyer's patches proposing an infectious or inflammatory model. However, the pathogenic mechanism including RV vaccine has remained unknown. (351)

2 Aims of the study

1. To further investigate the clinical picture of rotavirus infection in children, particularly to elucidate whether systemic spread of rotavirus is associated with a more severe clinical picture than infections confined to the intestines

2. To determine the effect of universal rotavirus vaccination on hospital admissions for acute gastroenteritis of all causes and for rotavirus gastroenteritis

3. To examine whether large-scale use of rotavirus vaccines exerts immunological pressure, by exploring the changes in the two outer capsid proteins VP7 and VP4 of circulating rotaviruses over a period of 20 years

4. To investigate the presence of rotavirus vaccine strains in children hospitalized with AGE in order to determine if the vaccine virus is associated with clinical AGE symptoms.

3 Materials and methods

3.1 Materials

3.1.1 Clinical material

The patients in each study group were children less than 16 years of age seen at the Tampere University Hospital because of acute gastroenteritis (AGE). The patients eligible for the study were either diagnosed with gastroenteritis (ICD-10 codes: A00-09) when admitted to the hospital or had gastroenteritis symptoms defined as three or more loose stools or two or more vomits or one loose stool and one vomit, in connection with another clinical diagnosis.

Collection of study material

The material dating from September 2006 to August 2008 was collected by Räsänen et al. to examine the role and epidemiology of different pathogens of AGE, especially norovirus and rotavirus. The results from these years have been published by Räsänen and co-workers (62,352).

The study material from the period 2009-2011 (period defined starting from 1 September ending 31 August) was initially collected to study the clinical significance of bocavirus in respiratory tract infections and AGE. Although children with either respiratory tract infection or AGE or both were enrolled in the original study, only children with identical inclusion criteria to those in the 2006-2008 gastroenteritis study were eligible for our study. The results relating to bocavirus have been published by Paloniemi et al. (353).

The study material from 2011-2012 was been obtained from an epidemiological survey conducted by Sanofi-Pasteur MSD. In this study, children hospitalized for AGE between 2009 and 2013 with an ICD-10 code indicating any disease related to gastroenteritis were eligible to be enrolled. The results have been published in part by Vesikari et al. (354).

The study material from September 2012 onward has been collected by the present author in the same settings and with the same methodology as in the studies conducted in 2006-2008 and 2009-2011 in children with AGE.

All study protocols were approved by the Ethics Committee of Pirkanmaa Hospital District and Tampere University Hospital. Written informed consent has been obtained from each child's parent or legal guardian.

Study groups

Group 1 (Study I)

From September 2006 to August 2008 1042 children less than 16 years of age with symptoms of AGE seen in the emergency department or admitted to a pediatric ward were recruited into the study at Tampere University Hospital. For this Study, both a serum and a stool sample were collected from 374 patients, 155 of whom were determined to be RV-positive in stools.

To study the clinical significance of rotavirus RNAemia and antigenemia in Study I, 131 RV-positive patients with available serum and whole blood samples in addition to the stool sample were included. The rotavirus-positive patients were further grouped according to their laboratory results, so that in the first subgroup there were 22 patients positive for rotavirus RNA in serum and whole blood with an ELISA positive serum. In the second subgroup there were 46 patients positive for RV RNA in serum and RV antigen in ELISA. The third subgroup consisted of 13 patients positive for RV RNA in serum, whereas in the fourth subgroup there were 50 patients with exclusively rotavirus positive stools.

Out of 219 rotavirus-negative patients with available stool and serum samples, 85 were detected to have other gastroenteritis virus in their stools, and these were selected as control patients.

Group 2 (Study II)

In Study II, two similar surveys for prospective surveillance of RVGE in the same setting and using the same methodology were conducted in the pre-NIP period from September 2006 to August 2008 and in the post-NIP period from September 2009 to August 2011. Children were recruited from the emergency department (ED) and pediatric ward for both surveys. In the pre-NIP years, 1193 patients were recruited and stool samples were obtained from 809 of them. In the post-NIP years, 495 children were recruited to the study and stool samples were obtained from 330.

From the second pre-NIP season, 65 patients infected in an extensive waterborne AGE outbreak in Nokia were excluded from the norovirus (NoV) analysis to better reflect a normal situation of endemic NoVGE.

Group 3 (Study III)

From 1992 onward, children were recruited into several epidemiological studies (study years 2006-2012) and rotavirus vaccine trials in Tampere (study years 1992-2004).

For the third study, we retrieved a total of 108 wild-type rotavirus G1P[8] strains collected in Tampere between 1992 and 2012. The stool specimens from the years 1992-2004 were from unvaccinated or placebo-vaccinated children who had symptomatic RVGE during vaccine trials. The study material consisted of 11 sample strains from 1992-1994 and 29 sample strains from 2002-2004. There were 33 sample strains from 2006-2008 and 35 strains from the post-NIP years 2009-2012. The specimens from the years 2006-2012 were collected from children seen in Tampere University Hospital emergency department or pediatric ward for RVGE.

Group 4 (Studies IV,V)

A prospective study on the viral etiology of AGE in children after universal mass vaccination was started in September 2009. For Study IV, we studied the origin of rotavirus strains (wild-type/vaccine-acquired) collected from 107 children positive for rotavirus in RT-PCR in the years 2009-2011. For Study V, similar research was done for 22 rotavirus-positive patients seen in 2012-2013.

The study material from both periods included all rotavirus positive children seen in the outpatient clinic or admitted to a pediatric ward and with available stool specimen.

3.1.2 Clinical methods

3.1.2.1 Sample collection

In all cases the time interval between sample collection and the onset of illness was less than 7 days.

Stool specimens (Studies I-V)

A stool specimen was collected during the outpatient clinic visit or, if the child was admitted, on the hospital ward. In study years 2009-2013, if the sample was not obtained before discharge, the parents were provided with a home kit and the sample could be sent to the laboratory of the Vaccine Research Center. In Study V, one child's parents were contacted 2.5 months after hospital admission, to give a follow-up stool specimen.

Serum and whole blood samples (Study I)

For Study I, an acute phase serum sample and a blood sample were collected during the outpatient clinic visit or on the pediatric ward only if the child's clinical treatment required collection of blood samples.

3.1.2.2 Clinical picture

While in hospital, information on patients' symptoms was collected from the hospital medical records. Before discharge, the parents received a diary card and instructions to record the following information: the duration of each symptom including starting day and ending day, maximum temperature (°C,) and maximum number of diarrheal stools/24 h, and maximum number of vomiting episodes/24 h. Parents were instructed to send the diary cards back to the study nurse after the child had fully recovered. If the cards were not returned within 4 weeks after discharge, the study nurse contacted the parents for a reminder.

For the statistical analyses, symptoms were graded according to the Vesikari 20-point scoring system for severity of rotavirus diarrhea (Table 4) (355).

Symptom		Score
Duration of diarrhea		
	1-4 days	1
	5 days	2
	≥6 days	3
Max no. Diarrheal stools/24h		
	1-3	1
	4-5	2
	≥6	3
Duration of vomiting		
	1	1
	2	2
	≥3	3
Max no. Vomiting episodes/24h		
	1	1
	2-4	2
	≥5	3
Fever		
	37.1-38.4°C	1
	38.5-38.9°C	2
	≥39.0°C	3
Dehydration		
	None	0
	1-5%	2
	≥6%	3
Treatment		
	None	0
	Rehydration	1
	Hospitalization	2

Table 4. "Vesikari" scoring system for severity of rotavirus diarrhea (355)

3.2 Laboratory methods

3.2.1 RNA Extraction (Studies I-V)

From stool, serum, and blood specimens, viral RNA was extracted 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.

3.2.2 Rotavirus

3.2.2.1 RT-PCR (Studies I-V)

Detection and G-typing of rotaviruses by RT-PCR

RT-PCR was the primary detection method for rotavirus in each study. The 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 (44,62).

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 $MgCl_2$ (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 $MgCl_2$, 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 H_20, 10.0 µl 5X Green GoTaq Flexi Buffer, 3.0 µl of 25 mM $MgCl_2$, 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 nucleotide sequences, positions and product sizes for both primer mixes are presented in Table 5.

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. Amplification products of different G-types differ in size and are recognized from the gel electrophoresis. The total length of first PCR product is 1062 base pair (bp).

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

Table 5. Lengths of H pool and C pool products of different rotavirus genotypes

P-typing of rotaviruses by RT-PCR

For the determination of rotavirus P-types, an RT-PCR assay to amplify the VP4 glycoprotein, gene 4, was performed, detecting P-genotypes P[4], P[6] and P[8] (356,357).

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 $MgCl_2$ (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 H_20, 3.5 µl of 5X Green GoTaq Flexi Buffer, 4.2 µl of 25 mM $MgCl_2$ (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 H_20, 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, whereas in the 2nd PCR the lengths for P[4], P[6], and P[8] are 289, 381 and 151 bp, respectively.

RT-PCR for VP6 glycoprotein

For the VP6 RT-PCR the method was provided by Dr. Max Ciarlet with Merck & Co. to detect both human and bovine origin VP6 glycoproteins using Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany). (358) The RT-PCR provides a partial 379 bp copy of the gene segment encoding for VP6.

Five microliters of extracted RNA was first denatured with Molecular Biology Grade H_20 (Sigma Aldrich) for 2 min at 95°C. For the RT reaction, a RT-PCR mix

containing 18 µl of Molecular Biology Grade H_20 (Sigma Aldrich), 10 µl of 5X One Step RT-PCR buffer, 2.0 µl of dNTP mix (10 µM dATP, dCTP, dTTP, dGTP), 2.0 µl of One Step RT-PCR enzyme mix, and 4.0 µl of both primers Rota VP6 fwd Bovine (forward primer, 5'GAYGGNGCDACNACATGGT3', nucleotides 747-765) and Rota VP6 rev Bovine (reverse primer, 5'GTCCARTTCATNCCTGGYGG3', nucleotides 1126-1107) (For Y (C,T), R(A,G), D(A,G,T) and N(A,G,C,T)) was added into the denatured sample-H_20 mix and run for 30 min at 50°C and 15 min at 95°C before being run for 40 cycles of 45 sec at 94°C, 45 sec at 54°C, and 1 min at 72°C, with a final extension for 10 min at 72°C.

The PCR products were run in a 2% agarose gel for 105 min at 100 V. All the positive bands were further sequenced in order to specify the origin of the protein (human/bovine).

3.2.2.2 Sequencing and sequence analyses (Studies III, IV, V)

The RT-PCR primers were further used as sequencing primers. Additionally, if required, due to short or unclear sequences for the VP7 protein, the primer H rev (5'AACTTGCCACCATTTTTTTCC3') and the primer G1 fwd (5'CAAGTACTCAAATCAATGATGG3') were also used for sequencing.

Gel-purified amplicons (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany) were sequenced using BigDye Terminator v1.1 Cycle Sequencing v1.1 Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Applied Biosystems, Foster City, CA) on an ABI Prism 310 Genetic Analyzer. Sequencing files were analyzed and consensus sequences were prepared using Sequencer 4.9.

Nucleotide sequences read from the chromatograms were aligned to published sequences from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/, nucleotide blast) (Studies III, IV, V)

In addition, for sequence analyses used in Study III, multiple consensus alignments were conducted using Clustal Omega. Statistical analyses and phylogenetic trees were constructed with the Neighbor-joining method using the Kimura two-parameter model, with MEGA (version 4.0) software.

3.2.2.3 ELISA (Studies I, IV, V)

Enzyme-linked immunosorbent assay (ELISA) testing was performed to detect rotavirus antigen (group specific proteins, especially the major inner capsid protein VP6), using the ProSpecT Rotavirus Kit (Oxoid Ltd, Basingstoke, Hampshire, United

Kingdom). The presence of RV antigen was determined from serum samples in Study I and from stool samples in Studies IV and V. Stools absorbed in a diaper were not tested for RV antigen, because the ELISA test could not be performed for technical reasons.

One hundred microliters of 10% stool suspensions or undiluted serum specimens were pipetted in two separate microwells coated with rotavirus specific rabbit polyclonal antibody, before adding a rotavirus specific antibody conjugated to a horseradish peroxidase enzyme in each microwell followed by incubation at room temperature for 60 min. The microwells were further washed for five times in a microplate washer using double-distilled H_20 and Wash Buffer.

Rotavirus antigen was captured between antibody on the solid phase and the enzyme-conjugated antibody.

Before sample reading, a substrate was added into each microwell and wells were incubated in the dark at room temperature for 10 min. The substrate reaction was stopped using a Stop Solution and the microwell plate was read by a Victor² 1420 Multilabel counter at 450nm within 30 min.

A sample with an optical density read by spectrophotometry with a value greater than 0.15 was considered as positive.

3.2.2.4 Cell culture (Studies IV, V)

For cell cultivation of rotavirus in studies IV and V, a 10% stool suspension was first vortexed with 1ml of minimum essential medium (MEM) supplemented for 15 min at 3000 x g. In study IV, the dilution was filtered using a 0.22 filter and diluted in MEM containing 10% fetal bovine serum "10% MEM" with trypsin (0.5 μg/ml) (Gibco), before activation for 30 min in a humidified 5% CO_2 incubator. MA104 cells were seeded in a 25 cm^2 tissue culture flask and six-well plate and further incubated in trypsin-MEM. The cells were infected with 2 ml of virus dilution and incubated for 1 h in a CO_2 incubator. After 24 h, the virus dilution was replaced with virus culture medium containing MEM and 0.5 μg/ml of trypsin. The cell lines were monitored daily for cytopathic effect. Cells showing extensive cytopathic effect were stored at -20°C. Cells were scraped, harvested, and centrifuged at 1100 rpm for 5 min (Passage 0). Two milliliters of supernatant obtained from the six-well plate and 4 ml of supernatant obtained from the 25cm^2 flask were each transferred into new 25 cm^2 flasks which were treated with 8 ml and 6 ml, respectively, of MEM with 0.5 μg/ml of trypsin. After 48 h, the cells were scraped, harvested, and centrifuged at 1100 rpm for 5 min, and stored at -20°C (Passage 1). The same protocol as for Passage 1 was repeated

for five times, cells were scraped after 48-72h and stored at -20°C until used in RT-PCR and sequencing.

In the fifth study, MA104 cells were seeded only in a 25 cm^2 flask. The dilution obtained from the stool suspension was diluted in 10% MEM with 10 μg/ml of trypsin. Next, MEM with 0.5 μg/ml of trypsin was used to rinse new flasks, but replaced with MEM containing Penicillin/streptomycin (1:100) and L-glutamine (1:100) as supplement. For the following passages, 5 ml of supernatant was added into new 80 cm2 flasks. The protocol was repeated three times and the supernatant obtained from each passage was stored at -20°C until used in RT-PCR and sequencing.

3.2.2.5 Purification of rotavirus from serum samples (Study III)

To detect possible rotavirus virions in the first study, serum samples from three patients with highly ELISA-positive serum were purified by ultracentrifugation of 4ml of serum at 100,000 x g for 1.5h at + 4°C. The pellets were suspended in 0.2 M Tris-HCl pH7.3 and purified on sucrose gradients (10%, 20%, 30%, 40%, 50%, 60%). Fractions of sucrose containing VP2 and VP6 proteins were pooled, dialyzed against PBS and concentrated by centrifugation on Amicon Ultra-30 filter units (Millipore Corporation). Total protein concentration was quantified using the Pierce BCA Protein Assay (Thermo Scientific, Rockford, USA) and the samples with the highest protein concentrations were chosen for examination in electron microscopy. (359)

3.2.2.6 Electron Microscopy (Study III)

For the detection of rotavirus virions from serum samples in the first study, serum sample was examined in electron microscopy. The samples were examined using an FEI Tecnai F12 electron microscope (Philips Electron Optics, Holland) with 18,500 x magnification following negative staining with 3% uranyl acetate, pH 4.6.

3.2.3 Tests for other viruses (Studies II, IV, V)

3.2.3.1 RNA viruses

Reverse transcription

Viral RNA was transcribed into cDNA before amplifying with random primers (Invitrogen, USA, Catalog number 48190-011).

For RT reaction, 15 µl of master mixture containing 4.0 µl Aqua sterilisata H_20, 4.0 µl 5x first strand buffer, 5 µM DTT, 200 nM dNTP mix, 0.5 µl RNaseOut™ (Invitrogen), 0.5 µl SuperScript™ II (Invitrogen), and 2.0 µl Random primers 300 ng/ µl (Invitrogen) was incubated with 5 µl of extracted RNA first for 60 min at 42°C and then for 15 min at 70°C.

The cDNA products were stored at -20°C until used in PCR reactions.

Human caliciviruses

RT-PCR for human caliciviruses

Human caliciviruses (noroviruses and sapoviruses) were detected by RT-PCR. The primer mixture p289H, I/p290H,I,J,K was used with additional primers p289IUB and p290IUB to detect each norovirus and sapovirus (360).

For the RT reaction, 2.5 µl of extracted RNA was denatured with RT mixture containing 22.9 µl of sterile water, 1X GeneAmp PCR buffer (Applied Biosystems), 1.5M GeneAmp $MgCl_2$ (Applied Biosystems), 400 µM dNTP mix, 10 U RNasin (Promega, USA), 70 U M-MLV Reverse Transcriptase RNase H- enzyme (Promega) and 16 ng/µl 289H,I,IUB reverse primer mixture (reverse primer 5'GATTACTCCARGTGGGAYTCMAC3') for 60 min at 42°C.

For the PCR-reaction, a PCR mixture containing 26.6 µl of sterile water, 5 U GoTaq DNA polymerase (Promega), 1x GoTaq Green buffer (Promega), 0.5 mM $MgCl_2$ (Promega) and a mixture of forward primers p290H,I,J,K,IUB (24 ng/µl) was added to the RT reaction and denatured at 94°C for 2 min and run for 40 cycles of 30sec at 94°C, 1 min 30 sec at 42°C, and 1 min at 72°C, with a final extension at 72°C for 10 min.

The PCR products were run in an agarose gel electrophoresis to confirm the correct size of the product. The length of PCR products positive for NoV is 319 bp and for

sapovirus it is 331 bp. All the positive bands were further sequenced in order to confirm the origin of the virus.

RT-PCR for norovirus genotyping

The norovirus genotypes were defined by the polymerase region A/capsid region C genotype. The RT-PCR targeting region C at the beginning of the capsid region in ORF2 was done with primers JV21 (forward primer, 5'CCNRCMYAACCATTRTACAT3'), JV24 (forward primer, 5'GTAAATGATGATGGCGTCTAA3') and JV24 mod (forward primer, 5'GTGAATGAAGATGGCGTCGA3').

A 317 bp fragment of synthesized cDNA was amplified with primers JV21, JV24, and JV24 mod. For the RT reaction, 5 µl of cDNA was denatured with PCR mixture consisting of 22.5 µl of sterile water, 0.5 µl GoTaq DNA polymerase, 5X GoTaq Green buffer, 1mM MgCl2, 200 µM dNTP mix, and 4 ng/µl mixture of each primer for 3 min at 94°C and for 40 cycles of 30 sec at 94°C, 90 sec at 49°C, and 60 sec at 72°C, with a final extension at 72°C for 10 min.

The PCR products were run in a 2% agarose gel for 90 min at 100V.

Sequencing

Nucleotide sequencing used the same primers as were used in RT-PCR. The sequencing methods were similar to those used for rotavirus sequencing. In addition to nucleotide blast programs, the Food-borne Viruses in Europe (FBVE) network NoV genotyping tool was used for the virus confirmation and genotyping (http://www.rivm.nl/bnwww).

Coronavirus

Human coronaviruses were studied using a two-step nested PCR method (361). For the first PCR reaction, 10 µl of cDNA was added to 40 µl of reaction mixture consisting of 1X Green GoTaq Flexi buffer, 2.5 mM of GoTaq $MgCl_2$, 200 µM dNTP mix, 2.5 U of GoTaq DNA polymerase and 0.5 µM of both primers 5'GWTGGGAYTATCCNAARTGTGA3' (forward primer) and 5'YRTCATCASWNARAATCATCAT3' (reverse primer), universal for all coronaviruses. The cDNA-PCR-mixture was denatured for 2 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 54°C, and 1 min at 72°C, with a final extension at 72°C for 5 min.

For nested PCR reaction, 2 µl of first PCR product was added to reaction mixture containing 1X Green GoTaq Flexi Buffer, 1.5 mM of GoTaq $MgCl_2$, 200 µM dNTP mix, 2.5 U of GoTaq DNA polymerase and 0.5 µM of each of three primers to distinguish coronavirus groups 1B, 2A and SARS. Primers for group 1B forward primer: 5'GTTGTTTATTCWAATGGTGG3' and reverse primer: 5'YCTATARCAATTATCATAMAG3' for group 2A forward primer: 5'WYTRCGTATTGTTAGTAGTTTRGT3' and reverse primer: 5'CGTATACTWARATCTTCAATCTT3' and for SARS forward primer: 5'TGCTGTAACTTATCACACCGT3' and reverse primer 5'CGGACATACTTGTCAGCTATCT3'. The nested PCR reaction was run for 2 min at 94°C, followed by 35 cycles of amplification for 30sec at 94°C, 30 sec at 53°C, and 30 sec at 72°C, with a final extension at 72°C for 5 min.

The PCR products were run in agarose gel electrophoresis to separate and identify the length of bands/type of coronaviruses (1B = 203 bp, 2a = 275 bp, SARS = 230 bp).

3.2.3.2 DNA viruses

Bocavirus

Human bocavirus (HBoV) ssDNA was amplified by nested PCR. The first PCR amplification produces a 959 bp amplicon of gene NS1 encoding for non-structural protein using primers HBoV NS1 1st fwd and HBoV NS1 1st rev. In the second (nested) PCR primer pair (HBoV NS1 2nd fwd/rev and Boca NS-1 fwd/rev) is used for amplifying regions inside the first PCR product, detecting all human bocaviruses and human bocavirus type 1 (353).

The HBoV primers and their nucleotides as following for HBoV NS1 fwd: 5'GGACGTGGTSCGTGGGAAC3', for HBoV NS1 rev: 5'GTCCTGTGAATGWGTAGGACAAAGG3', for HBoV NS1 2nd fwd: 5'CCWGTAATTATWTCCACTAACCA3', for HBoV NS1 2nd rev: 5'AGAGTACAKTCGTACTCATTRAA3', for Boca NS-1 fwd: 5'TATGGCCAAGGCAATCGTCCAAG3' and for Boca NS-1 rev: 5'GCCGCGTGAACATGAGAAACAGA3'.

PCR products are recognized and separated by gel electrophoresis.

Adenovirus

The presence of adenovirus in fecal specimens was tested using a ProSpecT™ Adenovirus immunoassay (362). The procedure was identical to the rotavirus ELISA, except that adenovirus-specific monoclonal antibody coated microwells were used.

3.2.4 Statistical analyses (Studies I-III)

All tests were performed in SPSS (version 20.0 (SPSS)) and were two-tailed, and a p value <0.05 was considered to be statistically significant.

"Vesikari" scoring system (Study I)

For the statistical analyses, symptoms were graded according to the Vesikari 20-point scoring system for severity of rotavirus diarrhea (Table 4) (355).

Statistical analyses were performed using the Kruskal-Wallis test to compare the distribution of age, level of fever, duration of diarrhea and vomiting, maximum number of vomiting episodes/24 h and maximum number of diarrheal stools/24 h in different groups. If the Kruskal-Wallis test showed statistical significance, the groups were further compared in pairs using the Mann-Whitney U test. The probabilities in different groups were calculated using the chi-square test.

Pre-NIP and post-NIP analyses (Study II)

Statistical analyses were performed using the Mann-Whitney U test to compare the age distribution of RVGE and using the chi-square test to calculate the reductions in RVGE between pre-NIP and post-NIP years.

Sequence analyses (Study III)

The statistical significances were assessed by bootstrap resampling analysis (2000 pseudo-replicates).

4 Results

4.1 Rotavirus infections

4.1.1 Clinical picture (Study I)

Of 131 children with rotavirus positive laboratory findings from stools, serum and whole blood specimens, 48 (37%) were treated as outpatients and 83 (63%) were hospitalized. The age range among these children was from 2 months to 11 years and 6 months and the mean age was 2 years and 1 month.

The 131 children with RVGE had diarrhea, vomiting and fever in 99%, 99% and 89%, of cases, respectively. Dehydration was evaluated in 121 of 131 patients and 92% of them had at least moderate dehydration. The mean Vesikari score for these 121 children seen in the outpatient clinic and hospital ward was 16 (with a range from 7 to 19). Often a score of 11 or higher is regarded as severe. By this definition, 113 (93%) of the 121 children had severe RVGE.

4.1.2 Rotavirus RNAemia and antigenemia (Study I)

Both a serum and a stool sample were obtained from 374 patients, 155 (41%) of whom had RV in the stools. Of these 155 children, 67% had RV RNA and 61% had RV antigen detected in serum. None of the 50 children negative for RV RNA in serum were positive for RV RNA in whole blood. In addition, none of 85 control patients in whom other gastroenteritis pathogens were detected was positive for RV RNA or RV antigen in any specimen. Of 131 rotavirus-positive children with available stool, serum, and whole blood laboratory results, 81 (62%) had RV RNA and 68 (52%) had RV antigen in serum.

Age distribution

The children positive for RV RNA also in serum were slightly younger than children in whom RV RNA was detected only in stools, but the difference was not statistically significant.

RV genotypes

The RV genotypes detected in stool, serum, and whole blood samples were concordant in each case. The predominant RV genotype detected in stools was G1P[8] (N=99, 76%), whereas the other detected genotypes G4P[8], G9P[8], G2P[4], and G3P[8] combined accounted for 24% (N=32). Although G1P[8] genotype was also more common overall in serum samples than were the other genotypes combined (71% of serum samples were positive for G1P[8] and 34% for non-G1P[8] genotypes), the presence of RV RNA in serum was not limited to any specific RV genotype. Furthermore, the G1P[8] genotype was also more often associated with RNAemia and antigenemia than were the other genotypes combined (p=0.001 and p=0.009, respectively).

Virus isolation

The total concentrations of purified serum samples were 22 mg/ml and 47 mg/ml from suspension samples and 2 mg/ml from pellet sample. However, no whole virions or inner core particles were visualized when these samples were studied in electron microscopy.

The serum samples were also cultivated for RV in MA104 cells, but the attempts were not successful.

4.1.3 Clinical severity of rotavirus cases with and without extraintestinal infection (Study I)

Diarrhea

Children with RV RNA in serum or blood did not have more severe diarrhea than children found to have RV RNA only in stools (p=0.423). Neither the maximum number of diarrheal stools/24 h nor the duration of diarrhea differed significantly among children when grouped according to their laboratory results (p=0.310, p=0.856, p=0.479, and p=0.603).

Vomiting

Severe vomiting (five or more episodes/24 h) was more common among children with positive serum ELISA than among those negative for RV antigen in serum (p=0.004). When the groups were compared in pairs, children positive for RV RNA in stools and

serum and RV antigen in serum, irrespective of their whole blood results had more severe vomiting than children positive for RV RNA only in stools (Table 6). In addition, children detected with RV RNA in serum, and according to the number of positive laboratory results, were more likely to suffer from severe vomiting than children who had RV RNA only in stools ($p=0.015$ and $p=0.037$).

However, the duration of vomiting did not differ in patient groups with different RV laboratory results. Moreover, although the presence of RNAemia or antigenemia did not have any statistically significant correlation with age, groupwise comparison showed that children with severe vomiting were older than children with non-severe vomiting ($p<0.001$).

	Vomiting	≥5 episodes/24h	Duration ≥3 days
Group 1 (N=22)	100 %	86 %	81 %
Group 2 (N=46)	100 %	72 %	76 %
Group 3 (N=13)	100 %	46 %	69 %
Group 4 (N=50)	98 %	50 %	62 %

Table 6. Prevalence of vomiting across rotavirus groups and the proportion of children with most severe vomiting by quantity and duration (≥5 vomiting episodes/24h and duration ≥3 days).

Fever

Patients positive for RV RNA in both stool and serum or with positive ELISA results were more likely to have fever (>37.1°C) than patients in whom RV was detected only in stools (p=0.013), as the probability of fever of any level differed between RV groups (p=0.024) (Table 7). Similarly, patients detected with RV antigen in serum more often had fever than patients with negative serum ELISA results (p=0.019). In addition, the probability of a higher level of fever rose according to the number of positive laboratory results (p=0.045). However, a statistically significant difference was found only between children positive for RV RNA in stool, serum, and whole blood, and ELISA-positive serum (Group 1) and children positive for RV only in stools (Group 4).

	No fever	37.1-38.4°C	38.5-38.9°C	≥39°C
Group 1 (N=22)	0	18.2% (4)	18.2% (4)	63.6% (14)
Group 2 (N=46)	4.3% (2)	19.5% (9)	28.3% (13)	47.8% (22)
Group 3 (N=13)	23.1% (3)	23.1% (3)	30.7% (4)	23.1% (3)
Group 4 (N=50)	18.0% (9)	22.0% (11)	16.0% (8)	44.0% (22)
Total	10.7%	20.6%	22.1%	46.6%

Table 7. Distribution of different levels of fever in rotavirus groups (%, (N)).

Dehydration

Information on dehydration was available on 121/131 children. As 92% (111) of these children had at least moderate dehydration, statistical significances were not calculated because of the small number of the remaining cases. However, the remaining 10 children without any dehydration were all of those detected with RV only in stools.

Vesikari score for severity of gastroenteritis

The Vesikari scores were calculated for the 121 patients with available information on dehydration. Children found to have RV also in serum, irrespective of their whole blood result, had more severe illness by the Vesikari score than children with RV only in stools (p=0.008 in patients with positive whole blood result and p=0.004 in patients with negative whole blood result). In addition, children with positive serum ELISA result had more severe illness than children with negative ELISA result in serum (p<0.001).

4.2 Effects of universal rotavirus vaccination in Finland

To estimate the effects of the universal rotavirus vaccination program in Finland, results from two similar studies were compared. The study data from pre-NIP years 2006-2008, when the rotavirus vaccine was available on the private market but coverage was less than 35%, was collected by Sirpa Räsänen and co-workers (62). The vaccine mainly used during those years was Rotarix™, and a 22% coverage was achieved in the first season (2006-2007) increasing up to 35% in the second season (2007-2008) (29% Rotarix™ and 6% RotaTeq®).

4.2.1 Hospitalizations and outpatient clinic visits (Study II, unpublished results from 2011-2013)

In the pre-NIP period from September 2006 to August 2008, of 809 recruited patients with a stool sample, 375 (46%) were treated as outpatients and 434 (54%) were hospitalized (62). In the post-NIP period from September 2009 to August 2011, a stool sample was obtained from 330 of recruited children, of whom 144 (44%) were treated at the outpatient clinic and 186 (56%) were hospitalized.

4.2.1.1 Hospitalizations and outpatient clinic visits for rotavirus gastroenteritis

Rotavirus burden of disease

RV was found in 128 (38%) of 341 stool samples obtained in the first pre-NIP season (2006-2007), and in 293 (63%) of 468 stool samples obtained in the second season 2007-2008 (62). During the first two study years after RotaTeq® was added into the NIP, from September 2009 to August 2010 and from September 2010 to August 2011, wild-type (not vaccine-acquired) RV was found in 43 samples each of 160 and 170 stool samples obtained (27% and 25%, respectively).

Comparing the pre-NIP years and first two post-NIP years, the total reduction in the number of RVGE cases seen in outpatient clinics and hospital was 80% (86 vs. 421 cases). Furthermore, the burden of RV disease has continued to decrease: in the most recent season, from September 2012 to August 2013, RV was found in 17 (9.2%) of 183 stool samples obtained from children seen at the outpatient clinic or in hospital wards with AGE (Fig. 5).

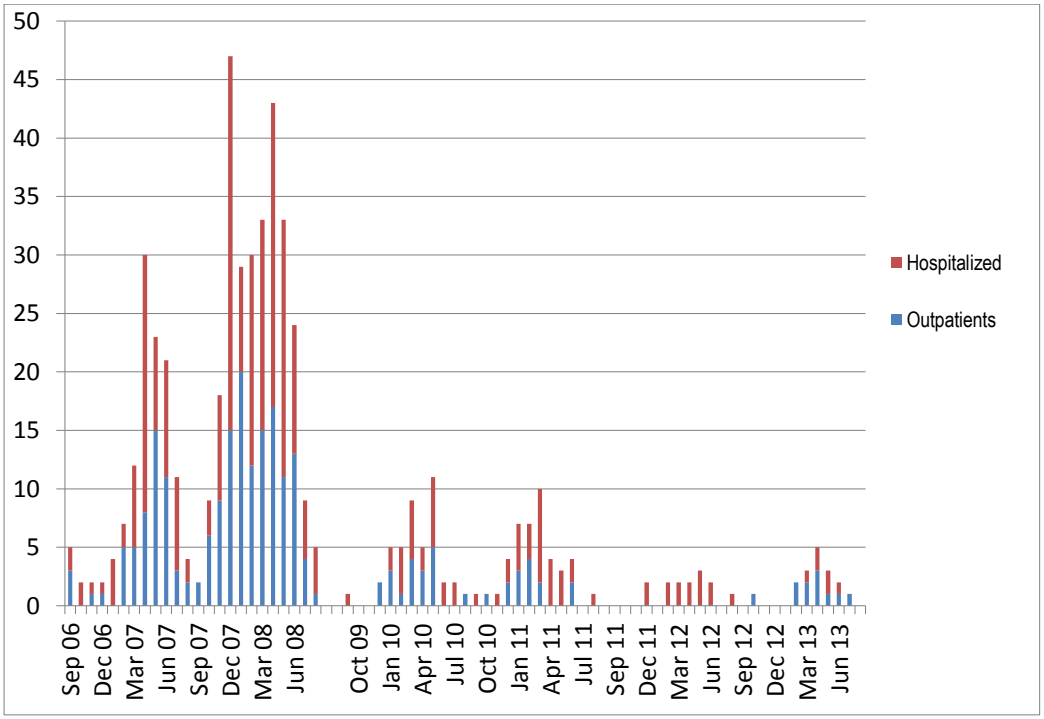

Figure 5. Timing of rotavirus seasons and hospitalizations for RVGE during pre-NIP seasons 2006-2008 and post-NIP seasons 2009-2013.

Outpatient clinic visits

During the pre-NIP years, 42% (177) of 421 children with RVGE were seen at the outpatient clinic (62). The distribution of children seen in the outpatient clinic remained similar in the post-NIP years when 40% (34) of all 86 children with wild-type RVGE were seen at the outpatient clinic. Thus, the number of RVGE cases seen at the outpatient clinic went down by 81% (34 vs. 177 cases) (Fig. 5). In the most recent season (2012-2013), 11 of 17 patients (65%) with RVGE were treated at the outpatient clinic; when this year was compared with the average of RV cases seen in the outpatient clinic in pre-NIP years, the reduction was 88%.

Hospitalizations

In the pre-NIP period, 54% (227) of 421 children with RVGE were seen in the hospital ward (62). Of these 227 children, 219 had non-hospital-acquired RV infections, whereas in 8 cases the RV infection was hospital-acquired. During the first

two post-NIP years, only 52 children required admission to a hospital ward because of RVGE, giving a 76% reduction in RVGE hospitalizations (52 vs. 219 cases) (Fig. 5). Moreover, since the mass use of RotaTeq® vaccine, no hospital-acquired RV infections have been detected in Tampere University Hospital. In the two following post-NIP years, from September 2011 to August 2012 and from September 2012 to August 2013, 24 and 6 patients, respectively, were hospitalized for RVGE. If the last year alone is compared with the average from pre-NIP years, the reduction in rotavirus-associated hospitalizations is 95%.

Age distribution

The age distribution of children with RVGE ranged from 2 months to 14 years 7 months in the two pre-NIP years and from 7 months to 14 years 6 months in the first two post-NIP years. In the post-NIP period, no infants less than 6 months of age were seen for RVGE due to wild-type RV. The age distribution of RVGE patients shifted toward older children in post-NIP years ($p<0.001$) (Fig 6). In the pre-NIP period the median age was 19 months, while after the introduction of NIP it was 24 months in 2009-2010 and 36 months in 2010-2011. In the most recent season, 2012-2013, the median age of children with RVGE was 45 months.

However, the proportion of RVGE cases decreased in all age groups, even among children too old to be vaccinated. In 2009-2010 patients less than one year of age, and in 2010-2011 patients less than 2 years of age were considered to be eligible for vaccination. The reduction of RVGE in children eligible for vaccination (including vaccine-originated infections) was 91% (RVGE burden of disease decreased from 178 cases in pre-NIP years to 16 cases in post-NIP years). In children too old to be vaccinated in the NIP, cases of RVGE were reduced by 72% (from 243 cases to 70 cases) (Fig. 6). None of six patients hospitalized in 2012 had received any RV vaccine (they were too old to receive the vaccine in the NIP).

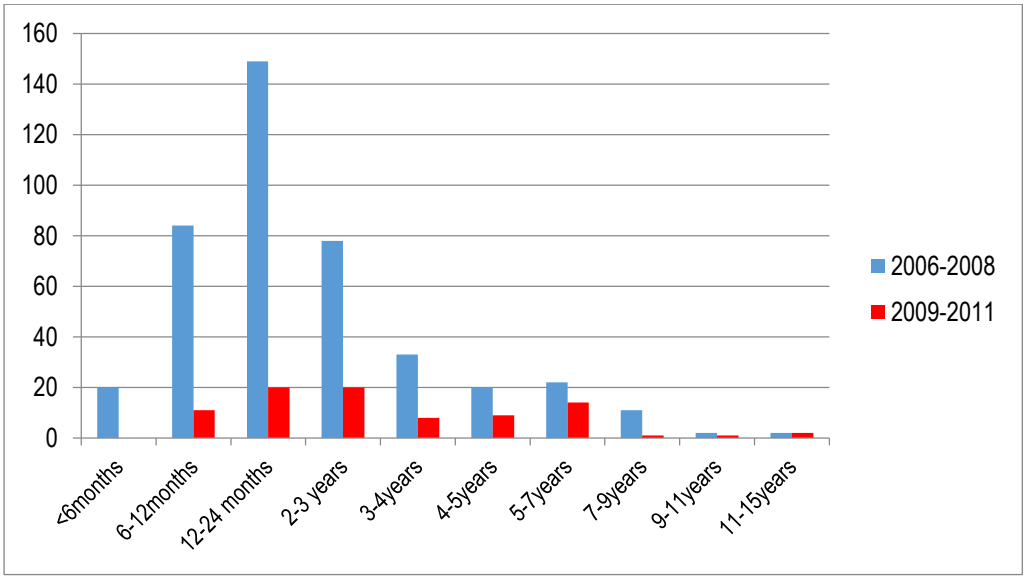

Figure 6. Age distribution of children with RVGE due to wild-type rotavirus infection in pre-NIP years 2006-2008 and post-NIP years 2009-2011.

Seasonal distribution

The seasonality and most active months of RV infections are known to fluctuate slightly from year to year. In Finland in the first pre-NIP period, from 2006 to 2007, the season was late and the majority of RVGE cases were seen between April and June, whereas in the next season, 2007-2008, the most active months started earlier in December but again continued until June. The majority of RVGE cases were seen between March and May in the first post-NIP season, 2009-2010, and between January and March in the second season 2010-2011 (Fig 5).

In the years 2011-2012 and 2012-2013, the few cases seen were spread over several months without any clear peak season.

4.2.1.2 Effects on all cases of acute gastroenteritis and other gastroenteritis with specific etiology (Study II)

All acute gastroenteritis cases

The reduction in all RVGE cases was seen as a 59% decrease in all AGE cases seen in both outpatient clinic and hospital ward (809 vs. 330 cases). The number of outpatient clinic visits for AGE of any cause was reduced by 62% (from 375 cases to 144 cases) and hospital ward admissions went down by 57% (from 434 cases to 186 cases).

Norovirus gastroenteritis cases

In the two pre-NIP years, NoV accounted for 26% (196 cases) of all AGE cases, of which 47% (92 cases) were seen in the hospital and 53% (104 cases) at the outpatient clinic (352). In the second pre-NIP season, children infected in the Nokia outbreak due to massive contamination of drinking water with sewage water were excluded from the analysis of NoVGE (had they been included, the proportion of NoVGE have been around 32%) Of all 330 cases of AGE in the post-NIP period 2009-2011, 34% (111 cases) were norovirus-positive. The absolute number of all NoVGE cases decreased slightly from 196 to 111 cases, but the proportion of NoVGE as a percentage of all AGE cases increased from 26% in the pre-NIP years to 34%, resulting in NoV being the most common causative agent of AGE in the post-NIP years. In addition, the proportion of NoVGE cases admitted to hospital increased from 47% to 69%, whereas the proportion of cases seen at the outpatient clinic decreased from 53% to 31%. In the most recent season 2012-2013, NoV accounted for 37% (66 cases) of all AGE cases and for 42% of all hospitalizations due to AGE.

The age distribution of children infected by NoV remained similar during the four study years. In the post-NIP period 84% of children with NoVGE were under 24 months of age and 41% were under 12 months of age. The age range was from 7 days to 15 years 7 months in the post-NIP period; in the pre-NIP period it was similar from 19 days to 13 years 8 months.

A clear seasonality of NoV infections was seen in the pre- and post-NIP periods. The majority of NoV infections occurred between January and April in each year (Fig 7).

Ninety-seven percent of NoVGE cases found in the post-NIP years were caused by genogroup GII noroviruses; more specifically, 65% of them were GII.4 strains. In the pre-NIP years the genotype GII.4 was even more common, representing 89% of all NoV cases. The other genotypes detected in the pre-NIP period were GII.1, GII.c, GI.6, and GII.2, which combined accounted for 11%, whereas in the post-NIP period

the proportion of other genotypes was larger with GII.b (14%), GII.7 (13%), GII.g (5%), GI.4 (2%), G1.3 (1%), and GII.e (1%) genotypes present.

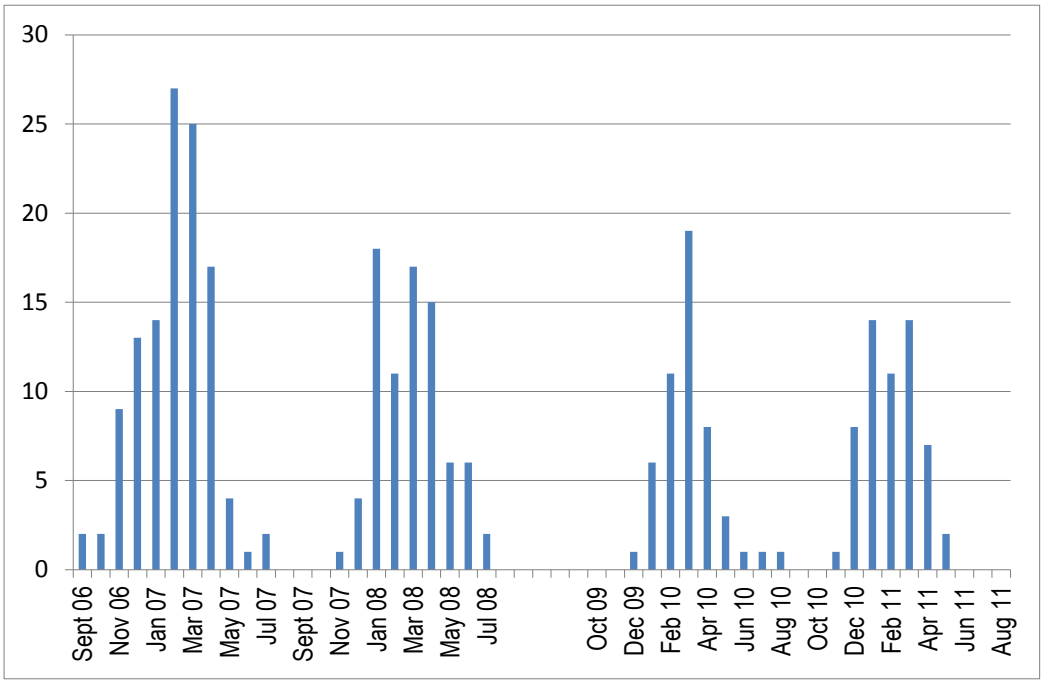

Figure 7. Prevalence and seasonality of NoVGE in pre-vaccination years 2006-2008 and post-vaccination years 2009-2011.

Sapovirus gastroenteritis

The absolute number and percentage of sapovirus-positive cases of gastroenteritis out of all gastroenteritis increased from 12 cases (1.6%) in the pre-NIP period to 23 cases (7.0%) in the post-NIP period.

Gastroenteritis of other etiology

In the pre-NIP period, 76% of all AGE cases were positive for either rotavirus, norovirus, or sapovirus, whereas in the post-NIP period, 67% were positive for these pathogens. The number of AGE cases caused by other pathogens (cases negative for RV, NoV, and sapovirus) actually decreased from 191 (23.6%) cases in the pre-NIP period to 108 cases (32.7%) in the post-NIP period.

Although a systematic research was not performed, some of the patients from pre-NIP study years as well as from post-NIP years were shown to have other viral agents such as human bocavirus, adenovirus, astrovirus, or coronavirus in their stools (353,361). In addition, we found patients shedding original vaccine viruses or parts of them in their stools (discussed in 4.2.2 Rotavirus infections in vaccinated children).

4.2.2 Rotavirus infections in vaccinated children

In the post-NIP years 2009-2011, 82 of all 330 children with AGE of any cause (25%), had been eligible for RV vaccination in NIP. Of the 86 wild-type rotavirus-infected children seen in those two post-NIP years, five had received at least one dose of either RotaTeq® or Rotarix™ vaccine.

In the first two post-NIP years, we detected a new vaccine-derived human-bovine double reassortant rotavirus in four vaccinated children (discussed later in Results). In addition, we found several vaccinated children, infected by another GE pathogen (most commonly norovirus) who were concomitantly shedding RotaTeq® vaccine viruses or parts of them.

Wild-type rotavirus infections (Study II, unpublished results from 2012-2013)

Four of the five vaccinated children with wild-type rotavirus infection had received RotaTeq® and one had received Rotarix™ vaccine. Of the RotaTeq® vaccinated children, three were fully vaccinated and were infected by the G4P[8] or G9P[8] genotype (Figure 8). One child had received only one dose of RotaTeq® and was found to be infected with the G4P[8] genotype, as was the child fully vaccinated with Rotarix™. Two of these breakthrough cases were admitted to a pediatric ward (fully vaccinated 9- and 10-month-old boys), while the others were treated at the outpatient clinic.

In the most recent season, 2012-2013, of the 17 patients with RVGE, 4 were fully vaccinated with RotaTeq® and detected to have wild-type RV genotypes G3P[8] (1 case), G9P[8] (1 case), and G12P[8] (2 cases) (Fig. 8). The age distribution of these children was from 8 months to 10 months. However, their clinical picture was fairly mild and hospital admission was not required.

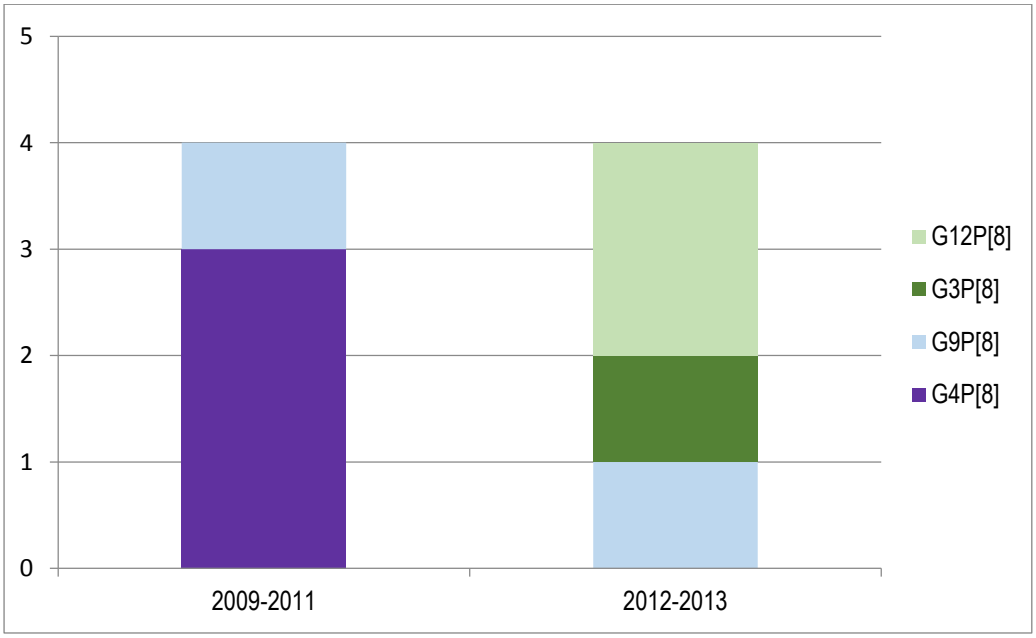

Figure 8. Genotype distribution of RotaTeq®-vaccinated children found to be infected with wild-type rotavirus. Except for one child in 2009-2011, infected by G4P[8], all children had received the full RotaTeq® regimen.

4.2.3 Effects on rotavirus genomics and vaccine-derived viruses

4.2.3.1 Genotype distributions (Study II)

The predominant RV genotype in both the pre-NIP period and the post-NIP period was G1P[8]. In the first pre-NIP season, 2006-2007, G1P[8] accounted for 40% and G9P[8] for 38% of RVGE cases. In the second pre-NIP season, G1P[8] was the predominant strain at 73%. The other genotypes detected in the two pre-NIP years were G4P[8] (10%), G2P[4] (8%), and G3P[8] (1%). In addition, in four cases more than one RV genotype was found in stools simultaneously and in one case a child was detected to have a rhesus rotavirus.

In the post-NIP period 2009-2011, the predominant genotypes were G1P[8] (44%) and G4P[8] (35%). Otherwise, the same genotypes were detected in the first two post-NIP years and in the pre-NIP period. After the first two post-NIP years, the proportional role of G3P[8] increased and that of G4P[8] decreased. In addition, we found the G12P[8] genotype for the first time between September 2012 and August 2013 (Fig. 9).

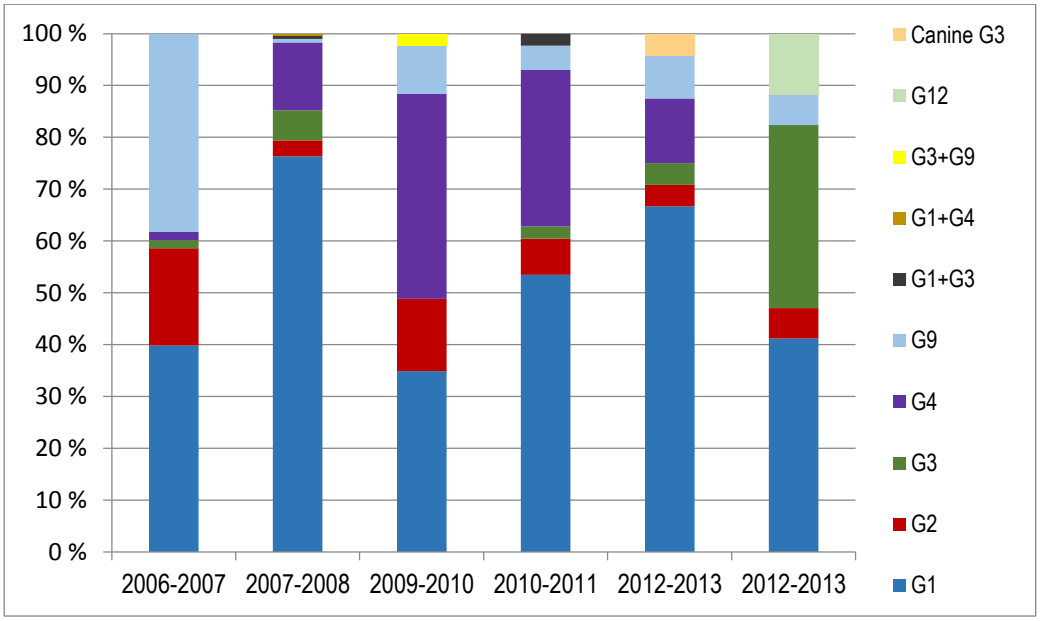

Figure 9. G-type distribution of RVGE cases detected in 2006-2008 and 2009-2013 in Tampere University Hospital.

4.2.3.2 Immunological pressure on VP7 and VP8* antigens (Study III)

To evaluate any existing vaccine-induced immunological pressure on the outer capsid proteins of the most prevalent G1P[8] rotavirus, we examined the nucleotide and amino acid sequences of the VP7 and VP8* antigens over a 20-year period, including the years when rotavirus vaccine was available (2006-2009) and years after it was adopted into the NIP (2009-2012).

VP7 sequences

At both at nucleotide and amino acid level, all 108 strains collected aligned into 12 published sequence strains, which further aligned into two different subgroups referred as sublineages G1-I and G1-II (Fig 10). Both of these two sublineages were present in each time period. The difference within strains aligning into the same sublineage was 1.3-3.6% nucleotide substitutions for the G1-I sublineage and 1.7-4.1% substitutions for the G1-II sublineage. The majority of nucleotide substitutions occurred between purines (A,T) or pyrimidines (C,G). The Rotarix™ VP7 sequence aligned with G1-II strains, whereas RotaTeq® VP7 sequence aligned into a completely distinguished G1-III sublineage. As at amino acid level the results, were identical with the nucleotide

level results and the two sublineages were present in each time period without shifting towards or away from the vaccine strains, it may be inferred that the use of Rotarix™ and RotaTeq® has not had any effect on the VP7 protein of circulating G1P[8] strains.

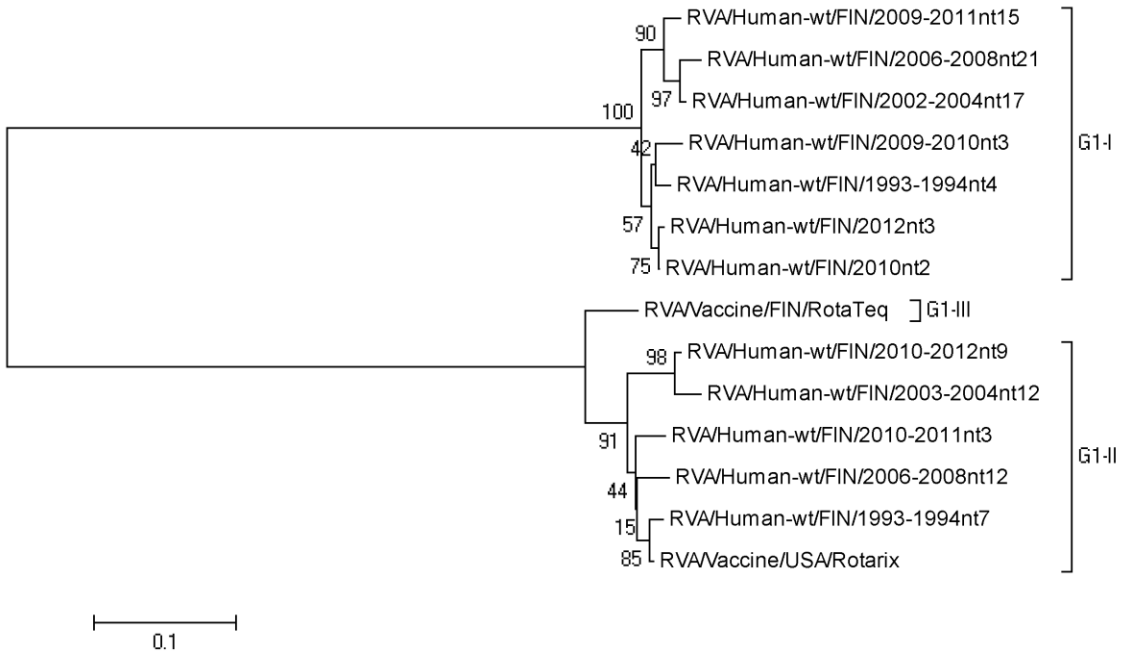

Figure 10. Phylogenetic dendrogram of VP7 of G1P[8] sequences at nucleotide level in the years 1992-2012 in Finland. Bootstrap values (2000 replicates). Intragenotypic lineages are given by square brackets on the right. Each strain is presented only once and includes all 100% identical strains. The strains are named according to their year of circulation followed by "nt" as nucleotide sequence and the number of identical strains in each time period.

VP8 sequences*

Like the VP7 sequences, the VP8* sequences aligned into two intragenotypic lineages P[8]-I and P[8]-III. Surprisingly, those two intragenotypic lineages were not present at the same time as the strains from 1992-1994 and 2006-2008 clustered in the same subgroup as Rotarix™ vaccine, defined as subgroup P[8]-I, whereas sample strains from 2002-2004 and 2009-2012 clustered in subgroup P[8]-III (Fig 11). The RotaTeq® vaccine aligned in subgroup P[8]-II.

At nucleotide level, strains from every pre-NIP period (1992-1994, 2002-2004 and 2006-2008) aligned all into two sublineage groups, whereas the strains from the post-

NIP period showed more diversity by aligning into four sublineage groups. However, at amino acid level, strains from 1992-1994 and 2006-2008 aligned into one sublineage each. The sequences from the P[8]-III subgroup were closer to the RotaTeq® strain representing subgroup P[8]-II than the strains from subgroup P[8]-I (4.4-5.0% substitutions vs. 6.1-6.6% substitutions from closest to farthest, respectively). The difference between G1 VP4 antigens of wild-type rotaviruses in subgroups P[8]-I and P[8]-III ranged from 6.5% to 7.6% (12 to 14 substitutions in a 184 aa fragment), however, not all of the nucleotide changes between strains led into changes at amino acid level.

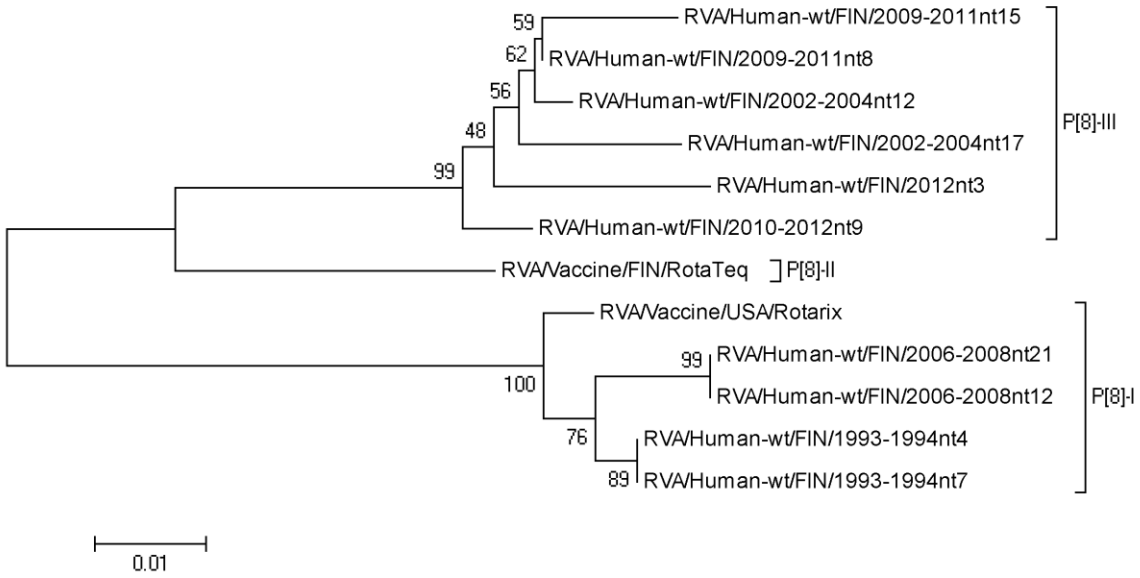

Figure 11. Phylogenetic dendrogram of VP8* of G1P[8] sequences at nucleotide level in the years 1992-2012 in Finland. Bootstrap values (2000 replicates). Intragenotypic lineages are given by square brackets on the right. Each strain is presented only once and includes all 100% identical strains. The strains are named according to their year of circulation followed by "nt" as nucleotide sequence and the number of identical strains in each time period.

All of those substitutions between strains from 2002-2004 and 2009-2012 versus strains from 1992-1994 and 2006-2008 occurred individually between amino acids 66 and 196 except for two consecutive amino acid changes in positions 120 and 121 aa, located in the fourth of five sequential neutralizing epitope regions of the Wa* protein. In addition, nine of these substitutions were located in the known serotype-specific neutralizing epitope regions (8-1 and 8-3) (Table 8). The amino acid substitutions in the one sequential neutralizing epitope and the two surface-exposed antigenic epitopes

shifted according to the phylogenetic tree, presented above, so that for the most part the strains from 2002-2004 and 2009-2012 resembled RotaTeq® VP8*, and the strains from 1992-1994 and 2006-2008 were more similar to the Rotarix™ VP8*.

Name/		8-1			8-3				IV	
Number	Year	146	190	196	113	125	131	135	120	121
RotaTeq®	2011	S	N	D	N	N	R	D	T	V
Rotarix™	1988	S	S	N	N	S	S	N	M	I
35	2009-2012	G	N	G	D	N	R	D	N	V
33	2006-2008	S	S	N	T	S	S	N	M	I
29	2002-2004	G	N	G	D	N	R	D	N	V
11	1992-1994	S	S	N	N	S	S	N	M	I

Table 8. Periodical amino acid changes in surface-exposed antigenic residues (8-1 and 8-3) and neutralizing epitope (IV) compared to RotaTeq® and Rotarix™ vaccines.

VP7 and VP8 sequences and antigenemia and RNAemia*

To determine the association of specific RV VP7 and VP4 sublineages and the presence of RNAemia and antigenemia and thus, the clinical severity of the disease - we studied the VP7 and VP8* sequences of G1 strains included in both Studies I and III. Only 8 of 33 children detected with G1 genotype rotavirus circulating between 2006-2008 had stool, serum and whole blood samples available and were included in both studies. Of these VP7 strains, four aligned into sublineage G1-I whereas four aligned into sublineage G1-II. All VP8* sequences detected between 2006 and 2008 aligned into P[8]-I sublineage.

4.2.3.3 Vaccine-derived double reassortant rotavirus (Studies IV-V)

Vaccine-derived double-reassortant rotavirus in vaccinated infants (Study IV)

In the two post-NIP years, we found 21 children shedding vaccine-originated RV RNA sequences (VP7, VP4, and/or VP6) in their stools. After RT-PCR of these gene segments, the nucleotide sequences were analyzed by sequencing.

Seventeen of these children were found to be shedding original RotaTeq® virus G1P7[5] or G6P[8] or parts of them, and were concomitantly detected to have another gastroenteritis pathogen. In addition, we found four children shedding a new human-bovine double reassortant G1P[8] rotavirus.

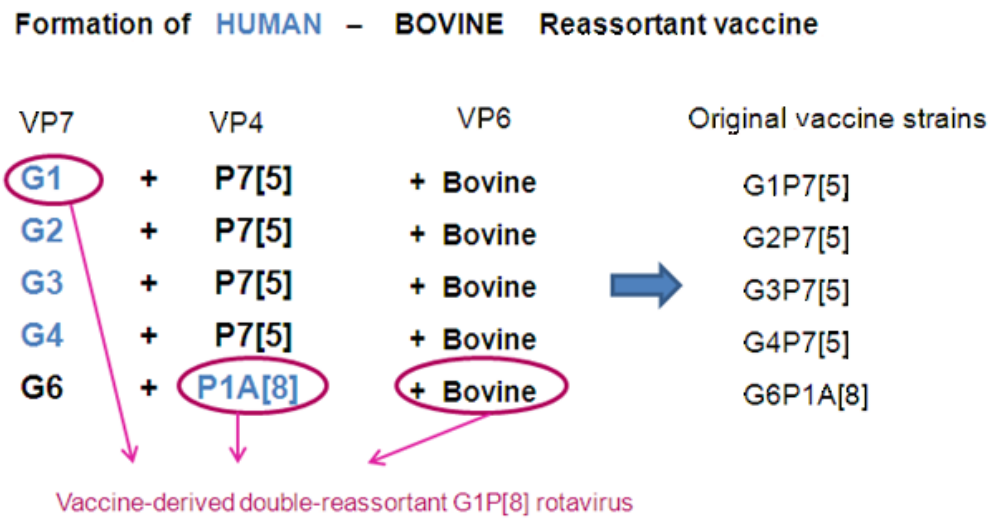

Figure 12. RotaTeq® vaccine strains and formation of vaccine-derived double-reassortant G1P[8] rotavirus. Proteins marked in blue are of human origin, whereas proteins marked in black are of bovine origin.

Detection

Children with vaccine-derived human-bovine double-reassortant G1P[8] rotavirus were detected to have VP7 antigen originating from the G1P7[5] vaccine strain and VP4 antigen originating from G6P[8] vaccine strain, and had VP6 protein from bovine origin (Fig. 12). All of these gene segments were 100% identical to the cognate gene

segments from RotaTeq®. Neither RT-PCR nor sequencing detected any other rotavirus types in their stool specimens.

Stool samples from children with vaccine-derived double reassortant G1P[8] rotavirus were studied for the presence of rotavirus antigen, and the one with a positive ELISA result was propagated successfully in MA104 cells for five passages. In the cell culture, the double-reassortant rotavirus remained stable as a double reassortant, and sequencing of the VP7, VP4, and VP6 gene segments was identical to that of the original isolates (Fig. 13).

Patients

All of these four patients with vaccine-derived double reassortant G1P[8] rotavirus had received the first or the second dose of RotaTeq® within one week before their onset of symptoms and attendance for health care. Three of these patients were negative for other gastroenteritis pathogens studied, and one had a concomitant norovirus infection. Of the three patients in whom exclusively vaccine-derived reassortant rotavirus was detected, all were suffering from diarrhea, vomiting, and fever. The children had grown and developed normally, and with the exception of one child who had had a cardiac surgery operation, did not have any previous hospital admissions or disease.

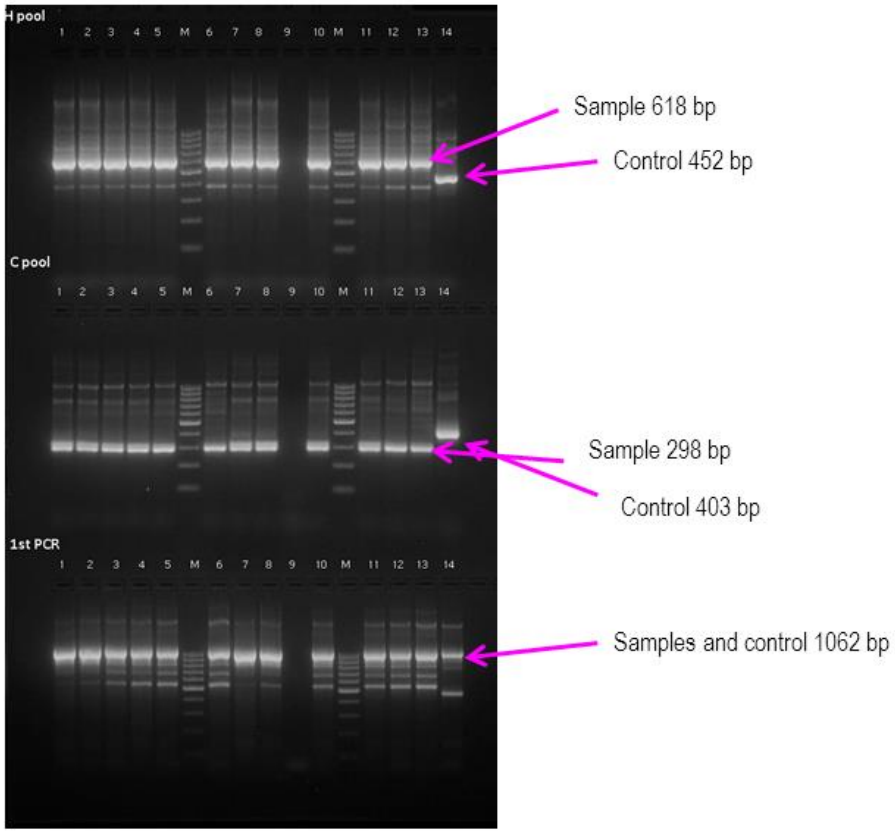

Figure 13. RT-PCR results from cell culture passages 0-5 from bottle (lanes 1-6) and plate (lanes 7, 8, 10-13) of VP7 gene of vaccine-derived double reassortant G1P[8]. Negative control (sterile water) in lane 9 and positive control (G4 rotavirus) in lane 14, with 100 bp DNA markers in "lane M".

Vaccine-derived double-reassortant rotavirus in unvaccinated children (Study V)

When the stool samples from the two latest post-NIP seasons (2011-2013) were analyzed, we found one child with vaccine-derived human-bovine reassortant G1P[8] rotavirus. This child was a 7-year-old schoolgirl hospitalized for AGE. The stability of the reassortant rotavirus was tested in MA104 cell culture and ELISA, as was done with reassortant cases found in earlier seasons (2009-2011). Two consecutive stool samples were ELISA-positive and the virus isolated from these remained stable in cell culture.

The child had not received any rotavirus vaccination, did not have any immunocompromising condition and had no other viral pathogen detected. The family did not have known contact with any infant who might have recently received rotavirus vaccine.

The child gave a follow-up stool specimen 14 weeks later for detection of any prolonged shedding of the virus, but this stool specimen was negative for rotavirus in RT-PCR. The patient's unvaccinated 11-year-old and 5-year-old siblings had had symptoms of gastroenteritis before the 7-year-olds onset of symptoms, but as their clinical picture was milder, they did not require any healthcare visit and their stool samples were not available.

5 Discussion

5.1 Rotavirus infections (Study I)

5.1.1 Clinical picture

In Study I, we examined the clinical picture of RVGE and the presence of RNAemia and antigenemia in children. The majority of children (93%) enrolled in the study had a Vesikari score of 11 or higher, indicating severe gastroenteritis, with diarrhea, vomiting, and fever. The percentage of children with severe RVGE was slightly higher than in other studies, which might be explained by patient selection as only those children with eligible stool, serum, and whole blood samples were included in the final analysis, and it is the most severe cases that are referred to Tampere University Hospital. However, using a different cut-off point for severe gastroenteritis (e.g., score ≥15/20), a clearer distinction between truly severe and milder cases could have been made. The clinical picture of RVGE was described in the late 1970s in the studies of Vesikari et al. and Mäki et al., who showed the clinical picture of RVGE to be more severe than that of "non-rotavirus" gastroenteritis (4,363).

The number and proportion of children with RV RNAemia and antigenemia were similar to previous studies (109-111), as 67% of children with RVGE had RV RNAemia and 61% RV antigenemia.

Our study supports the notion that RV viremia may contribute to the clinical manifestations of RV infections (109,110,112,113) as children positive for RV RNA and antigen were more often showed quantitatively severe vomiting and higher levels of fever than patients with RV RNA only in stools. Since the diarrhea is more a local reaction in the intestines than vomiting which requires activation in the brain stem, it seems plausible that children with RV-induced emesis and more severe illness have stronger and/or deeper infection in their intestines, and this assumption is supported by the restricting effect of pre-existing antibodies and previous observations of an overall milder clinical picture in subsequent RV infections (10). Children who were positive for RV RNA in serum were slightly younger than those who were negative, which may suggest that they more often had a primary rotavirus infection. The serum antibody levels were not studied to determine possible subsequent rotavirus infection, but it seems plausible that immunity developed from previous infections could reduce

the spread of the virus and restrict the infection to the intestines. This concept is supported by a previous finding of an inverse association between baseline serum IgG titers and levels of serum antigen and serum RNA (113).

5.1.2 Pathophysiology/genotype distribution and virus isolation

In a previous study by Ray et al., the levels of RNA in serum and stools were found not to be associated, and, similar to our results, children infected with a G1P[8] genotype were more likely to have antigenemia than children infected with other RV genogroups (71% vs. 34%) (113). We were unable to study the association between specific P-types and extraintestinal spread as 95% of children were infected by a P[8] strain and P[4] was detected in only six children infected by a G2P[4] RV strain.

Recent studies have raised the possibility that host-dependent histo-blood group antigens (HBGA) (A-type) may be associated with susceptibility of certain cells to specific human rotavirus strains. The HBGAs are present on red blood cells, mucosal secretions and epithelia, and are divided into three phenotypes Lewis (Le) antigen Le(a+b-), Le(a-b+) and Le(a-b-). Recently, it was discovered that only Lewis-positive children could be infected by P[8] strains, whereas P[6] strains infect Lewis-negative children. Lewis b positive/negative children induce different neutralization titers to specific human RV strains, and the Lewis b phenotype might also reflect the presence of RNAemia or antigenemia. (Oral presentation: Nordgren et al. Lewis-negative phenotype is a strong restriction factor for genotype P[8] rotavirus infections in Burkina Faso, Africa and Nicaragua, Central America. 5th European Rotavirus Biology Meeting, 6-9 Oct 2013, Valencia, Spain).

In a study by Chiappini et al., minor changes in two outer capsid proteins, VP7 and VP4 at the amino acid level, were observed between rotaviruses found in serum and stool, suggesting possible genetic pressure for the virus to modify for dissemination from the intestines (364). However, the combined results from our Studies I and III indicated that the presence of RNAemia is not associated with any specific VP7 or VP8* sublineage, as strains from both sublineages G1-I and G1-II were detected in children with RV RNA in serum and only P[8]-I strains were detected circulating between 2006 and 2008 (unpublished data).

Previous findings of correlation between antigen levels in stools and serum but not between RNA in stools and serum support the possibility of extraintestinal replication of the virus (113). Extraintestinal replication has been detected in multiple extraintestinal sites such as mesenteric lymph nodes, liver and lungs (365,366). However, the presence of RV RNA might be fully explainable by excess production of

free viral proteins or release of noninfectious RV particles (DLPs) from the intestines into the serum without any extraintestinal replication.

In a study by Blutt et al., antigenemia was found to be directly related to the presence of infectious virus in blood (123). In our study, we tried to isolate and visualize the whole virion or inner core particles in electron microscopy, but the attempts were not successful. So far, Blutt and co-workers are the only ones to have successfully cultivated and visualized infectious rotavirus from serum using HT-29 cells and serial blind passages and multiple dilutions of sera (123). Like our attempts with two ELISA positive serum samples, previous isolation and cultivation have been largely unsuccessful, possibly due to the presence of serum inhibiting factors and too few infectious particles (121).

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5.2 Effects of universal rotavirus vaccination in Finland

So far, rotavirus vaccination has been added to the NIP of 53 countries worldwide, the majority of them using Rotarix™ vaccine (287). As Finland has been exclusively using RotaTeq® vaccine since September 2009 and the vaccination coverage is uniquely high, rotavirus surveillance in Finland is very important and may provide the best results on the impact of rotavirus vaccine, specifically RotaTeq®. The nearest comparison is the United States, which has a lower coverage about 80% nationwide, whereas in Finland the estimated coverage is about 95%. The catchment population of Tampere University Hospital is about one tenth of the Finnish population, and the experiences in Tampere may be generalized for the whole country.

In Finland, before the RotaTeq® vaccine was added to the NIP, cost-effectiveness analysis estimated the annual costs of RVGE in children under 5 years of age without vaccination to be 4.2 million euros. The price of universal mass vaccination with RotaTeq® is 2.6 million annually. The vaccination was assessed as cost-effective even at a higher price level, and is more cost-effective now. (367)

5.2.1 Hospitalizations and outpatient clinic visits

5.2.1.1 Hospitalizations and outpatient clinic visits for RVGE

To evaluate the impact of the RotaTeq® vaccine, we compared the results from two similar studies from the pre- and post-vaccination periods, each for 2 years, and using the same settings and methodology.

We detected a significant reduction in outpatient clinic visits and hospitalization (81% and 76%, respectively) for RVGE in the 2-year post-NIP period. However, as Rotarix™ was already available in the post-NIP period with 22% coverage in the first season (2006-2007) increasing to 29% in the second season (2007-2008) (RotaTeq® coverage 6%), the actual effect on RVGE cases is even greater. Although we did not observe a further reduction in RVGE between the first and the second post-NIP years, results from more recent years have indicated that the number of RVGE cases is continuing to decrease. In the latest season (2012-2013), the total reduction in all RVGE cases was 92% compared to the average of the two pre-NIP years. Similar reductions with the exclusive use of RotaTeq® have been observed in the USA (288,300,368); in a study by Wang and co-authors, health-care visits were reduced by 88-94% even with an incomplete immunization regimen (301). On the other hand, it has been observed in the United States that rotavirus activity follows a biennial pattern: after a high impact of vaccination on RVGE in the first year (2008 season), rotavirus activity went up the next year, followed by another year of low activity, followed by another rise. (289)

The significant reduction in hospitalizations for RVGE was shown as a disappearance of nosocomial infections in post-NIP seasons, apparently because the small number of hospitalized cases interrupted transmission among contacts. Most likely for the same reason, we observed a significant decrease (72%) in RVGE cases among unvaccinated children. Although the decrease was significant in every age group, the median age of children infected by RV shifted towards older children in post-NIP years. The reduction in cases among unvaccinated children in our study was higher than observed with RotaTeq® vaccine in USA (42-45%) (288). In addition, in Australia, with the combined use of both vaccines, the reduction in cases among children too old to be vaccinated in the NIP was 50% (298). One reason for the greater effect in Finland may be the level of vaccination coverage (≥95%), which is higher than anywhere else. Still, it is possible that in the future, following the accumulation of susceptible children in older groups, the level of indirect protection in Finland may decrease.

Since the introduction of universal mass vaccination with RotaTeq®, no wild-type RV infections have occurred in infants below the age of 6-months. In the post-NIP years, the few cases of RVGE in such infants leading to an outpatient clinic visit or hospitalization have been caused by a vaccine-derived double reassortant rotavirus G1P[8] (discussed later).

5.2.1.2 Effects on all cases of acute gastroenteritis and other gastroenteritis with specific etiology (Study II)

As in other countries with rotavirus vaccine in a NIP, the reductions in all cases of RVGE were shown as a decrease in the total number of AGE cases seen in the outpatient clinic and on the hospital ward. In addition, as recently observed in other studies (369,370), norovirus has become the leading causative agent of AGE in small children. It should be noted that, norovirus has become predominant because the proportion of cases in which it is the causative pathogen is increasing, but not the absolute number.

5.2.1.3 Rotavirus infections in vaccinated children

In the first two post-NIP years (2009-2011), wild-type RVGE cases in children eligible for vaccination in the NIP were reduced by 93% (91% if including vaccine-derived cases). Among these cases, there were five children who were partly or completely immunized. In the most recent season (2012-2013), four children in whom wild-type rotavirus was detected were fully vaccinated with RotaTeq®. Although it was not possible to study the protection rate in partially vaccinated children, the proportion of vaccinated children in all RVGE cases in the most recent season is surprisingly high (24%), as the protective effect after one or two vaccine doses combined with low numbers of circulating virus seems sufficient to protect from RV infection even with incomplete vaccination regimen (301).

All eight fully/partially RotaTeq® vaccinated children with wild-type RV infection in post-NIP seasons 2009-2013 were found to have either RV genotype G9P[8], G4P[8], G3P[8], or G12P[8]. Although in the prelicensure studies RotaTeq® vaccine was found to be efficacious against all common genotypes (G1-G4 and G9), the protection was actually highest against G1 strains, and in several studies the protective effect against G2, G3, and G4 strains was lower than for other genotypes, or was not calculated separately because of the small number of these genotypes circulating (278,279,371). However, it is too early to conclude whether the prevalence of these

genotypes in vaccinated children is associated with lower protection induced by the vaccine or is a result of natural shifting of predominant genotypes. Of note, however, G1 continued to be common, even predominant in all four postvaccination seasons in Finland (discussed below), and its absence in cases in vaccinated children appears striking.

5.2.1.4 Genotype distribution

Like studies in other countries, during 2-year pre-NIP period and 4-year post-NIP surveillance period, we observed some changes in the predominant RV genotypes. Although those changes were proportionally most significant in the most recent season (2012-2013) (disappearance of G4 strains, increase in G3 and G12 strains), the absolute number of all RVGE cases is remarkably small (N=17). Moreover, it is worth noticing that the G1 genotype has remained one of the predominant genotypes (35-76% of all RV strains annually) over the total 6-year surveillance. The emergence of novel genotypes such as G8 and G12, has also been noticed elsewhere (317).

Bucardo and co-workers studied all 11 gene segments of wild-type G1P[8] rotaviruses detected in RotaTeq® vaccinated children. Surprisingly, the NSP2 gene segment from two patients was found to be identical to the cognate gene segment in RotaTeq® vaccine. (334) In our studies, we did not examine all 11 gene segments, and may possibly have missed such new reassortants. However, in future, a full-genome PCR will be performed on all RV strains detected after universal mass vaccination.

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5.2.2 Effects on rotavirus genomics and vaccine-derived viruses

5.2.2.1 Immunological pressure on VP7 and VP8* antigens (Study III)

We examined the potential changes in the nucleotide and amino acid sequences of two outer capsid proteins VP7 and VP8* of the predominant G1 rotaviruses. As both of those proteins are presented in the RotaTeq® vaccine in both human and bovine origin, we studied the phylogeny of G1 strains over a 20-year period to detect possible vaccine-induced selective pressure (320,372).

Similar to previous findings from other countries, we detected several G1 VP7 sublineages circulating at the same time in each study period (60,321,324). Interestingly, in Brazil and parts of Germany countries with wider use of Rotarix™

vaccine, changes in the antigenic regions of G2 proteins at the amino acid level have been observed (373). Similarly, Matthijnssens et al. presented study results indicating changes in the antigenic regions of G1 VP7 strains (Poster presentation: Matthijnssens J et al., Selective rotavirus vaccine pressure against P[8] strains, and possible immune evasion by specific G1 lineages after vaccine introduction in Belgium. European Society for Pediatric Infectious Disease, 9-13 June 2009, Brussels, Belgium). However, no statistically significant association with the vaccine was found and the results have not been described further in the literature. In our study, we did not find any association with either of the two rotavirus vaccines, as strains from G1-I and G1-II sublineages were circulating at the same time, and no strains belonging to the same sublineage as RotaTeq®, G1-III, were detected during the study period.

For the VP8* sublineages we observed the circulation over time of two sublineages, P[8]-I and P[8]-III. In our study, these two sublineages were not detected at the same time, although the P[8]-III sublineage is considered to be the most prominent P[8] sublineage. Both sublineages were circulating before the launch of the rotavirus vaccines, but after two years when Rotarix™ was used with a 29% vaccine coverage in Finland, the VP8* sublineage changed from the same sublineage as where Rotarix™ aligns, P[8]-I, to a different lineage P[8]-III. Since 2009, all circulating RV strains have belonged to the P[8]-III sublineage. Although the change between sublineages has a temporal association with the use of the Rotarix™ vaccine, a clear correlation cannot be drawn as both sublineages were already circulating before the vaccines were available. However, the same observation of dominance of P[8]-III strains was noticed in Brazil after the Rotarix™ vaccine was implemented in a NIP (Poster presentation: Silva, MFM et al. VP8 P[8] lineages of group A rotaviruses circulating over 20 years in Brazil. 11th International Symposium on dsRNA viruses, 27 Nov-1 Dec 2012, Puerto Rico). Whether the VP8* protein of Rotarix™ has perhaps resulted in sufficiently strong neutralizing antibody responses to cause the shift in VP8* sublineages in Finland, or whether the shift may just represent normal genetic fluctuation of a prominent P[8] lineage, cannot be concluded.

RotaTeq® VP8* aligns into a completely different P[8]-II sublineage, which is phylogenetically distant from the P[8]-I and P[8]-III sublineages. During the whole 20-year study period, we did not detect any circulating G1 P[8]-II strains. At the amino acid level, the strains circulating between 2009 and 2012 are closer to the RotaTeq® than to the Rotarix™ vaccine.

However, although the circulating strains from 2009-2012 are closer to the RotaTeq® vaccine than to Rotarix™ in their antigenic epitopes, the alignment of RotaTeq® into completely different sublineages in both VP7 and VP8* proteins (G1-III and P[8]-II, respectively) than circulating G1 strains potentially indicates the absence of vaccine-induced selective pressure. In other words, it seems clear that at

least the RotaTeq® vaccine has not induced any selective pressure on G1 strains even with the 95-97% vaccine coverage in Finland. Sequencing of G1 strains of RotaTeq®-vaccinated children would provide a better understanding on the immunological aspect of circulating G1P[8] strains, but, remarkably, none of the fully or partly vaccinated children in whom a wild-type rotavirus was found had been infected by a G1 rotavirus.

5.2.2.2 Vaccine-derived double reassortant rotavirus (IV, V)

Stool samples from post-NIP seasons were studied by sequencing VP7, VP4, and VP6 proteins to detect possible vaccine-originated viruses. The VP6 protein included in the RotaTeq® vaccine is of bovine origin, and the origin of the virus can be detected by RT-PCR and by sequencing. In study IV, we found four recently vaccinated children with AGE who were shedding a new vaccine-derived double reassortant rotavirus G1P[8] and one child with original vaccine virus G6P[8] in stools. Of these five patients, only one was detected concomitantly to have another gastroenteritis pathogen (norovirus) which could explain the symptoms of acute gastroenteritis.

The stability and origin of new vaccine-derived double reassortant rotavirus G1P[8] was verified by cell culturing ELISA-positive stool sample. In our study, the new reassortant virus remained stable and 100% identical to the original isolates. This finding confirms that the vdG1P[8] double-reassortant is a truly new stable virus which has a potential for transmission to contacts. Unlike other studies (331-333), our study is the first to describe successful cultivation of this new reassortant. We did not study the other gene segments of the double-reassortant viruses, as they were detected soon after immunization and we did not except to find any wild-type – vaccine strain reassortants. However, RT-PCR and sequencing of the VP3 gene (which is of bovine origin in G1P[5] and of human origin in G6P[8]) could have provided more information about the formation of this new reassortant.

In the pre-licensure studies of WC3-reassortant vaccine using plaque assay and electropherotyping, shedding of RotaTeq® vaccine viruses has been a relatively uncommon phenomenon (277,278). In more recent studies using more sensitive methods, 21-94% of children have been detected as shedding vaccine viruses at some point after the first immunization (325,326). The formation of vaccine-derived double reassortant appears to be much less frequent than shedding of original vaccine strains in stools. Detection of the new double reassortant G1P[8] was reported for the first time even before RotaTeq® vaccine was licensed in 2004 (274). In our Study IV, vdG1P[8] was detected in two of the children after the second dose of vaccine. In such cases, it seems that the first immunization has not been efficacious enough to limit the multiplication of the vaccine virus and give protection against vaccine-originated RV

disease. Perhaps, in such cases, the first vaccination did not "take" due to the presence of maternally acquired antibodies, and the second dose was effectively the first successful vaccination for the infant, or the protection given by the first dose was not enough to prevent the symptoms of AGE upon the second vaccination. (187,374) Similarly, in Study II, an incomplete vaccine regimen in some cases did not induce an immune response strong enough to protect the children from wild-type RV infection.

Soon after study IV, we detected the same vaccine-derived double reassortant G1P[8] in an unvaccinated 7-year-old girl (Study V). Potential sibling transmission of vaccine-derived virus has been previously described by Payne et al. and Boom et al. (331,333). In our study, the source of vdG1P[8] reassortant could not been identified as the child did not have any known contacts with recently vaccinated children. Prolonged shedding of vaccine viruses has been reported earlier in immunocompetent children (330). However, as passaging of the virus in cell culture showed the vdG1P[8] to be equally as stable and infectious as the reassortants detected in Study IV, it is possible that this virus was circulating in the environment. The virulence of this vdG1P[8] reassortant has not been studied, but, if spread in the community, it could have the potential to cause cases of rotavirus gastroenteritis.

In addition, as both VP7 and VP8* antigens in the vdG1P[8] reassortant belong to sublineages G1-III and P[8]-II (Study III), respectively, which have not been circulating in the environment in Finland, they might potentially have higher virulence than wild-type strains. We studied the serum and whole blood sample of one ELISA-positive child in Study IV and did not detect any RV RNA or antigen in either of those samples. Still, we may assume the number of viral particles to be sufficient to induce similar extraintestinal spread as in Study I. As it is not likely that these children could have encountered a natural primary infection before the first immunization, which could have induced protective neutralizing antibodies, and restricted extraintestinal spread of the virus it seems possible that either pre-existing maternal antibodies are sufficient to restrict the infection to the intestines, or the rotavirus reassortant with a bovine backbone is not virulent enough to spread to extraintestinal sites.

Previously, detection of vdG1P[8] was associated with immunocompromised children (332,333), as the prolonged shedding could more easily result in formation of this new reassortant in their intestines. However, in our Studies IV and V, none of the children have been diagnosed with an immunocompromising condition.

In study IV, we also detected several children with AGE shedding original vaccine strains or parts of them concomitantly with other gastroenteritis pathogens. Interestingly, all children detected with vaccine P[8] (vdG1P[8] or G6P[8]) had vaccine-originated RV as the only AGE pathogen, whereas children in whom original vaccine strains (G1P[5]) also had other GE pathogens detected that were likely to explain the symptoms of AGE. This raises the possibility that P[8] alone may be

associated with AGE symptoms and could also be the component resulting in increased virulence of the double reassortant. In the pre-licensure studies evaluating the final composition of RotaTeq® vaccine, fever was found to be more frequent in children immunized with the pentavalent vaccine (G1-G4, P[8]) than in those immunized with quadrivalent vaccine (G1, G2, G3 and G4) (277).

So far, in total, we have detected only five cases of these new reassortants in the post-NIP seasons. Compared with the estimated number of children immunized with RotaTeq®, the percentage of children who develop this reassortant and AGE is remarkably small. However, the true number of these vaccine-derived reassortants could be much higher, as mild cases of gastroenteritis associated with vaccination are not detected in a hospital-based survey.

As reported in Nicaragua, RotaTeq® vaccine strains have the potential to form new reassortants with wild-type strains (334). To study these new reassortants and possible new variants of vaccine-derived reassortants, continuous monitoring of the genetic composition of the full genome of rotaviruses in the post-NIP period is needed.

6 Conclusions and future prospects

In this thesis, the overall aim was to evaluate the effects of universal mass vaccination with a human-bovine rotavirus vaccine (RotaTeq®) in Finland. For background, some aspects related to the clinical picture of rotavirus gastroenteritis (RVGE) were also studied.

While the pathogenesis of RVGE has been elucidated for many years, the extraintestinal spread of RV is a relatively recent discovery. To examine the association of clinical severity with extraintestinal spread of RV as compared with infection confined into the intestines, we studied stool, serum, and whole blood samples from rotavirus-infected children. First, we found that 67% of RVGE patients in hospital had evidence of systemic spread, as they were detected with RV RNA in serum and 61% had RV antigen in their serum. Children in whom extraintestinal spread of the virus was detected were more likely to have a more severe clinical picture, as determined by level of fever and severity of vomiting, than were children in whom RV RNA was detected only in stools. Younger children with primary rotavirus infection seem to be more likely to have RV RNA detected in serum. Altogether, the relatively common occurrence of extraintestinal spread and its association with a more severe clinical picture only emphasizes the need for preventing RVGE by vaccination.

This study investigated the impact of universal mass vaccination with RotaTeq® by comparing epidemiological data from pre- and post-vaccination years in similar hospital settings. We observed that outpatient clinic visits and hospitalizations for RVGE were reduced dramatically, by 81% and 76%, respectively, in the two post-vaccination years. Preliminary data from further surveillance until 2013 indicates that the reduction is being sustained. The reduction was seen as a 59% decrease in all AGE cases, leading to a relative increase and further predominance of norovirus as a major causative agent of AGE in children seen in hospital. This has raised the question of the need for a norovirus vaccine as the next research target.

The extensive use of the RotaTeq® vaccine has not resulted in any major shift in the prevalent wildtype RV strains, which may be in contrast to widespread use of the G1P[8] human RV vaccine Rotarix™. However, interestingly, vaccinated children are often infected by genotypes other than G1P[8], which may be a reflection of the immunodominance of G1 in the pentavalent combination with P[8]. As for G1P[8], the RotaTeq® vaccine did not appear to have caused any selective pressure upon the two outer capsid proteins, VP7 and VP8*.

Vaccination with RotaTeq® was found to be associated with formation of new vaccine-derived double-reassortant rotaviruses, which have the potential to cause AGE symptoms in vaccinated children and to infect unvaccinated contacts. In addition, RV strains included into the vaccine have been shown to reassort with wild-type RV strains, causing new vaccine-wild-type RV reassortants. These observations support the continued surveillance of circulating RVs at the molecular level.

All together, the large-scale use of RotaTeq® vaccine in the Finnish NIP has been highly successful - arguably the most successful rotavirus vaccination program anywhere in the world. The potential vaccine-related safety issues, including vaccine-derived viruses, risk of intussusception, and circovirus contamination, do not outweigh the remarkable achievements of this program.

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9 Original communications

Rotavirus Antigenemia in Children is Associated With More Severe Clinical Manifestations of Acute Gastroenteritis

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Background: Rotavirus (RV) antigenemia and RNAemia are common findings in rotavirus-infected children. Sporadic associations between RV antigenemia and extraintestinal manifestations of RV infection have been observed. We examined the clinical severity of RV gastroenteritis in patients with and without RV antigenemia or RNAemia.

Methods: Stool, serum and whole blood samples were collected from children seen with acute gastroenteritis in Tampere University Hospital and studied for RV using reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay. Only exclusively RV-positive specimens were included into this study. The patients were divided into groups according to RV findings from stool, serum and blood specimens. Clinical manifestations were graded according to 20-point Vesikari scoring system.

Results: Of 374 children, 155 (41%) had RV in their stools. Of these 155 children, 105 (67%) were found to have RV RNA in the serum; of those, 94 (90%) had also RV enzyme-linked immunosorbent assay antigen. Thus antigenemia occurred in 61% (94 cases) of RV-infected children all of whom had concomitant RNAemia. Neither antigenemia nor RNAemia were detected in 85 patients with non-RV gastroenteritis. Patients who had RV RNA and RV antigen in both serum and stools were more likely to have a higher level of fever and more severe vomiting than patients who had RV only in stools. G1 genogroup RV was more often associated with RNAemia and antigenemia than other genogroups combined.

Conclusion: Rotavirus antigenemia and viremia are commonly detected in children hospitalized for RV gastroenteritis and may be associated with increased severity of fever and vomiting.

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Rotaviruses (RVs) are a major cause of severe gastroenteritis in young children, as associated with an estimated 450,000 deaths worldwide.[1] Clinical symptoms of primary rotavirus infection last from 4 to 7 days and include watery diarrhea, vomiting as well as fever. Second infections with RV are clinically milder or even asymptomatic.[2,3]

Initially, rotavirus infection was thought to be limited to the gastrointestinal tract, with rotaviruses replicating in the mature enterocytes at the tip of the villi of the small intestine.[4] However, it is now recognized that RV infection can spread from the intestines into circulation, as RV RNA and RV antigen have been detected in blood (serum) of infected children.[5] Rotavirus infection may be associated with systemic symptoms and extraintestinal manifestations such as febrile seizures and encephalitis as well as with elevated transaminases and interleukin 8 and interleukin 10 levels.[6–13]

Rotavirus RNA and antigen(s) (mainly VP6) have been detected in 58–72% and 33–90%, respectively, of sera of RV-infected children.[5,14–19] Several reports have described detection of RV RNA or antigens in multiple extraintestinal organs, such as heart, kidney, spleen, testes, bladder and liver.[20–23] A case report described a death due to systemic RV infection, where RV VP6 and NSP4 genes were detected in liver, intestine and brain in addition to RV antigens detected in brain, heart, liver, small intestine and cerebrospinal fluid.[23,24]

Even though presence of RV RNA and RV antigens in serum implies RV viremia with infectious RV particles in blood, the isolation and culture of infectious RV from antigen-positive and RNA-positive human sera have been largely unsuccessful, possibly due to insufficient amount of infectious viral particles and presence of serum-inhibiting factors.[25,26] In a study by Blutt et al[19] using a modified virus isolation technique, infectious virus was detected in HT-29 cells using immunofluorescence staining for rotavirus structural and nonstructural proteins. In the same study, the detection of antigenemia was found to be directly related to the presence of viremia. Still, the site of viral replication remains unclear, and certain RV strains might have higher susceptibility to infect extraintestinal cells.[27,28]

We wanted to examine if patients detected with RV RNA and RV antigen in stool, serum and blood sample had more severe gastroenteritis symptoms (diarrhea, vomiting and fever) than the patients with only intestinal infection (RV RNA detected only in stools). In addition, we examined if particular RV genotypes were associated with antigenemia and RNAemia.

MATERIALS AND METHODS

Clinical Methods

The prospective study was conducted at Tampere University Hospital from September 2006 to August 2008. The study was approved by the Ethics Committee of Pirkanmaa Hospital District. The methods have been described previously when the results of the epidemiological study were reported.[29–31]

Patients with acute gastroenteritis <16 years of age, who were seen in the emergency department of the hospital or were admitted to a pediatric ward, were eligible for the study, subject to obtaining informed consent from the parents. While in hospital, information was collected on fever, diarrhea and vomiting in the hospital medical records.

Before discharge, the parents received a diary card and instructions to record possible still ongoing fever, vomiting and/or diarrhea. Parents were instructed to send the diary cards back to the study nurse after the child had fully recovered. If the cards were not returned within 4 weeks after the discharge, the study nurse contacted the parents for a reminder.

In the diary card, information on the duration of each symptom (starting day and ending day), maximum temperature (°C) and maximum number of diarrheal stools per 24 hours and maximum

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number of vomiting episodes per 24 hours was continued to be collected.

A stool specimen was collected during the outpatient clinic visit or at the hospital ward if the child was admitted. If the treatment required collection of blood samples, an acute phase serum sample and a blood sample were collected and used in this study. In all cases, the time interval between sample collection and the onset of illness was <7 days.

Laboratory Methods

All stool, serum and blood specimens were tested for the presence of RV using a reverse transcription polymerase chain reaction (RT-PCR) method, as described previously.[31] All stool samples were also tested for the presence of other (but not all) gastroenteritis pathogens such as human caliciviruses (including norovirus and sapovirus), adenovirus, bocavirus and coronavirus. After the detection of RV, the G and P genotypes were determined by nucleotide sequencing of the gene segments encoding for the VP7 and VP4 antigens.[31–33]

The presence of RV antigen as evidence of RV antigenemia was determined from serum samples with an enzyme-linked immunosorbent assay (ELISA), using the IDEIA Rotavirus kit (Oxoid Ltd, Hampshire, United Kingdom), unless the sample had been exhausted in PCR. The same ELISA test was used to detect rotavirus in stools. Stools absorbed in a diaper were not tested for RV antigen, because the ELISA test could not be performed technically. A sample with an optical density read by spectrophotometry at a wavelength of 450 nm with a value >0.15 was considered as positive.

Virus Isolation Attempts

Attempts were made to isolate RV from serum samples of 2 children with RV RNA-positive serum and whole blood and ELISA-positive serum as described previously.[34] The total protein concentration was quantified using the Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL) and the 2 samples with highest protein concentration were studied in electron microscopy.

Statistical Analyses

Only patients with an available stool and serum specimen were included in this study. The patients were divided into groups according to laboratory results from stool, serum and blood samples. For the statistical analyses, symptoms (diarrhea, vomiting and fever) were graded according to the Vesikari 20-point scoring system for severity of rotavirus diarrhea.[35]

Statistical analyses were performed using the Kruskal-Wallis test to compare the distribution of age, level of fever, duration of diarrhea and vomiting, maximum number of vomiting episodes per 24 hours and diarrheal stools per 24 hours in different groups. If the Kruskal-Wallis test showed statistical significance, the groups were further compared in pairs using the Mann-Whitney U test. The probabilities in different groups were calculated using the χ^2 test. All tests were performed in SPSS [version 20.0 (SPSS)] and were 2-tailed and a $P < 0.05$ was considered to be statistically significant.

RESULTS

In the 2-year period from September 1, 2006 through August 31, 2008, a total of 1042 patients were recruited into the study at Tampere University Hospital. Both a serum and a stool sample were obtained from 374 patients, of whom 155 (41%) had RV in the stools. Of those 155 children with a RV-positive stool specimen, 105 cases (67%) were found to have RV RNA in the serum.

Of the 105 stool and serum PCR-positive cases, 94 (90%) had RV antigen in serum by ELISA, 9% (9 cases) were ELISA negative and in the 2 cases serum sample could not be tested using ELISA. In the total material, antigenemia occurred in 61% (94 of 153) of RV-infected children all of whom had concomitant RNAemia.

Whole blood samples were obtained from 87 of the 105 patients that were positive for RV RNA in stool and serum. Twenty-eight of 87 (32%) patients positive for RV RNA in stool and serum were detected with RV RNA in blood. Of the 28 patients, 22 had RV antigen in serum, 4 were serum ELISA negative and in 2 patients serum ELISA could not be done. None of the 50 patients negative for RV RNA in serum was positive for RV RNA in whole blood.

Eighty-five nonrotavirus gastroenteritis patients were selected from 219 patients negative for RV RNA in stools as control patients, as they were detected with other gastroenteritis viruses, such as norovirus, bocavirus and adenovirus, in their stools explaining their GE symptoms. None of 85 control patients were positive for RV ELISA antigen or RV RNA in any serum or blood sample.

To calculate the differences between groups, the patients with missing results from ELISA or PCR from whole blood samples were excluded to reduce any bias (N = 7 and N = 18, respectively). Four patients detected with RV RNA in stool, serum and blood specimens but negative in RV ELISA from serum were also excluded from statistical analyses due to an insufficient number in this group.

Of RV stool-positive subjects, the following groups were formed for subsequent calculations:

(1) RV RNA-positive serum, ELISA-positive serum and RNA-positive whole blood (N = 22);
(2) RV RNA-positive serum, ELISA-positive serum, RNA-negative whole blood (N = 46);
(3) RV RNA-positive serum, ELISA-negative serum and RNA-negative whole blood (N = 13);
(4) RV RNA-negative serum, ELISA-negative serum and RNA-negative whole blood (N = 50).

These groups were compared all together and in pairs. In addition, patients detected with RV RNA in serum (groups 1–3) were compared with patients detected with RV RNA only in stool.

Of the 131 children with available stool and serum specimens (patients in groups 1–4), 48 (37%) were treated as outpatients and 83 (63%) were hospitalized. The ratio of hospitalized children to outpatients was not different in the 4 groups presented above ($P = 0.91$).

Age

The age distribution of children with RV-positive stools was from 2 months to 11 years and 6 months (mean: 2 years and 1 month, standard deviation: 1 year and 7 months). The children who had RV RNA also in serum were slightly younger than those detected with RV RNA only in stools, but the age difference between these groups was not significant ($P = 0.78$; Table 1).

RV Genotypes and Antigenemia

The RV genotypes detected in stool, serum and whole blood samples were concordant in each case. The predominant RV genotype detected in stools was G1P[8] (N = 99, 76%). Other detected genotypes were G4P[8] (N = 11, 8.4%), G9P[8] (N = 8, 6.1%), G2P[4] (N = 6, 4.6%) and G3P[8] (N = 7, 5.3%).

The presence of RV RNA in serum was not limited to any RV genotype. G1P[8] genotype was not only more common overall but also proportionally more serum-positive cases were associated with G1P[8] genotype. Of 99 G1P[8] RVs detected in stools,

TABLE 1. RV Laboratory Results of Serum and Whole Blood Specimens and Mean and Median Ages of Children Detected With RV RNA in Stools

	Group 1 (N = 22)	Group 2 (N = 46)	Group 3 (N = 13)	Group 4 (N = 50)	Total
Serum RNA	Positive	positive	Positive	Negative	
Serum ELISA antigen	Positive	positive	Negative	Negative	
Whole blood RNA	Positive	negative	Negative	Negative	
Mean	1 year 10 months	1 year 11 months	1 year 11 months	2 years 4 months	2 years 1 month
Median	1 year 8 months	1 year 7 months	0 years 10 months	1 year 11 months	1 year 8 months

Group 1 includes children with RV RNA and ELISA-positive serum and RV RNA-positive blood (N = 22), group 2 children with RV RNA and ELISA-positive serum (N = 46), group 3 children with RV RNA-positive serum (N = 13) and group 4 children positive for RV RNA only in stools (N = 50).

70 (71%) were detected also in serum, whereas of the non-G1P[8] genotypes, the same genotype was also found in serum in only 34% (11 cases). G9P[8] was detected in serum in 4 (50%) cases, G2P[4] in 2 (33%) cases, G4P[8] in 4 (36%) cases and G3P[8] in 1 (14%) case ($P < 0.001$).

As G1P[8] genotype accounted for 86.5% of cases with RV RNA in both serum and stool sample and only for 58% of cases with RV RNA only in stool, also the difference in genotype distribution according to presence of RV RNA in serum or RV ELISA antigen in serum was statistically significant ($P = 0.001$ and $P = 0.009$, respectively), indicating G1P[8] genotype to be more often associated with RNAemia and antigenemia than pooled other genotypes.

Diarrhea

For the grading of diarrhea, scores were given by the maximum number of diarrheal stools per 24 hours during the GE period (No episodes = 0 point, 1–3 episodes = 1 point, 4–5 episodes = 2 points and 6 episodes or more = 3 points) and by duration of diarrhea in days (1–4 days = 1 point, 5 days= 2 points and 6 days or more = 3 points). Of 131 children, 130 (99%) had diarrhea. In 73% of 130 cases, the duration of diarrhea was 6 days or more and, typically, children had >5 diarrheal stools per 24 hours (N = 100, 76%).

The presence of RV RNA in serum or blood did not have any association with the maximum number of diarrheal stools per 24 hours or with duration of diarrhea ($P = 0.310$ and $P = 0.856$), nor did the groups differ according to presence of RV RNA in serum (from group 1 to 4; $P = 0.479$ and $P = 0.603$). In addition, no significant difference in the probability of severe diarrhea (6 diarrheal stools per 24 hours) across RV groups was found ($P = 0.423$).

Vomiting

For evaluation of vomiting, scores were given for maximum number of vomiting episodes per 24 hours (no episodes = 0 point, 1 episode = 1 point, 2–4 episodes = 2 points and ≥5 episodes = 3 points) and for duration of vomiting in days (1 day = 1 point, 2 days = 2 points and ≥3 days = 3 points).

Apart from 1 child detected with RV RNA only in stool, all children (N = 130) suffered from vomiting. In 71% of these cases, the duration of vomiting was 3 days or more and 67% children had more than 4 vomiting episodes per 24 hours.

There was no difference in the duration of vomiting according to presence or absence of RV RNA in serum ($P = 0.312$ and $P = 0.634$, respectively). However, a statistically significant difference was found in maximum number of vomiting episodes per 24 hours across RV groups ($P = 0.011$; Table 2). Patients with positive serum ELISA result suffered more often from severe vomiting than those with negative serum ELISA result ($P = 0.004$). Compared in pairs, the difference was also found between groups 1 and 4 and also groups 2 and 4 (i = 0.011 and $P = 0.038$, respectively), indicating that RV RNA positivity in both serum and whole blood was associated with more severe. Of 130 children with vomiting, 88 (68%) had severe vomiting (≥5 episodes per 24 hours). Compared with patients who had RV only in stools, children detected with RV RNA in serum, and according to the number of positive laboratory results, were more likely to have severe vomiting (from group 4 to group 1; $P = 0.015$ and $P = 0.037$, respectively; Table 2). Moreover, children with severe vomiting were older than children with non-severe vomiting ($P < 0.001$). No other age-related association with symptoms was found.

Fever

For statistical comparisons, the level of fever was scored as following: <37.0°C = 0 point, 37.1–38.4°C = 1 point, 38.5–38.9°C = 2 points and ≥39°C = 3 points. Of all 131 children, 117 (89.3%) had fever (>37.1°C), and of those 52% had a maximum temperature >38.9°C. The probability of any fever (>37.1°C) differed between RV groups ($P = 0.024$), so that patients positive for RV RNA in both stool and serum (groups 1–3) or with positive serum ELISA result were more likely to have fever than patients who were detected with RV only in stool ($P = 0.013$); similarly, patients with RV ELISA antigen in serum had more often fever than patients with negative serum ELISA result ($P = 0.019$; Table 3).

Distribution of the level of fever was different across RV groups ($P = 0.045$), as the probability of higher fever rose according to the number of positive laboratory results (from group 4 to group 1). However, a statistically significant difference was found only between RV groups 1 and 4 (RV RNA positive in stool, serum and whole blood sample and ELISA-positive serum vs. RV RNA positive only in stools and ELISA-negative serum).

TABLE 2. Distribution of Number of Vomiting Episodes per 24 hours in RV Groups

	Group 1	Group 2	Group 3	Group 4	Total
No episodes	0	0	0	1	1
1 episode	0	1	3	7	11
2–4 episodes	3	12	4	17	36
≥5 episodes	19	33	6	25	83
Total	22	46	13	50	131

The groups of RV stool-positive subjects were formed according to the number of positive RV laboratory results from serum and whole blood.

TABLE 3. Distribution of Fever in Patients With Positive or Negative RV ELISA Antigen in Serum (N = 68 and N = 63, respectively)

	Serum ELISA Positive	Serum ELISA Negative
No fever	2 (3%)	12 (19%)
37.1–38.4°C	13 (19%)	14 (22%)
38.5–38.9°C	17 (25%)	12 (19%)
≥39°C	36 (53%)	25 (40%)
	68 (100%)	63 (100%)

TABLE 4. Vesikari Scores in Study Groups With RV RNA-positive Stool Sample

	Group 1	Group 2	Group 3	Group 4	Total
N	22	46	13	50	131
Mean	17,5	16,66	14,83	15,43	16,1
Median	18	17	15	16	17
Minumum	14	11	12	7	7
Maximum	19	19	18	19	19

The groups of RV stool-positive subjects were formed according to the number of positive RV laboratory results from serum and whole blood. Group 1 includes children with RV RNA and ELISA-positive serum and RV RNA-positive blood (N=22), group 2 children with RV RNA and ELISA-positive serum (N=46), group 3 children with RV RNA-positive serum (N=13) and group 4 children positive for RV RNA only in stools (N=50).

Dehydration

Information on dehydration was available for 121 of 131 patients. Ten children had no dehydration, and all 10 had RV RNA only in stool, whereas 111 children had at least moderate dehydration and all had RV RNA concomitantly in stool, serum and blood. However, because of small number of cases, these differences were not statistically significant.

Vesikari Score

The Vesikari scores were calculated from 121 of 131 children with all available information. The median Vesikari score for children seen in the University Hospital with rotavirus gastroenteritis was 16 (from 7 to 19; Table 4). Children with more positive laboratory results for RV in serum and blood had more severe illness by the score than those with no RV RNA in serum and blood ($P = 0.004$). The difference was statistically significant also between groups 1 and 4 and groups 2 and 4 ($P = 0.008$ and $P = 0.04$, respectively). In addition, the difference was statistically significant when children were compared according to their ELISA results (groups 1 and 2 had more severe illness than groups 3 and 4; $P < 0.001$).

Virus isolation Attempts

No whole virions or inner core particles could be found in electron microscopy. In addition, we tried to propagate the original and purified serum samples in MA104 cells, but the attempts were not successful.

DISCUSSION

In this study, we examined the impact on clinical picture of RV gastroenteritis in children with concomitant RV RNAemia and antigenemia. Previous studies have shown that rotavirus RNA and proteins (ELISA antigen, mainly VP6) are commonly detected in the sera of children infected with rotavirus[5,14,15] and that antigenemia is also related to the presence of infectious virus in blood.[36] In this study, we only attempted to cultivate RV from 2 children strongly positive in serum PCR and ELISA in MA104 cells but could not detect infectious RV.[14] Blutt et al.[19] were able to cultivate infectious rotavirus from serum in HT-29 cells, after serial blind passages and testing of multiple dilutions of sera. They also demonstrated antigenemia to be predictive of viremia, that is, a marker of infectious RV in blood. Still, the source of virus and the site of viral replication that results in RV viremia remain unclear.[17,37,38]

Studies in mouse models have demonstrated RV viremia to be plasma associated, even though cell-associated viremia has not been excluded.[36,37,39,40] Recent studies have raised the possibility of host-dependent histo-blood group antigens (A-type), present on red blood cells, mucosal secretions and epithelia, to be associated with susceptibility to specific human rotavirus strains.[27,28] RV gene expression has been found in several immune cell types in animal models, suggesting that several cell types to be involved in the pathogenesis of viremia.[37,40–42] Moreover, particular RV strains may have greater infectivity for certain cell types.[27,28]

Ray et al.[17] studied the level of rotavirus antigen and RNA copies in serum for possible association with genotype distribution. Similar to our results, children infected with G1 genogroup were more likely to have antigenemia than children infected with other genogroups, even though the serum antigen levels were not dependent on the infecting RV G-type. In another study by Chiappiani et al,[43] minor changes in 2 outer capsid proteins, VP7 and VP4, were observed between serum and stool samples at the amino acid level, suggesting possible genetic pressure for the virus to modify for dissemination from the intestines. Sometimes discordance between RV genotypes detected in serum and stool samples has been observed.[44] In our study, antigenemia and RNAemia were particularly common with G1P[8] genotype, but we did not find a discordance between RV genotype of serum and stool samples. However, a systematic research on possible nucleotide level or amino acid level changes between RVs in serum and stool samples were not performed.

Our study supports the notion that RV viremia may contribute to the clinical manifestations of RV infection[5,14,16,17] and, specifically, rotavirus antigenemia may correlate with more severe symptoms such as fever or convulsion.[8,45,46] We observed that patients detected with RV RNA and antigen in serum were more likely to have high fever and frequent vomiting, but we did not find any association with RV viremia or antigenemia and diarrhea. RV diarrhea has been associated with several pathogenetic mechanisms, particularly with the activation of crypt cells, by viral nonstructural protein NSP4 leading to release of serotonin from enterochromaffin (enteroendocrine) cells, changes in the intracellular calcium concentration and stimulation of the enteric nervous system, all leading to induced fluid loss from the epithelial cells in intestines.[47–50] Even though the pathogenesis of vomiting also involves RV-induced secretion of serotonin from enterochromaffin cells, it results into activation of vagal afferent nerves, connected to the brain structures associated with vomiting.[51,52] Because the diarrhea is more local reaction in the intestines than vomiting which requires activation in the brain stem, it seems plausible that children with RV-induced emesis and more severe illness have stronger and/or deeper infection in their intestines possibly resulting in more powerful activation, reflecting also in RNAemia and antigenemia, than those with RV RNA only in stools in whom diarrhea may be the predominant symptom. However, this is only speculation and further studies on RV disease and pathogenesis of clinical symptoms and certain RV strains are needed. Cytokines are known to be involved in the pathogenesis of RV infection as well as elevated prostaglandin (especially E_2 and F_2) levels. Sugata et al. [8] found correlations between 2 of 6 analyzed cytokines and RV antigen levels and patients with fever,[53,54] suggesting that the grade of systemic rotavirus infection contributes to the systemic manifestations of disease. In addition, treatment with COX inhibitor (aspirin) has been shown to reduce the duration of diarrhea and level of fever in RV-infected children.[55] However, the severity of rotavirus gastroenteritis in children in our study was more severe than usually in RV infections, probably because severe gastroenteritis cases are more likely to be referred to University Hospital. This may have enabled us to find the correlation between RV antigenemia and vomiting and fever.

Animal studies suggest that at least occasionally RV replication and further dissemination of viral particles may happen in multiple extraintestinal sites such as the mesenteric lymph nodes, liver and lungs.[17,37,38] Extraintestinal RV replication might also happen in

blood vessels Still, the presence of RV RNA and protein in serum might be fully explainable by excess production of free viral proteins or release of noninfectious RV particles from the intestines into serum without any extraintestinal viral replication.[14,42]

In summary, RV RNAemia and antigenemia are common in severe RV gastroenteritis and contribute to the severity of RV gastroenteritis by increasing vomiting and fever by an unknown mechanism. Both antigenemia and RNAemia appear to be particularly common in RV G1P[8] genotype infections.

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Current Abstract

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Incidence and Clinical Characteristics of Herpes Zoster Among Children in the Varicella Vaccine Era, 2005–2009

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Herpes zoster (HZ) is caused by reactivation of varicella zoster virus (VZV) that remains latent in the sensory nerve ganglia after primary infection. HZ occurs more commonly in elderly adults, oftentimes with postherpetic neuralgia. HZ occurs less frequently among children, typically causing mild disease with minimal pain. Although varicella vaccine use has significantly reduced the incidence of varicella among US children, the contribution of vaccine virus to HZ incidence rates is unknown. Similar to wild-type VZV, vaccination establishes a latent infection and can reactivate, causing HZ. Therefore, in vaccinated children, HZ can be caused by vaccine-strain VZV or by wild-type VZV. Attribution of the causal virus type requires laboratory confirmation and genotyping. To further characterize the epidemiology of childhood HZ in the varicella vaccine era, the VZV strain of HZ cases among children <18 years in a managed care plan was determined and demographic and clinical characteristics by vaccination status and VZV type were assessed. Because the presentation of HZ may be changing in the vaccine era, the positive predictive value (PPV) of the providers' diagnoses of HZ was also assessed.

The study was conducted at Kaiser Permanente Northwest (KPNW), a health maintenance organization serving approximately 475,000 members in Oregon and Washington, 144,000 of whom were <18 years of age; Kaiser Permanente Northwest's electronic medical record system contains information on all care received by members and covered by Kaiser Permanente Northwest. One-dose varicella vaccine coverage among 24-month-old children increased from 70% in 2005 to 81% in 2009. Potential study subjects were patients who were 0–17 years of age diagnosed with HZ and assigned *International Classification of Diseases, Ninth Revision (ICD-9*) code 053 by a primary care provider between May 2005 and September 2009. All specimens were tested at the National VZV Laboratory at the Centers for Disease Control and Prevention using 4 fluorescent resonant energy transfer polymerase chain reaction protocols to determine the type of VZV.

Specimens were collected from all 322 enrolled subjects; 309 (96%) were adequate. VZV was identified in 254 (82%), of which 214 (84%) were wild-type, 38 (15%) were vaccine-strain and 2 (0.8%) were possible vaccine/wild-type virus recombinants. Of 254 subjects with VZV-positive specimens, 83 (33%) were vaccinated and 171 (67%) were unvaccinated. Of the 83 vaccinated subjects, 43 (52%) had wild-type HZ, 38 (46%) had vaccine-strain HZ and 2 (2%) had HZ from possible recombinant VZV. Among vaccinated subjects, the proportion of vaccine-strain HZ did not change over the study period ($P = 0.72$). A history of clinical varicella was reported by 4 (11%) of 38 subjects with vaccine-strain HZ, 22 (51%) of 43 vaccinated subjects with wild-type HZ and 164 (96%) of 171 subjects with wild-type HZ. Five percent of subjects reported a previous HZ episode.

Vaccinated subjects with vaccine-strain HZ were significantly younger at diagnosis than the other groups ($P < 0.0001$). The median age at diagnosis for vaccinated subjects was 9 years (range, 1–17 years), including 2 years (range, 1–14) for vaccinated subjects with vaccine-strain HZ and 13 years (range, 2–17) for vaccinated subjects with wild-type HZ. For unvaccinated subjects with wild-type HZ, the median age at diagnosis was 14 years (range, 3–17). Characteristic HZ rash was reported for almost all subjects with no difference by vaccination status. Subjects with vaccine-strain HZ were more likely to have lumbar (37%, $P = 0.004$) and cervical (26%, $P = 0.03$) dermatomal involvement, whereas vaccinated and unvaccinated subjects with wild-type HZ mainly had thoracic involvement (63% and 67%, respectively; $P < 0.0001$).

Among the 30 subjects with vaccine-strain HZ and known vaccination location, 16 (53%) had rash on the extremity where vaccine had been administered, 7 (23%) had rash on a different dermatome ipsilateral to the vaccination, 4 (13%) had rash on the corresponding dermatome contralateral to the vaccination and 3 (10%) had rash on a different dermatome contralateral to the vaccination. Although some clinical features suggested a milder presentation among subjects with vaccine-strain HZ, except for itchiness, clinical features did not differ significantly between subjects with vaccine-strain HZ and vaccinated and unvaccinated subjects with wild-type HZ. No serious HZ-related complications were reported.

The overall PPV of a "definite" primary care provider diagnosis of HZ was 89.2% [95% confidence interval (CI): 85.0–92.6%]. The overall PPV of a "possible" primary care provider diagnosis of HZ was 66.7% (95% CI: 57.6–74.8%). When stratified by varicella vaccine status, the PPV for "definite" diagnosis was higher for unvaccinated subjects (93.2%; 95% CI: 88.7–96.2%) than for vaccinated subjects (79.3%; 95% CI: 68.6–87.6%); the PPV for "possible" diagnosis was also higher for unvaccinated (76.9%; 95% CI: 63.2–87.4%) than for vaccinated subjects (58.8%; 95% CI: 46.3–70.5%).

The overall incidence of laboratory-confirmed HZ was 112 per 100,000 person-years. Vaccinated children had a 79% lower incidence of HZ than unvaccinated children (48 vs. 230/100,000 person-years, $P < 0.001$). These lower incidence rates were present in the 3–9 and 10–17 age groups. However, among children 1–2 years, incidence was higher among vaccinated children ($P = 0.01$).

Comment: This population-based study provides more evidence that childhood varicella vaccination reduces HZ risk. HZ incidence due to vaccine-strain VZV was lower than that due to wild-type VZV. The potential for vaccine-strain VZV reactivation at younger ages and clinical characteristics of HZ among 1-and 2-dose varicella vaccine recipients remain important areas for research. Ongoing monitoring of HZ incidence is warranted for understanding the varicella vaccination program's impact on HZ epidemiology.

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ORIGINAL ARTICLE

Major reduction of rotavirus, but not norovirus, gastroenteritis in children seen in hospital after the introduction of RotaTeq vaccine into the National Immunization Programme in Finland

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Abstract Universal rotavirus (RV) vaccination is expected to reduce hospitalizations for acute gastroenteritis (GE) of children by eliminating most of severe RVGE, but it does not have any effect on norovirus (NV), the second most common causative agent of GE in children. After the introduction of the RV vaccine into the National Immunization Programme (NIP) of Finland in 2009, we conducted a prospective 2-year survey of GE in children seen in Tampere University Hospital either as outpatients or inpatients and compared the results with a similar 2-year survey conducted prior to NIP in the years 2006–2008. Compared with the pre-NIP 2-year period, in 2009–2011, hospitalizations for RVGE were reduced by 76 % and outpatient clinic visits were reduced by 81 %. NVGE showed a slight decreasing trend and accounted for 34 % of all cases of GE seen in hospital in pursuance of RVGE having decreased to 26 % (down from 52 %). In cases admitted to the hospital ward, RV accounted for 28 % and NV accounted for 37 %.The impact of RV vaccination was reflected as a 57 % decrease in all hospital admissions and 62 % decrease in all outpatient clinic visits for GE of any cause. *Conclusion*: RV vaccination in NIP has led to a major reduction of hospital admissions and clinic visits due to RVGE, but has had no effect on NVGE. After 2 years of NIP, NV has become the leading cause of acute GE in children seen in hospital.

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Introduction

Rotaviruses (RVs) and noroviruses (NVs) are the two most common causative agents of acute gastroenteritis (GE) in children <5 years of age in Finland [17, 27]. In all resource-rich countries combined, RVs and NVs cause annually an estimated 1,500,000 episodes of GE requiring a hospital visit [22].

A major reduction in severe RVGE has already happened or is expected to happen in countries with extensive use of vaccines against RV [21]. As a consequence, while overall severe GE is expected to decrease, the proportional role of NVs in childhood GE is likely to increase for NV to become the leading cause of GE requiring in-hospital admission [10, 12].

In Finland, RV vaccines were licensed in 2006, and the vaccination coverage rose from 0 to about 30 % between 2006 and 2008. In this pre-National Immunization Programme (NIP) period, we conducted a 2-year prospective study on RVs and NVs as causative agents of GE in children and found RVs to account for 52 % and NVs to account for 25 % of GE seen in hospital [27].

In the season 2008–2009, no prospective surveillance was ongoing. RV vaccination with exclusive use of bovine–human reassortant RV vaccine RotaTeq® (RV5, Merck & Co. Inc.; in Europe, Sanofi Pasteur MSD) was included into the Finnish NIP on 1 September 2009. The coverage of vaccination rose quickly to over 90 % and reached a level 95–97 %, similar to other vaccines in NIP (source: National Institute for Health and Welfare [THL], Finland). The present study, following the same methodology as the pre-NIP

study, was started at the same time with the introduction of RotaTeq into the NIP and continued for a 2-year period 2009–2011. This enabled us to compare the absolute numbers and proportions of RVs and NVs in acute GE (AGE) seen in the hospital before and after universal RV vaccination.

Materials and methods

Clinical methods

The prospective study was conducted at Tampere University Hospital from September 2009 to August 2011. The hospital is the pediatric referral center for the Pirkanmaa Hospital District, a mainly urban area with a birth cohort of approximately 6,000 children. The study was approved by the Ethics Committee of Pirkanmaa Hospital District.

All children under 16 years of age seen in the emergency room (ER) or admitted to a pediatric ward with AGE were eligible for enrolment. Prior to enrolment, a parent or legal guardian provided a written informed consent for participation.

Parents were interviewed about their child's symptoms before the hospital visit and about their child's RV vaccination. A study nurse confirmed the vaccination status from the records of the respective well baby clinic. A stool specimen was collected during the hospital visit in the ER or at the hospital ward.

If the child had required more than one ER visit or hospitalization due to AGE during the study period and if there were more than seven symptom-free days between them, they were considered as two separate episodes.

Laboratory methods

All stool specimens were tested for the presence of RV and human caliciviruses (including NVs and sapoviruses [SaVs]) using a reverse transcription polymerase chain reaction (RT-PCR) method, as described previously [8, 15, 25–27].

After the detection of RV, the G and P genotypes were determined by nucleotide sequencing of the gene segments encoding for the VP7 and VP4 antigens. The gene segment encoding for VP6 protein was also sequenced to determine the presence of vaccine-derived virus [13].

After the detection of human caliciviruses, RT-PCR typing targeting region C at the beginning of the NV capsid region in open reading frame 2 was done with primers JV21, JV24, and JV24mod [3, 35]. The NV genotypes were defined as the polymerase region A/capsid region C genotype.

RV-positive and NV-positive PCR products were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA).

Nucleotide sequences read from the chromatograms were aligned to published sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and from the Food-borne Viruses in Europe network (http://www.rivm.nl; National Institute of Public Health and the Environment, The Netherlands).

Statistical analyses

Statistical analyses were performed using the Mann–Whitney *U* test to compare the age distributions of RVGE and using the chi-square test to calculate the reductions in RVGE between the two study years and two reference years; both tests were performed in SPSS, version 20.0 (SPSS). All tests were two-tailed and a p value <0.05was considered to be statistically significant.

Reference years

The results were compared to reference years of 2006–2007 and 2007–2008 (both seasons from September to August), during which prospective surveillance for RV AGE had been conducted in the same setting using the same methodology [27, 29]. In the second study year in late 2007, an extensive waterborne AGE outbreak occurred in the town of Nokia caused by massive contamination of drinking water by sewage water [27, 28]. We excluded 65 patients associated with this outbreak from the comparative NV analysis to better reflect a normal situation of endemic NVGE [7, 16, 20, 36]. For the RV analysis, those patients were not excluded.

Results

In the 2-year period from 1 September 2009 through 31 August 2011, a total of 495 patients were recruited for the study at Tampere University Hospital. Stool samples were obtained from 330 children (66 % of those recruited)—160 in the first season (September 2009–August 2010) and 170 in the second season (September 2010–August 2011). Of these 330 children, 144 (44 %) were treated in the ER and 186 (56 %) were admitted to a pediatric ward.

For comparison, in the 2-year period of 2006–2008, 1,193 patients were recruited and stool samples were obtained from 809 (68 % of those recruited) children; of whom 434 (54 %) were hospitalized and 375 (46 %) were treated as outpatients.

Rotavirus gastroenteritis

In 2009–2011, of the 330 cases with AGE with a stool specimen, 86 (26 %) were found to have a wild-type RV in stools; 34 of those were treated as outpatients and 52 were hospitalized. Compared to reference years 2006–2007 and

2007–2008 (combined), this means an 81 % reduction of RV AGE in the outpatient clinic (34 vs. 177 cases) and a 76 % reduction in hospital ward admissions (52 vs. 219 cases) (Fig. 1).

The total reduction of all RVGE cases was 80 % during the study years and the proportion of RVGE cases of all AGE cases was decreased from 52 % (421 cases) in the two reference years to 26 % (86 cases) in the two study years combined. The total reduction was statistically significant with $p<0.001$.

RV was found in 43 cases each (27 and 25 %, respectively) of 160 and 170 stool samples obtained in the first and the second season after NIP, respectively. In the first RV epidemic season, the majority of RV-positive AGE cases were seen relatively late between March 2010 and May 2010, whereas in the second season, the most active months were between January 2011 and March 2011 (Fig. 1).

The age distribution of the children with RVGE was from 7 months to 14 years 6 months. The age distribution of RVGE patients shifted towards older children each year ($p<0.001$). In 2006–2008, the median age was 19 months, while in 2009–2010, it was 24 months, and in 2010–2011, it was 36 months. Still, the proportion of RVGE cases decreased in every age group, even among children too old for vaccination. The reduction in the age groups eligible for vaccination (patients <1 year of age in 2009–2010 and patients <2 years of age in 2010–2011) was 91 % (16 vs. 178 patients), and in children too old to be vaccinated in NIP, it was 72 % (70 vs. 243 patients). The age distribution of RV-positive cases during 2006–2008 and 2009–2011 are shown in Fig. 2.

The predominant RV types in the two seasons 2009 to 2010 and 2010 to 2011, combined, were G1P[8] ($n=38$, 44 %) and G4P[8] ($n=30$, 35 %). In the first season, genotype G4P[8] ($n=18$, 42 %) was slightly more common than G1P[8] ($n=15$, 35 %), but in the second season, genotype G1P[8] was more predominant with 53 % ($n=23$) over G4P[8] ($n=13$, 30 %). Other common RV genotypes G2P[4], G3P[8], and G9P[8] were all seen to a lesser extent ($n=9$, $n=1$, and $n=6$, respectively, counted from all RV-positive AGE cases in the two seasons combined). In two cases, more than one RV type was found in stools simultaneously: in one case, G1P[8] with G3P[8] and, in the other, G3P[8] with G9P[8]. Other than the predominance of G9P[8] genotype in the season of 2006–2007, no great changes in the genotype distribution were observed during the study years compared to the reference years (data not shown).

Among the 86 wild-type RV-positive cases, there were 4 children who had received at least 1 dose of RotaTeq® and 1 child who had received Rotarix™ before the introduction of NIP. Three of those who had received RotaTeq® were fully vaccinated (two were detected with G4P[8] in the stools and one was detected with G9P[8] in the stools) and one had received only one dose and was detected with G4P[8] RV. The child who had received Rotarix™ was also fully vaccinated with two doses and was detected with the G4P[8] genotype. Two of the four breakthrough cases, 9- and 10-month-old fully vaccinated boys (RotaTeq®), were admitted to the pediatric ward and the other two were seen in the ER only.

We identified three cases of GE in young infants shedding a human–bovine double reassortant G1P[8] vaccine virus. This human–bovine double reassortant was also detected from one patient infected concomitantly with NV. Furthermore, one patient was detected with RotaTeq® vaccine virus G6P[8] and 16 patients were detected to shed the original vaccine virus G1P[5] or just the VP7 G1 part of it separately or detected with several VP4 proteins. No patients were detected with Rotarix™ vaccine strain after 2006–2008. The vaccine-associated cases have been reported separately [13].

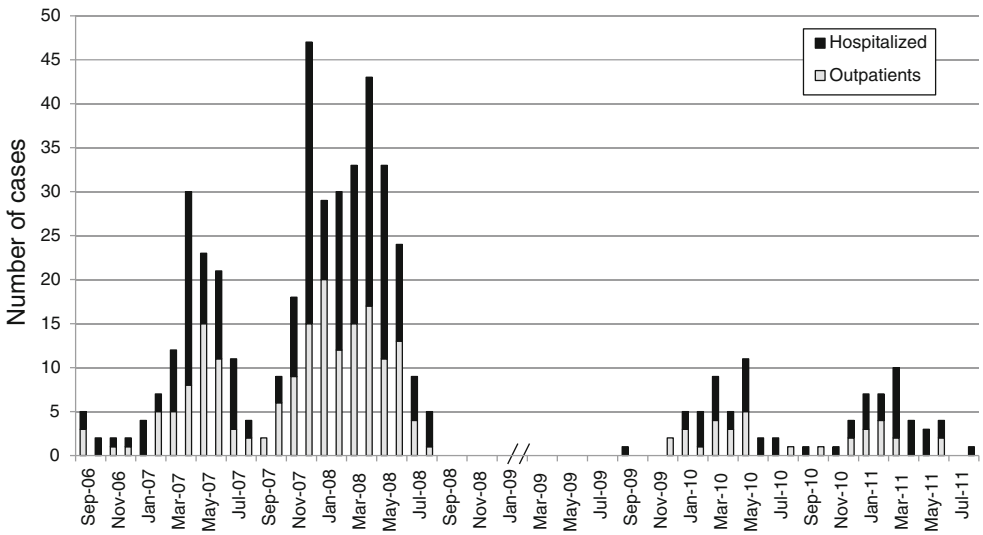

Fig. 1 Timing of the RV seasons between September 2006 and August 2008 and September 2009 and August 2011 and treatment needed in the 507 children seen at Tampere University Hospital because of acute RVGE

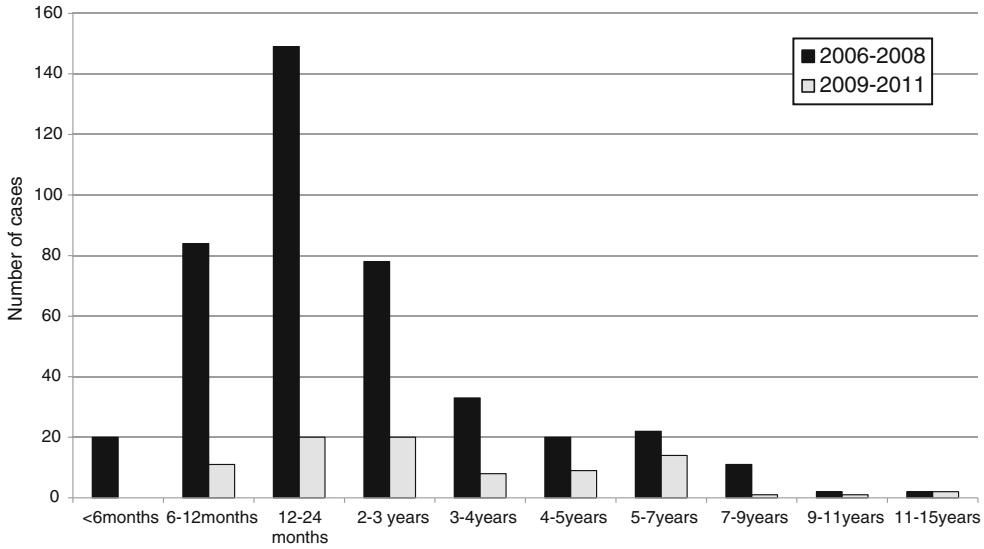

Fig. 2 Reduction of RVGE between 2006–2008 and 2009–2011 in different age groups

Norovirus gastroenteritis

Of all 330 cases of GE in 2009–2011, 111 (33.6 %) were NV-positive. In the first year, NV was found in 52 (33 %) of 160 stool samples and, in the second season, in 59 (35 %) of 170 stool samples (Fig. 3). SaV was found in a total of 23 (7.0 %) cases, 13 (8.1 % of 160) of these in the first season and 10 (5.9 % of 170) in the second season.

Of the NV-positive cases, only one was a mixed infection with RV (more specifically with G2P[6]). Three of 23 (13 %) SaV-positive cases were mixed infections with RVs. There were no cases with NV and SaV in the stools at the same time. The reduction of GE positive for NV, SaV, and RV is shown in Fig. 4.

In the reference years 2006–2008, there were 196 cases (excluding the outbreak mentioned in the "Materials and methods" section) of NVGE as compared with 111 cases in the study years 2009–2011. Of the 111 NV-positive cases, 69 (62 %) were admitted to the hospital and 42 (38 %) were treated as outpatients. Even though the absolute number of NV-positive cases decreased slightly (196 vs. 111 cases), the proportion of NVGE of all AGE increased (26 vs. 33.6 %) from the reference years. Moreover, the proportion of NV-positive cases that were admitted to the hospital increased from 47 % (92) in the reference years to 62 % (69) in the study years (Fig. 4).

Compared to the reference years, the proportion and the absolute number of SaV-positive GE increased from 1.6 % (12 cases) to 7.0 % (23 cases). A clear seasonality was seen in the NVGE both in the study years and in the reference years (Fig. 3). The most active months when the majority of NV-positive cases were seen were between January and April in each year.

Of all 111 NV-positive cases, 108 (97 %) were genogroup GII strains and 72 (65 %) were genotype GII.4 (37 (71 % of 52 cases) and 35 (59 % of 59 cases) in the first and the second study years, respectively). In the reference years, genotype GII.4 was even more common with 89 % proportion (175 of 196 cases). The other genotypes detected were GII.b (14 %, $n=15$), GII.7 (13 %, $n=14$), GII.g (5 %, $n=6$),

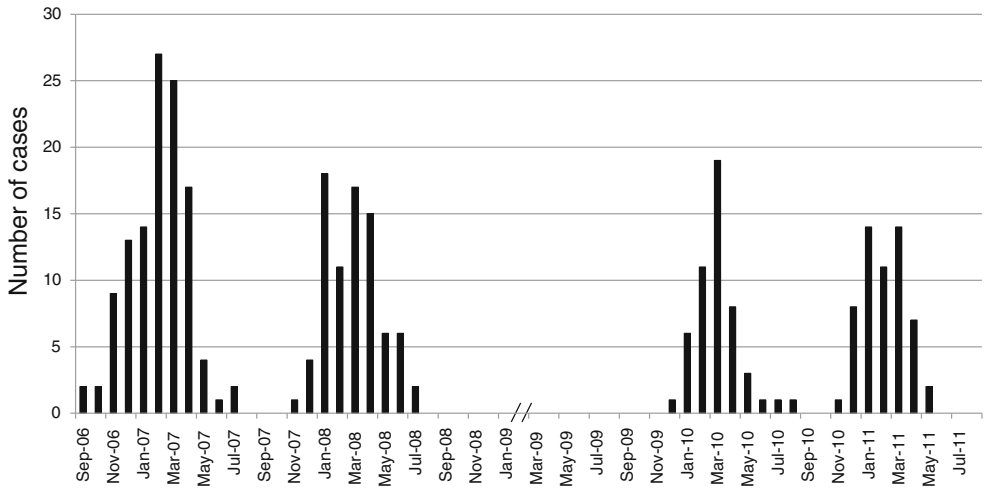

Fig. 3 Seasonality of the NVs seen in AGE in children between 2006–2008 and 2009–2011 in Tampere University Hospital

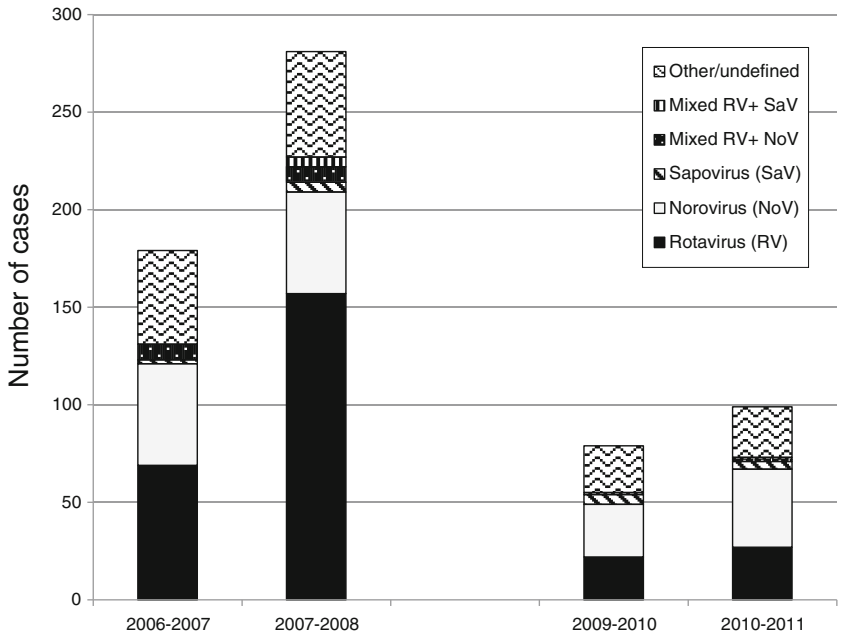

Fig. 4 Viruses detected in the stools of children admitted to the hospital for AGE in 2006–2008 and 2009–2011

GI.4 (2 %, n=2), GI.3 (1 %, n=1), and GII.e (1 %, n=1). Additional genotypes detected in the reference years included GII.1, GII.c, GI.6, and GII.2, all of which accounted for <1 % each and none of which were detected in the study years 2009–2011.

The age distribution of NV-positive children was similar between the study years and the reference years. The age range was from 7 days to 15 years 7 months (19 days to 13 years 8 months in reference years), with a median age of 12 months (15 months in reference years). Eighty-four of 111 cases (76 %) of children were under 24 months of age, and 41 % was under 12 months of age.

Gastroenteritis with neither RV nor NV

In the study years 2009–2011, of the 330 cases of AGE, 222 (67 %) were positive for RV, NV, or SaV alone or as a mixed infection, whereas in the reference years 2006–2008, 603 (76 %) were positive for RV, NV, or SaV alone or as a mixed infection. The absolute number of GE cases due to other pathogens decreased from 191 cases in the reference years to 108 cases in the study years. Of 108 cases, 53 % (57 cases) detected in the two study years was admitted to a pediatric ward and 47 % (51 cases) was treated as outpatients. RV, NV, or SaV could be detected in the stools in 70 % of all 186 cases admitted to a pediatric ward due to GE and, conversely, 30 % was negative for these viruses (Fig. 4). The absolute number of children who were admitted due to GE and were negative for RV, NV, or SaV decreased from 101 cases in the reference years to 57 cases in the study years. Even though a systematic search for other GE viruses was not performed, some of these patients were found to have other viral agents such as human bocavirus, adenovirus, astrovirus, and coronavirus in their stools [30, 31]. In the emergency department, 35 % (51 of 144 cases) of the children seen for GE symptoms were negative for RV, NV, or SaV. All hospital admissions due to all AGE decreased by 57 % from 434 cases in the reference years to 186 cases in the study years, and the proportion of outpatient clinic visits decreased by 62 % from 375 to 144 cases.

Discussion

In this study, we examined the impact of the National RV Immunization Programme (NIP) on hospitalizations and outpatient clinic visits due to GE in one hospital. The coverage population of the Tampere University Hospital is about one tenth of Finland, and the results may be generalized for the whole country.

We detected a significant reduction in outpatient AGE visits and hospital admissions due to RV (81 and 76 %, respectively) in the 2-year post-NIP period in the entire children population. Similar reductions with the exclusive use of RotaTeq have been observed previously from the USA [32, 33, 37] and from countries using both two available RV vaccines [2, 4, 23].

We did not observe further reduction in RVGE between first (2009–2010) and second (2010–2011) seasons. This might be because the second season post-NIP (2010–2011) might have been a strong epidemic season of RV, resulting in more RV infection pressure; just like in the two reference years, season 2007–2008 was a high epidemic season compared to 2006–2007.

Our study supports the evidence of herd protection in children too old to be vaccinated that have been observed in three studies from the USA after the widespread use of RV

vaccines [5, 9, 37]. We observed that the reduction in RVGE cases was statistically significant in every age group. The decrease in hospitalizations in children too old to be vaccinated was 72 %, similar to the findings from the USA (70–79 %) [5, 9, 37]. We found no cases of wild-type RVGE in children <6 months of age. In contrast, in the USA, no reduction in infants <3 months of age, who had been too young to be vaccinated, was observed [5, 9, 37]. Additionally, we observed that the median age distribution of RVGE cases had shifted toward older children.

The high level of herd protection in our study probably resulted from high vaccine coverage. In Austria, no evidence of herd protection was found with vaccine coverage of 57 % in 2007 (RotaTeq) [2]. The reason for herd protection is probably interruption of RV transmission among all children. Exposure of unvaccinated children to vaccine virus shed in the stools of vaccinated infants is possible but unlikely to explain herd protection. Such transmission of vaccine-acquired virus resulting to a symptomatic RVGE has been reported in several countries [24]. In our study, we detected shedding of vaccine virus in a number of children, but all were recently vaccinated and none was unvaccinated.

After the introduction of the RV vaccine into the vaccination program, several studies have detected unusual non-vaccine-included RV strains, such as G8 or G12, or changes in the genotype distribution [14]. However, none of these changes were observed in our study.

In addition, we observed that the impact of RV vaccination was reflected as decreased hospital admissions and outpatient clinic visits for GE of any cause. Compared to the pre-NIP period, there was a 57 % reduction in cases admitted to the hospital ward for all GE. The reduction was higher than the reduction rates observed in previous studies from the USA (29–52 %) [5, 6, 37].

In addition, we observed a reduction of 62 % in all outpatient clinic visits for GE of any cause. Interestingly, such a reduction in all outpatient clinic visits has not been reported from countries where the protective effect of RV vaccination in unvaccinated children has been observed [10, 12].

The important role of NV as a causative agent of endemic (not outbreak-associated) GE in children was first discovered in Finland in connection with an efficacy trial of RotaShield vaccine [18]. In the same study, it was observed that RV vaccine (RotaShield) did not have any effect on NVGE. In that sense, the present findings on the impact of universal RV vaccination on NVGE are (only) confirmatory, and we conclude that the RotaTeq vaccination program does not reduce NVGE. The slight decrease observed in the study vs. reference years may well be explained by natural annual variation. In a decade, there has been considerable year to year variation of NVGE, although the winter epidemic has occurred every year [26].

In reverse, other viruses and notably NVs could theoretically replace RV after its elimination by universal vaccination and fill the available niche as a major causative agent of AGE in children. Our results strongly suggest that such a replacement is not happening. Overall hospitalizations have been reduced according to the share of RV, and NV has become a leading cause of GE only in relative terms, without any increase in absolute numbers.

The reduction of all hospitalizations (57 %) and outpatient clinic visits (62 %) due to GE is well in line with what was observed in the prelicensure efficacy trial (REST) of the RotaTeq vaccine in Finland. RotaTeq reduced all-cause GE requiring medical intervention by 65 % over a period of 3.1 years [34]. To compare the numbers, it should be noted that the present population-based study also includes children who were eligible for vaccination but did not receive it (initially about 10 %, decreasing to 5 % over time).

The present study focused on NV and was not intended as a full etiological examination of GE. Hospitalizations due to GE not associated with RV or NV seemed to decrease somewhat in comparison with the reference years. This observation should be viewed with caution, and a detailed etiological study of GE viruses should be performed before conclusions. However, even though the RV vaccine had no effect on NVGE, it is nevertheless possible that RV vaccination might have a "nonspecific" effect on non-RV-associated GE. Some suggestive evidence of RV vaccine (RotaShield) effect on adenovirus GE was seen in the study in the 1990s [19].

The new leading role of NV as the main causative agent of AGE in children supports the concept of developing an NV vaccine for use in children [11]. Such a vaccine is foreseen and being developed [1].

Conclusion

RV in the NIP of Finland had an immediate and major impact on RVGE cases seen in hospital, i.e., severe RVGE. The age distribution of children with RVGE has shifted upwards at the same time as a statistically significant decrease in every age group was observed as an evidence of herd protection. The impact of RV vaccination was reflected in a decrease of all hospital admissions and outpatient clinic visits for GE, while NV has become the leading cause of AGE in children seen in hospital.

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Conflict of interest Maria Hemming, Sirpa Räsänen, Leena Huhti, Minna Paloniemi, and Marjo Salminen have no conflict of interest to disclose. Timo Vesikari has been the principal investigator of clinical trials of rotavirus vaccines produced by Merck and GlaxoSmithKline and is a member of the advisory boards of Sanofi Pasteur MSD, Merck, Pfizer, Novartis, and GSK.

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Review

Genetic diversity of G1P[8] rotavirus VP7 and VP8* antigens in Finland over a 20-year period: No evidence for selection pressure by universal mass vaccination with RotaTeq® vaccine

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ABSTRACT

Two live-attenuated oral vaccines (Rotarix™ and Rotateq®) against rotavirus gastroenteritis were licensed in 2006 and have been introduced into National Immunization Programs (NIPs) of several countries. Large scale use of rotavirus vaccines might cause antigenic pressure on circulating rotavirus types or lead to selection of new rotaviruses thus decreasing vaccine efficacy.

We examined the nucleotide and amino acid sequences of the surface proteins VP7 and VP4 (cleaved to VP8* and VP5*) of a total of 108 G1P[8] rotavirus strains collected over a 20-year period from 1992, including the years 2006–2009 when rotavirus vaccine (mainly Rotarix™) was available, and the years 2009–2012 after implementation of RotaTeq® vaccine into the NIP of Finland.

In G1 VP7 no changes at amino acid level were observed. In VP8* periodical fluctuation of the sublineage over the study period was found with multiple changes both at nucleotide and amino acid levels. Most amino acid changes were in the dominant antigenic epitopes of VP8*. A change in VP8* sublineage occurred between 2008 and 2009, with a temporal correlation to the use of Rotarix™ up to 30% coverage in the period. In contrast, no antigenic changes in the VP8* protein appeared to be correlated to the exclusive use of RotaTeq® vaccine after 2009.

Nevertheless, long-term surveillance of antigenic changes in VP4 and also VP7 proteins in wild-type rotavirus strains is warranted in countries with large scale use of the currently licensed live oral rotavirus vaccines.

Contents

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1. Introduction

Rotaviruses (RVs) are major causative agents of acute gastroenteritis in infants and young children worldwide (Parashar et al., 2006). RVs have a segmented double-stranded RNA genome consisting of 11 gene segments within a non-enveloped, multilayered protein capsid. Six of the 11 gene segments code for structural proteins (VP1–VP4, VP6 and VP7), which are arranged into three concentric layers surrounding the rotavirus genome, while the other 5 gene segments code for non-structural proteins (NSP). The outermost layer of the virus particle is composed of VP7 and VP4 proteins both of which induce neutralizing antibodies (Malik et al., 2008). Glycoprotein VP7 (34 kDa) forms the smooth external surface of the outer shell and contains two structurally defined antigenic epitopes (7-1 and 7-2), which are targets of neutralizing antibodies (Aoki et al., 2009).

The non-glycosylated spike protein VP4 (88 kDa) is implicated in several important functions, including attachment to the cell, penetration, hemagglutination, and virulence (Ruggeri and Greenberg, 1991). VP4 is activated by proteolytic trypsin cleavage to produce VP8* and VP5* proteins. VP8* has five sequential neutralizing epitopes which induce cross-lineage neutralizing antibodies and four surface-exposed antigenic epitopes (8-1 to 8-4) that induce serotype-specific neutralizing antibodies. VP5* has five surface-exposed antigenic epitopes (5-1 to 5-5), which show more cross-reactive neutralization among strains belonging to different VP4 serotypes (Larralde et al., 1991; Dormitzer et al., 2002, 2004; Kovacs-Nolan et al., 2003).

The VP7 and VP4 antigens are used to classify rotaviruses to G- and P-genotypes, respectively (Matthijnssens et al., 2011). The most common circulating rotavirus genotype around the world and in Finland is G1P[8]; yet the genetic diversity within circulating G1P[8] rotavirus strains is great with several intragenotypic lineages and their sublineages (Rasanen et al., 2011; Matthijnssens et al., 2011; Maunula and von Bonsdorff, 1998). As a whole, the diversity of rotaviruses is generated by several mechanisms, including point mutations and gene rearrangements (Kirkwood, 2010).

Large scale use of rotavirus vaccination has been proposed to potentially cause genetic drift of the virus genome or even new reassortments leading to antigenically new strains, thus decreasing the vaccine efficacy (Kirkwood, 2010; Hoshino et al., 2004). Two live-attenuated oral vaccines against rotavirus gastroenteritis have been available since 2006. The monovalent vaccine (Rotarix™, GlaxoSmithKline) is derived from human rotavirus type G1P[8] whereas the pentavalent (RV5) rotavirus vaccine, licensed as RotaTeq® (Merck&Co., Inc.), is a human-bovine reassortant vaccine containing four reassortant viruses of human G-types (G1–G4) and one reassortant for human P-type (P[8]) reassorted on a bovine G6P[5] rotavirus backbone. Both vaccines were found in large prelicensure trials to be safe and efficacious against severe rotavirus disease (Vesikari et al., 2006; Bernstein et al., 1998; Vesikari et al., 2010).

In Belgium, Rotarix™ was introduced in 2006 with a reimbursement plan and an 85% coverage was reached (Braeckman et al., 2011). Possible vaccine induced selective pressure on G1P[8] rotavirus strains was studied after introduction of Rotarix™; the study found changes in the prevalent VP7 sublineage of the wild-type G1, with strains more distinct from Rotarix™ becoming more prevalent (Zeller et al., 2010, Zeller, M., J. Matthijnssens, M. Rahman, M. Van Ranst,. Possible immune evasion by G1 lineage I after vaccine introduction in Belgium? Poster Presentation, Third European Rotavirus Biology Meeting, Loch Lomond, Scotland. 2009).

In Finland, rotavirus vaccines became available in 2006 in the private market, and Rotarix™ was the most commonly used vaccine until September 2009 (coverage up to 30%), when rotavirus vaccination was introduced into the National Immunization Program (NIP) with exclusive use of RotaTeq® vaccine. As the implementation of RotaTeq® might also induce a selective pressure on VP7 and VP4 lineages of the prevalent G1P[8] rotavirus, we were interested to examine the potential modifications in these proteins attributable to the use of pentavalent human-bovine reassortant vaccine RotaTeq®. At the same time, for comparison, we examined a collection of G1P[8] rotavirus strains, the predominant rotavirus genotype from 1992 (periods 1992–1994, 2002–2004, 2006–2008 and 2009–2012), including the period before rotavirus vaccination, the interim period 2006–2009 when Rotarix™ vaccine was used with a moderately high coverage and the incidence of G1P[8] rotaviruses in Finland was 63%, and the period 2009–2012 with use of RotaTeq® in NIP when the incidence of G1P[8] on the average was 44% (Räsänen et al., 2011, Hemming et al., 2013; Vesikari et al., 1999).

2. Materials and methods

2.1. Study samples

For this study, a total of 108 wild-type (not vaccine acquired) rotavirus G1P[8] strains were selected from several prospective studies conducted between 1992 and 2012 in Tampere. The study periods are defined to last from September 1 in 1 year to August 31 the following year. All the stool specimens had been collected from unvaccinated or placebo vaccinated children who had had rotavirus gastroenteritis, including 11 sample strains from 1992–1994 (6 from 1993, 5 from 1994), 29 samples from 2002–2004 (2 from 2002, 13 from 2003, 14 from 2004), 33 samples from 2006–2008 (4 from 2006, 15 from 2007, 14 from 2008), and 35 samples from the post-NIP period 2009–2012 (3 from 2009, 14 from 2010, 12 from 2011, 6 from 2012). The specimens from the post-NIP period were collected at the Tampere University Hospital from children hospitalized or seen in the outpatient clinic for RVGE and the material included all detected wild-type G1 strains of which the VP7 and VP4 sequences could be determined.

RotaTeq® vaccine strain VP7 and VP4 sequences were taken from three patients shedding identical G1P[8] double reassortant rotavirus after RotaTeq® vaccination and are identical with the published sequences of RotaTeq® by Matthijnssens et al. (Hemming and Vesikari, 2012; Matthijnssens et al., 2010). The nucleotide sequences of the VP7 and VP4 of Rotarix™ were determined from a commercially obtained dose of Rotarix™ and deposited in GenBank by Zeller et al. (Accession no; JN849114.1 and JN849113.1, respectively) (Zeller et al., 2012). For the VP4 sequence analysis, human rotavirus VP4 P[8]b from strain MMC71 (Accession no; EU979382.1) was used as a reference strain in sequence alignments (Nagashima et al., 2009).

2.2. RT-PCR and nucleotide sequencing

The stool specimens were studied by reverse transcription-polymerase chain reaction (RT-PCR) to detect rotavirus and further to determine the genotype. Viral RNAs were extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RT-PCR for the VP7 and the VP4 gene segments were performed as described previously. (Rasanen et al., 2011; Gouvea et al., 1990; Pang et al., 1999; Das et al., 1994; Simmonds et al., 2008) The VP7 gene segment was amplified using the forward primer Beg9 (5′-GGCTTTAAAAGAGA-GAATTTCCGTCTGG-3′) and the reverse primer End9 (5′-GGTCA-CATCATACAATTCTAATCTAAG-3′), and additionally, if required

due to short or unclear sequences, also using the primer H rev (5′-AACTTGCCACCATTTTTTCC-3′) and the primer G1 fwd (5′- CAAGTACTCAAATCAATGATGG-3′). For the section of VP4 coding for the tryptic cleavage product VP8*, the gene segment was amplified using primers VP4 fwd and VP4 rev (5′-TATGCTCCAGTNAATTGG-3′ and 5′- CTATTGTTAGAGGTTAGAGTC-3′, respectively, for N = [A,C,G,T]). Gel purified amplicons were sequenced using BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Applied Biosystems, Foster City, CA) on an ABI Prism 310 Genetic Analyzer.

2.3. Sequence analyses

Sequencing files were analyzed and consensus sequences were prepared using Sequencer (4.9). Multiple consensus alignments were conducted using the Clustal Omega. In the phylogenetic analysis a 860 bp fragment of VP7 and a 552 bp fragment of VP8* were aligned to published sequences from GenBank. Statistical analyses and phylogenetic trees were constructed with Neighbor-joining method using the Kimura-two parameter model, with the MEGA version 4.0 software (Tamura et al., 2007) .The statistical significance was assessed by bootstrap resampling analysis (2000 pseudo-replicates). The sample strains were compared to VP7 and VP4 gene segments from both Rotarix™ and RotaTeq®-vaccine. If the child participated in various studies at the same time, or RNA was extracted several times, the available sequence from additional extraction was compared to the original extraction to exclude the non reproducibility.

2.4. Nomenclature and reference strains

The obtained strains are named according to recommended guidelines by Matthijnssens et al. (Matthijnssens et al., 2011). The common name used in nomenclature represents the year of detection compared to availability of rotavirus vaccine in Finland, "POSTVC" indicating study periods after RotaTeq® vaccine implementation into NIP of Finland (2009–2012), "INTVC" indicating study periods with commercially available rotavirus vaccines (RotaTeq® and Rotarix™) before NIP (2006–2008) and "PREVC" indicating study periods before rotavirus vaccines (1992–1994 and 2002–2004). Nomenclature of all obtained strains in both nucleotide and amino acid analyses is uniform so that "N" in common name of nucleotide analyses has been replaced with "A" in amino acid analyses.

The accession numbers to reference strains are published reference strains with highest match (99–100%) to the actually obtained strains, with a maximum of six sporadic nucleotide changes compared to the original sequences. The actually obtained strains have not been deposited into GenBank due to absence of required information in consent. The Results have been calculated from the actually obtained sequences, not from reference strains. In addition, the phylogenetic trees have been formed from the actually obtained sequences.

Nucleotide sequences of G1 rotavirus strains and accession numbers used in this study are the following

RVA/Vaccine/FIN/RotaTeqFIN17/2011/G1P[5]:GU565057.1,
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]: JN849114.1,
RVA/Human-wt/FIN/POSTVC7-1Nx/xxxx/G1P[8]: GU377135.2,
RVA/Human-wt/FIN/POSTVC7-2Nx/xxxx/G1P[8]: FJ948838.1,
RVA/Human-wt/FIN/POSTVC7-3Nx/xxxx/G1P[8]: GU979202.1,
RVA/Human-wt/FIN/POSTVC7-4Nx/xxxx/G1P[8]: JF490444.1,
RVA/Human-wt/FIN/POSTVC7-5Nx/xxxx/G1P[8]: JN258368.1,
RVA/Human-wt/FIN/POSTVC7-6Nx/xxxx/G1P[8]: JX027828.1,
RVA/Human-wt/FIN/INTVC07-1Nx/xxxx/G1P[8]: FJ948848.1,
RVA/Human-wt/FIN/INTVC07-2Nx/xxxx/G1P[8]: HQ392250.1,
RVA/Human-wt/FIN/PREVC07-1Nx/xxxx/G1P[8]: JN232069.1,
RVA/Human-wt/FIN/PREVC07-2Nx/xxxx/G1P[8]: JN232064.1,
RVA/Human-wt/FIN/PREVC07-3Nx/xxxx/G1P[8]: M93006.1,
RVA/Human-wt/FIN/PREVC07-4Nx/xxxx/G1P[8]: AF480263.1
X = 1–9,a-b,1993–2012.
(http://www.ncbi.nlm.nih.gov/Genbank/index.html)

Reference VP4 nucleotide sequences of P[8] rotavirus strains and accession numbers used in this study are the following:

RVA/Vaccine/FIN/RotaTeqFIN/2011/G6P[8]: GU565044.1
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]: JN849113.1,
RVA/Human-wt/FIN/POSTVC-1Nx/xxxx/G1P[8]: JN580432.1,
RVA/Human-wt/FIN/POSTVC-2Nx/xxxx/G1P[8]: JQ248943.1,
RVA/Human-wt/FIN/POSTVC-3Nx/xxxx/G1P[8]: FN179467.1,
RVA/Human-wt/FIN/POSTVC-4Nx/xxxx/G1P[8]: JQ613166.1,
RVA/Human-wt/FIN/INTVC1-1Nx/xxxx/G1P[8]: JN849119.1,
RVA/Human-wt/FIN/INTVC2-2Nx/xxxx/G1P[8]: GU392991.1,
RVA/Human-wt/FIN/PREVC1-1Nx/xxxx/G1P[8]: DQ857910.1,
RVA/Human-wt/FIN/PREVC1-2Nx/xxxx/G1P[8]:: FN179467.1,
RVA/Human-wt/FIN/PREVC2-1Nx/xxxx/G1P[8]:: HQ392417.1,
RVA/Human-wt/FIN/PREVC2-2Nx/xxxx/G1P[8]:: JN849151.1,
X = 1–9, a-b, 1993–2012
(http://www.ncbi.nlm.nih.gov/Genbank/index.html)

3. Results

3.1. Analysis of the VP7 sequences

At nucleotide level, wild-type G1 VP7 sequences (*N* = 108) from the study were aligned to 12 published sequence strains from GenBank. These 12 strains aligned to two different subgroups referred to as sublineages G1-I and G1-II, both of which were found in samples from each time period (Fig. 1). At nucleotide level, the difference between the wild-type viruses representing the two sublineages, G1-I and G1-II, was from 1.3% to 3.6% (13/1007 and 36/1007) substitutions for G1-I sublineage strains and from 1.7% to 4.1% (17/1004 and 42/1004) substitutions for G1-II sublineage strains, from the closest to the farthest homogeneity between strains, respectively .The majority of nucleotide substitutions between intralineage strains occurred between purines (A,T) or pyrimidines (C,G). As reported before, Rotarix™ VP7 sequence aligned with the G1-II strains, whereas RotaTeq® VP7 sequence corresponded to the G1-III sublineage (Zeller et al., 2012). However, at amino acid level, the wild-type strains from both intralineages were identical, aligning into the same G1 sublineages (G1-I and G1-II) as at nucleotide level, indicating that no amino acid changes in the VP7 protein had occurred during the 20-year period, including the time after licensure and use of Rotarix™ from 2007 to 2009 and RotaTeq® in NIP since 2009, as both two sublineages (G1-I and G1-II) have been circulating during the study period.

3.2. Analysis of the VP8* sequences

The 108 wild-type rotaviruses of the total study period from 1992 to 2012 clustered into two intragenotypic lineages of VP8* (P[8]-I and P[8]-III). Interestingly, samples from years 1992 to1994 (*N* = 11) and 2006 to 2008 (*N* = 33) clustered both at nucleotide level and at amino acid level into the same subgroup with Rotarix™ vaccine, defined as subgroup P[8]-I, whereas samples from years 2002 to 2004 (*N* = 29) and 2009 to 2012 (*N* = 35) clustered into the subgroup P[8]-III. VP8* of RotaTeq® vaccine aligned with the subgroup P[8]-II (Fig. 2a and b). Both subgroup P[8]-I and P[8]-III containing study samples clustered separately from P[8]b VP4 (OP354-like) gene from strain MMC71 which represents the rare sublineage P[8]-IV. OP354-like VP4 strain aligns

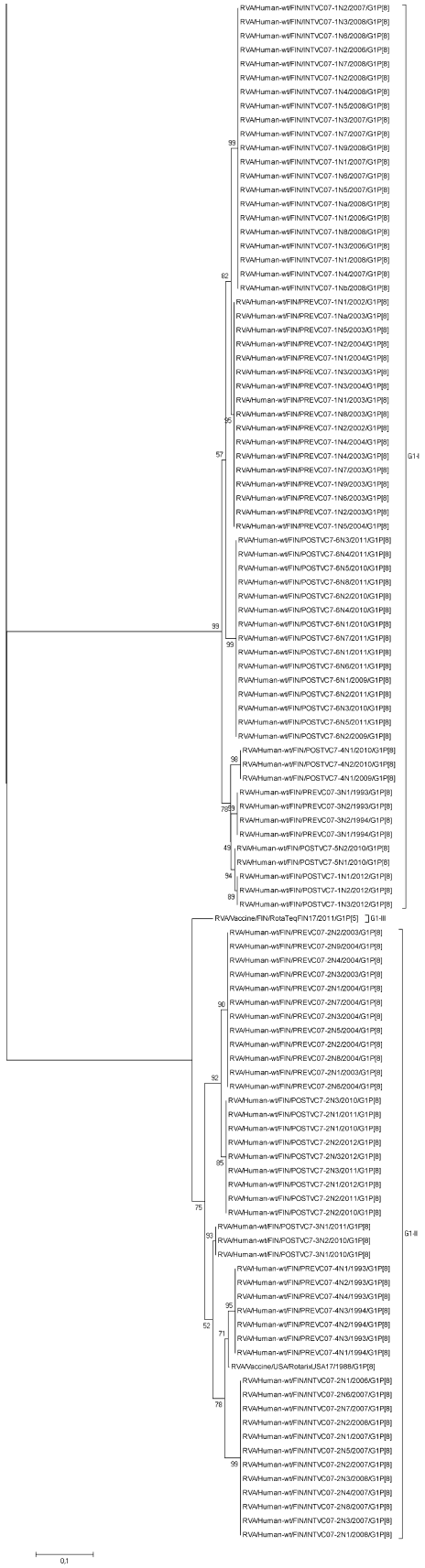

Fig. 1. Phylogenetic dendrogram of VP7 of G1P[8] sequences at nucleotide level in the years 1992–2012 in Finland. Bootstrap values (2000 replicates). Intragenotypic lineages are given by squared brackets to the right.

phylogenetically distinct, between P[8]- and P[4]- strains (data not shown) (Nagashima et al., 2009).

At nucleotide level, the difference between the wild-type viruses representing the subgroups (P[8]-I and P[8]-III) was from 7.4% to 9.6% (41/552 and 53/552) substitutions, from the closest to the farthest homogeneity between strains, respectively (Fig. 2a). Strains from every pre-NIP time period 1992–1994, 2002–2004 and 2006–2008 aligned all into two intrasublineage groups (Fig. 2a), whereas the strains from post-NIP seasons 2009–2012 showed more diversity by aligning into four intrasublineage subgroups.

At amino acid level, the wild-type G1 VP4 strains clustering to the subgroup P[8]-I (11 samples from 1992 to 1994 and 33 from 2006 to 2008) from two different pre-NIP time periods, aligning both into two intrasublineage groups at nucleotide level, were identical with each other and aligned into two separateintrasublineage groups accordingly to the two time periods so that only one group from those two time periods was observed. The difference between strains belonging to two time periods (1992–1994 and 2006–2008) was one amino acid at the position 113 (Asn → Thr) in the antigenic region 8-3 (Fig. 2b and 3.)

Strains clustering to the subgroup P[8]-III (29 samples from 2002 to 2004 and 35 from 2009 to 2012) aligned into six intrasublineage groups. The samples from 2002–2004 ($N = 29$) aligned into two subgroups, with a difference of two amino acids (amino acid positions 78 and 217)(Fig. 2a and b). The 35 strains from the post-NIP period 2009–2012 showed more diversity than those from the pre-NIP period by aligning into four intrasublineage subgroups. However, only strains "POSTVC-3" were circulating in every post-NIP rotavirus season (from September 2009 to August 2012). The difference between the other post-NIP strains (POSTVC-1, POSTVC-2, POSTVC-4) compared with the strains POSTVC-3, was from 1 to 2 sporadic amino acid changes in positions aa 91, aa 95 and/or aa 104 (Fig. 3) However, none of these substitutions was located in the antigenic regions (Kovacs-Nolan et al., 2003; Dormitzer et al., 2004).

The difference between wild-type strains of the subgroup P[8]-III and RotaTeq® vaccine strain representing the P[8]-II lineage was from 4.4% to 5.0%, from 9 to 11 substitutions in the 184 aa fragment, while the difference between the strains clustering into the subgroup P[8]-I and RotaTeq® was from 6.1% to 6.6%; therefore the sequences from subgroup P[8]-III were closer to that of RotaTeq® P[8] reassortant than the sequences from subgroup P[8]-I (Zeller et al., 2012). The difference between the wild-type rotaviruses in subgroup P[8]-I and Rotarix™-strain was from 0.5% to 1.0% (different amino acid in position 168 alone or together with aa 113).

The difference between G1 VP4 antigens in wild-type rotaviruses of the subgroups P[8]-I and P[8]-III was from 6.5% to 7.6% (from 12 substitutions to 14 substitutions in 184 aa fragment), from the closest to the farthest homogeneity, respectively, and 9 of the substitutions were located in the known serotype-specific neutralizing epitope regions. All amino acid substitutions occurred individually between amino acids 66–196 except for two consecutive amino acid changes in positions 120 and 121 aa (Fig. 3), located in the fourth (V115G123) of five sequential neutralization epitopes in the Wa* protein (Kovacs-Nolan et al., 2003).

The other sporadic substitutions occurred in two of four surface-exposed antigenic epitopes (8-1 and 8-3) of VP8 (Fig. 3) (Dormitzer et al., 2002, 2004). The amino acid substitutions in the one sequential neutralization epitope and the two surface-exposed antigenic epitopes shift according to the phylogenetic tree so that for the most part the strains from 2002 to 2004 and 2009 to 2012 were more similar to the RotaTeq VP8* sequence, and the strains from 1992 to 1994 and 2006 to 2008 resembled the VP8* sequence of Rotarix™ (Fig. 2b) (Fig. 3).

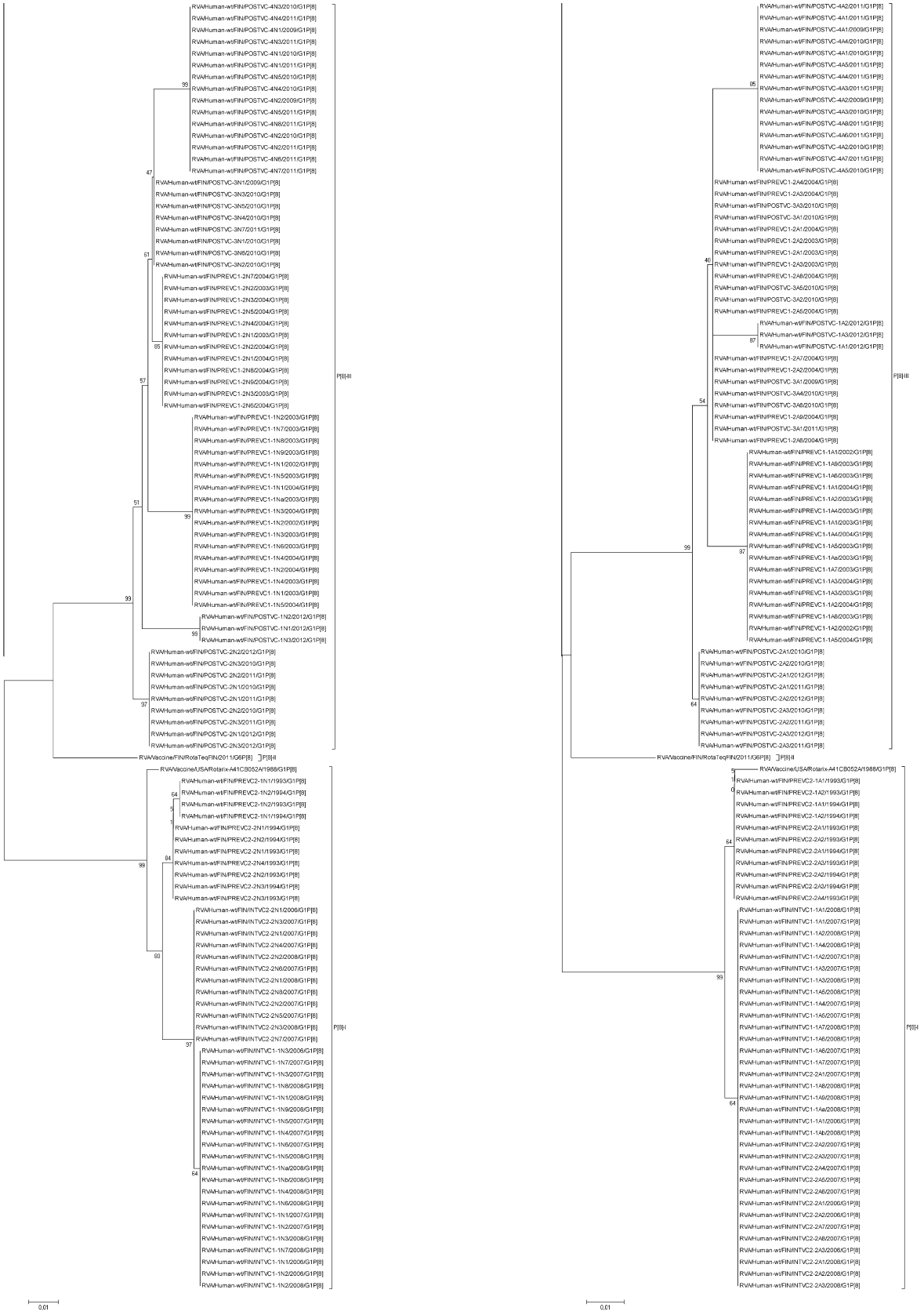

Fig. 2. (a) Phylogenetic dendrogram of VP8* of G1P[8] sequences at nucleotide level during 1992–2012 in Finland. Bootstrap values (2000 replicates). Intragenotypic lineages are given by squared brackets to the right. (b) Phylogenetic dendrogram obtained from amino acid sequence analyses on VP8* genes of G1 rotaviruses in Finland during 1992–2012. Intragenotypic lineages are given by squared brackets to the right.

Fig. 2 (*continued*)

	Number	Year	8-1											8-2		8-3									8-4		
			100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
RotaTeq	1	2011	D	S	S	N	S	N	A	N	L	N	D	E	R	N	P	V	D	N	R	N	D	D	N	T	N
Rotarix	1	1988	D	S	S	N	S	S	A	N	L	N	N	E	R	N	P	V	D	S	S	N	D	N	N	T	N
POSTVC-1Ax/xxxx/G1P[8]	3	2012	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
POSTVC-2Ax/xxxx/G1P[8]	9	2010-2012	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
POSTVC-3Ax/xxxx/G1P[8]	8	2009-2011	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
POSTVC-4Ax/xxxx/G1P[8]	15	2009-2011	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
INTVCx-xAx/xxxx/G1P[8]	33	2006-2008	D	S	S	N	S	S	A	N	L	N	N	E	R	T	P	V	D	S	S	N	D	N	N	T	N
PREVC1-1Ax/xxxx/G1P[8]	17	2002-2004	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
PREVC1-2Ax/xxxx/G1P[8]	12	2003-2004	D	G	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N
PREVC2-xAx/xxxx/G1P[8]	11	1992-1994	D	S	S	N	S	S	A	N	L	N	N	E	R	N	P	V	D	S	S	N	D	N	N	T	N

	Number	Year	IV								
			115	116	117	118	119	120	121	122	123
RotaTeq	1	2011	V	D	R	Q	Y	T	V	F	G
Rotarix	1	1988	V	D	R	Q	Y	M	I	F	G
POSTVC-1Ax/xxxx/G1P[8]	3	2012	V	D	R	Q	Y	N	V	F	G
POSTVC-2Ax/xxxx/G1P[8]	9	2010-2012	V	D	R	Q	Y	N	V	F	G
POSTVC-3Ax/xxxx/G1P[8]	8	2009-2011	V	D	R	Q	Y	N	V	F	G
POSTVC-4Ax/xxxx/G1P[8]	15	2009-2011	V	D	R	Q	Y	N	V	F	G
INTVCx-xAx/xxxx/G1P[8]	33	2006-2008	V	D	R	Q	Y	M	I	F	G
PREVC1-1Ax/xxxx/G1P[8]	17	2002-2004	V	D	R	Q	Y	N	V	F	G
PREVC1-2Ax/xxxx/G1P[8]	12	2003-2004	V	D	R	Q	Y	N	V	F	G
PREVC2-xAx/xxxx/G1P[8]	11	1992-1994	V	D	R	Q	Y	M	I	F	G

Fig. 3. Alignment of 4 surface-exposed antigenic residues (8-1 to 8-4) and 1 neutralizing epitope (IV) between both vaccine strains and detected VP8* strains. Every detected strain is presented only once. N = number of detected strains, Year = Seasons of circulation.

4. Discussion

We analyzed the sequences of the VP7 and the VP4 proteins from G1 rotaviruses circulating in Finland over a 20-year study period trying to see if the use of rotavirus vaccines in recent years might have had an effect on the antigenic determinants of the prevalent wild-type G1P[8] rotavirus strains. Amino acid changes in the immunodominant regions of these surface antigens could result in loss of vaccine-induced protection since the vaccine induced antiVP7 and VP4 antibodies might not effectively neutralize the corresponding antigens of different subgroups. (Hoshino et al., 2004; Matthijnssens et al., 2009).

Interestingly, for the VP7 protein, despite minor substitutions at nucleotide level, we did not detect any changes at the amino acid level over the whole study period. On the other hand, in Belgium after the mass use of Rotarix™, a shift in the prevalent G1 sublineage due to several amino acid substitutions in VP7 gene has been observed, even though no statistical association to the vaccination could be found (J. Matthijnssens, M. Zeller, N. Verstappen, M. Rahman, M. Van Ranst, Selective rotavirus vaccine pressure against P[8] strains, and possible immune evasion by specific G1 lineages after vaccine introduction in Belgium. Poster Presentation P707, ESPID June 9–13, 2009, Brussels, Belgium). However, similar to our results, circulation of several sublineages of VP7 at the same time has been reported from many countries in the 2000s (Arista et al., 2006, 2007; Phan et al., 2007; Trinh et al., 2007; Dey et al., 2009; Le et al., 2010; Pietsch et al., 2011). In a recent study by Tatte and Chitambar, with a greater variety of intragenotypic strains, changes in the antigenic regions of VP7 of Wa G1 were observed in rotaviruses circulating among adults (Tatte and Chitambar, 2012). Previous studies from countries with wider use of Rotarix™, such as Brazil and parts of Germany, have shown amino acid substitutions in the antigenic regions of G2 strains, genetically far from the Rotarix™ vaccine strain (Matthijnssens et al., 2010). Even though, in contrast to other studies on the epidemiology of human G1P[8] strains, we did not observe any amino acid changes in the G1P[8] strains circulating in Finland, the possibility of vaccine-induced selective pressure on other rotavirus genotypes and in countries with greater variety of intragenotypic lineages, needs further research (Arista et al., 2006; Bányai et al., 2009; Pietsch et al., 2011; Tatte and Chitambar, 2012).

For the VP4 protein, we observed a great number of substitutions both at nucleotide and amino acid level, including changes in the variable region containing the serotype-specific neutralizing epitopes. Previous studies have described world-wide circulation of four VP4 P[8] lineages (P[8]-I – P[8]-IV) with a divergence of 8.6–13% in the amino acid sequence (Maunula and von Bonsdorff, 1998). Even though the P[8]-III lineage is considered to be the most prominent lineage throughout the world, no chronological changes in this or other P[8] sublineages have been reported. The reasons for such periodical shifts of the sublineages, as seen in our study, are not known. Potentially immunological pressure from large scale use of rotavirus vaccines could be a factor in the recent shifts of the P[8] sublineages.

The parental strain of Rotarix™ G1P[8] 89–12, was developed in 1989 from the P[8]-II lineage (Bernstein et al., 1998; Ward and Bernstein, 2009). However Rotarix™ strain aligns to the P[8]-I lineage. This may be because in the propagation of the strain 89–12 virus it was found to contain two plaque variants, and one these was selected to develop Rotarix™. Apparently the second plaque variant was of P[8]-I lineage.

From May 2006 to 2007 the only available rotavirus vaccine in Finland was Rotarix™, and it remained the most commonly used rotavirus vaccine until RotaTeq® vaccine was introduced into the NIP of Finland as of September 1, 2009. The vaccine coverage of Rotarix™ reached 22% during the first period 2006–2007 and rose to 29% in the second period 2007–2008 (Rasanen et al., 2011).

Two different P[8] sublineages (P[8]-III and P[8]-I) were circulating one at a time between 2000 and 2007, before the widespread use of rotavirus vaccines. The samples from post-NIP period 2009 to 2012 align back into the P[8]-III sublineage. In addition, those samples show more intralineage diversity than samples from pre-NIP years. This may represent normal genetic fluctuation of the prominent P[8] subgroup, or, possibly, may be associated with the use of Rotarix™ vaccine in 2007–2009, as the sequence alters toward greater divergence from the P[8] of Rotarix™. Interestingly, the same observation of dominance of P[8]-III strain after mass vaccination with Rotarix™ has been made in Brazil. (Poster presentation: Silva MFM et al. VP8 P[8] lineages of group A rotaviruses circulating over 20 years in Brazil, 11th International Symposium on dsRNA viruses, 27Nov–1Dec 2012, Puerto Rico)

After the introduction of RotaTeq® into the NIP in September 2009, the vaccine coverage reached quickly95–97%, high enough to potentially exert selective immunological pressure on wild-type strains. However, we found that the VP7 remained stable for both G1-I and G1-II lineages, and the VP4 sequence altered between different lineages (P[8]-I and P[8]-III), and of these the more recent one resembles more the P[8] of RotaTeq® vaccine than the earlier one. Thus, we could not see any sign of antigenic drifting or shifting due to RotaTeq® use. The apparent lack of immunological pressure by RotaTeq® might be associated with relatively modest (as compared with Rotarix™) neutralizing antibody responses against the G1 VP7 and P[8] VP4 antigens after vaccination with RotaTeq® (Vesikari et al., 2006). Although sequencing rotavirus strains from vaccinated, infected children would provide a better understanding on the immunological aspect of circulating G1P[8] strains, none of the few children with potential vaccine failure after implementation of RotaTeq® into NIP of Finland have been detected with G1P[8] rotavirus (Hemming et al., 2013).

A large proportion of the modifications between sublineages P[8]-I and P[8]-III occur in the cross-neutralizing linear epitope region and surface-exposed antigen epitope regions. Antibodies directed against VP4 (VP8*) neutralize the virus by inhibition of binding to the cell surface (Ludert et al., 2002; Ruggeri and Greenberg, 1991). Both P[8]-I and P[8]-III strains have been reported to mount a homologous immune response with comparable IgG-titers (100–1600). In the same study, the reactivity of antisera to the peptide B (defined as 84–180 aa of VP8*) of VP8* was higher and not lineage specific, compared to reactivity against peptide A (defined as 1–102 aa of VP8*). (Contreras et al., 2011) However, the amino acid substitutions between the P[8]-I and P[8]-III lineages referred to in the study of Contreras et al. are not totally equivalent to the substitutions detected in our study. (Contreras et al., 2011)

In conclusion, we describe periodical shifts of the P[8]-lineages of G1 rotaviruses over a 20-year period of time. In the years 2006–2009, the use of Rotarix™ might have had an effect on appearance and diversity of a new VP8* sublineage of wild-type G1P[8]. In the last three-year period 2009–2012 the changes in the VP8* protein of the circulating wild-type strains at the nucleotide or amino acid level were apparently not related to the use of RotaTeq®. While the history of rotavirus vaccination in Finland and availability of rotavirus strains over a long period of time provided an opportunity to make preliminary observation on possible antigenic pressure on circulating wild-type rotaviruses, it still should be recognized that the follow-up period is short. Clearly, long-term surveillance of antigenic changes in VP4 and also VP7 proteins in wild-type rotavirus strains is warranted in countries with large scale use of either Rotarix™ or RotaTeq®live oral rotavirus vaccines.

Conflict of interest

Maria Hemming has no conflict of interest to disclose. Timo Vesikari has been PI of clinical trials of rotavirus vaccines produced by Merck and GlaxoSmithKline, and is a member of advisory boards of SanofiPasteur-MSD, Merck, GSK and Pfizer.

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VACCINE-DERIVED HUMAN-BOVINE DOUBLE REASSORTANT ROTAVIRUS IN INFANTS WITH ACUTE GASTROENTERITIS

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Abstract: We describe 3 cases of acute gastroenteritis in healthy infants after vaccination with RotaTeq, shedding a G1P[8] human-bovine double reassortant rotavirus in stools. Such a double reassortant virus appears stable in vitro and may explain diarrheal symptoms in a small percentage of RotaTeq recipients, and might also be transmitted to contacts in the environment.

Key Words: RotaTeq, rotavirus vaccine, diarrhea

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RotaTeq (RV5, Merck & Co. Inc., Whitehouse Station, NJ; in Europe Sanofi Pasteur-MSD, Lyon, France) is an oral live attenuated human-bovine reassortant vaccine that contains 5 reassortants on bovine G6P[5] rotavirus backbone, 4 of which express the human VP7 antigen G1,G2,G3 or G4, respectively. One reassortant expresses human VP4 P1A[8] and retains VP7 type G6 from the bovine rotavirus parent strain WC3.[1–3] RotaTeq was licensed in 2006 following a large trial called RotaTeq Efficacy and Safety Trial, involving 70,000 infants.[1] In the United States, the incorporation of RotaTeq into the routine immunization program has been highly successful in preventing hospitalizations and emergency department (ED) visits due to wild-type rotavirus gastroenteritis.[4–6] Rotavirus vaccination was introduced into the Finnish National Immunization Program as of September 1, 2009, and RotaTeq has been exclusively used in Finland since then. In Finland, RotaTeq is given in 3 doses at ages 2, 3 and 5 months. The vaccination coverage is about 96%, or approximately 56,000 vaccinated children annually (Source: National Institute of Health and Welfare [THL], Finland).

RotaTeq is generally regarded as a very safe vaccine. However, a small increase in diarrhea and vomiting in the first week after the first dose has been observed in large materials.[2,3] Recently, diarrhea in children after RotaTeq vaccination was reported from the United States[7,8] and Australia (Kirkwood CD, Donato C, Ch'ng LS, et al. Identification of RotaTeq vaccine in paediatric patients with acute gastroenteritis following routine vaccination. Paper presented at: 6th International Conference on Vaccines for Enteric Diseases, Cannes, France, 2011.) The diarrhea was associated with the formation of a new human-bovine double reassortant between G1P[5] and G6P[8] strains from the pentavalent vaccine.[8] We describe 3 cases of acute gastroenteritis (AGE) after RotaTeq vaccination in healthy infants, all of whom shed such a G1P[8] double reassortant in the absence of other viral pathogens.

METHODS

A prospective study on the etiology of AGE in children started on September 01, 2009, in Tampere University Hospital concurrently with the introduction of RotaTeq vaccine and was conducted until August 31, 2011. The study was approved by the Ethics Committee of Pirkanmaa Hospital District. Patients with AGE less than 16 years of age, who were seen in the ED of the hospital or were admitted to a pediatric ward, were eligible for the study, subject to obtaining informed consent from the parents.

Stool specimens were collected from 316 patients and examined at the Vaccine Research Center of the University of Tampere Medical School. The stools were collected in the outpatient clinic or, if the patient was hospitalized, in the ward, and studied by reverse transcription-polymerase chain reaction (RT-PCR) methods for several gastroenteritis viruses, including rotavirus. Of all enrolled subjects, 79 (25%) were rotavirus-positive and, of these, 17 had received at least 1 dose of RotaTeq vaccine. Rotavirus G and P genotypes were determined by nucleotide sequencing of the gene segments encoding for the VP7 and VP4 antigens,[9] respectively, and of the gene segment encoding for VP6 to determine the presence of vaccine-derived virus.

For the VP6 RT-PCR, a method was provided by Max Ciarlet, based on the method described by Iturriza-Gómara et al,[10] and adapted at Merck Research Laboratories. Briefly, the 379 base pair (bp) amplicons of the VP6 genes were obtained using primers Rota VP6-Fs (forward primer): 5′GAYGGNGCDACNACATGGT3′ (nt 747–765) and Rota VP6-Rs (reverse primer): 5′GTCCARTTCATNCCTGGYGG3′ (nt 1107–1126) synthesized in Sigma-Genosys Ltd. For Y(C,T), R(A,G), D(A,G,T) and N(A,G,C,T). The oligonucleotides were designed based on the sequence of the Wa (human) VP6 (access no: K02086) and WC3 (bovine) VP6 (access no: AF411322) genes.

The RT-PCR primers were further used as sequencing primers. Gel-purified amplicons (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany) were sequenced using BigDye Terminator v1.1 Cycle Sequencing v1.1 Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Applied Biosystems, Foster City, CA) on an ABI Prism 310 Genetic Analyzer. Nucleotide sequences read from the chromatograms were aligned to published sequences from GenBank.

Stool specimens from patients who had received RotaTeq vaccine were also tested for presence of rotavirus antigen by enzyme-linked immunosorbent assay (ELISA), using the IDEIA Rotavirus Kit (Oxoid Ltd, Basingstoke, Hampshire, United Kingdom). The rotavirus ELISA-positive stool specimen from patient 3 was propagated in MA104 cells as described previously.[11] For other gastroenteritis viruses, norovirus, sapovirus and bocavirus were tested as described previously.[12,13] Coronavirus was tested as described by Risku et al. (Human bocavirus types 1, 2 and 3 in acute gastroenteritis of childhood. Paper submitted to *Acta Pediatrica*).

CASE REPORTS

Patient 1

A 2-month-old girl had received the first dose of RotaTeq vaccine in March 2010, 7 days before the onset of diarrhea. She was taken to the ED on the third day of illness after she had had diarrhea for 2 days (day 1: 15 loose stools; day 2: 5 loose stools). In the ED, she was pale and sleepy, had blood in her stools and a rectal temperature of 37.8°C. The extremities were warm, the mouth and tongue were moist, but she refused to drink. The estimated dehydration rate was 1 to 2%. The patient was rehydrated with an oral rehydration solution and discharged, but at home the diarrhea continued for another 5 days.

The infant had reached full gestational age but had had cardiac surgery 1 week after birth due to tetralogy of Fallot. She was growing and developing well, was breastfed and, except for the follow-up of the heart surgery, did not have any hospitalizations or diseases.

Patient 2

A 3-month-old boy had received the second dose of RotaTeq vaccine in April 2011, 1 day before the onset of diarrhea. He was taken to the ED on the eighth day of illness when his general condition was getting worse. The infant had approximately 10 loose stools of greenish or yellowish color per day. On admission he had a rectal temperature of 37.3°C, the abdomen was tender, and there was slight reddish rash on the abdomen. He had instant recoil on skin turgor test, and his general condition was good. He had no breastfeeding problem and was discharged.

The infant had had no side effects after the first dose of RotaTeq vaccine. He was born in the 37th week and was growing and developing normally. Concomitantly with the second dose of RotaTeq, he had received Infanrix Penta (GlaxoSmithKline) vaccine containing the diphtheria, tetanus, pertussis, *Haemophilus influenzae* type b and inactivated polio virus components.

Patient 3

A 2-month-old girl had received the first dose of RotaTeq vaccine in August 2011, 5 days before she was taken to the ED. Before admission, she had had vomiting for 1 day, diarrhea with a maximum of 12 loose stools a day and a rectal temperature up to 38.1°C. On admission, she had a moist mouth and tongue and instant recoil on skin turgor test, but a sunken fontanel. The infant was irritable and, due to vomiting, had problems with breastfeeding; therefore, overnight admission was required. She was discharged the next day, still having a temperature of 37.8°C but no diarrhea or vomiting. The fecal specimen was collected on the second day in hospital. The infant was full term, was growing and developing normally and had no previous medications, diseases or hospitalizations.

Other Cases

The same G1P[8] double reassortant human-bovine rotavirus as in patients 1–3 was also found in a 3-month-old girl who had received her second dose of RotaTeq 16 days before hospital admission because of gastroenteritis. In this case, norovirus was detected in the same stool specimen, and it was assumed that the principal cause of gastroenteritis symptoms was norovirus, which had infected all family members earlier the same week.

Additionally, 1 gastroenteritis patient was found to shed a single reassortant vaccine virus G6P[8]. This 2-month-old boy had received the first dose of RotaTeq vaccine 5 days before he was taken to the ED due to forceful vomiting. Although the vomiting had started only 1 day before, an overnight admission was required, and the infant was rehydrated with an oral rehydration solution before discharge. In the hospital, the child had no fever or diarrhea. Tests for other gastroenteritis viruses were negative.

In the same study material, we also detected 8 patients who had received RotaTeq and were shedding the G1P[5] vaccine strain. All these patients had simultaneously other gastroenteritis viruses in their stools. Four of these patients shed the vaccine virus identical to the G1P[5]-strain. In the other cases, the VP4 was either negative on RT-PCR, or 2 VP4 strains were detected by sequencing. The detection of 2 VP4s would indicate the presence of more than 1 vaccine virus, most likely G1P[5] and G6P[8] reassortants separately.

LABORATORY FINDINGS

In patients 1–3, only 1 rotavirus was identified in the stool specimens by RT-PCR. In each case, the virus was a bovine rotavirus as determined by VP6 sequencing. Genotyping by RT-PCR confirmed that in each case the G-type was G1 and P-type was P[8].

In all 3 specimens, analysis of nucleotide sequences revealed that an 860 bp fragment of the VP7 gene and a 570 bp fragment of the VP4 gene were 100% identical to cognate gene segments from the corresponding G1P[5] and G6P[8] viruses in RotaTeq. Also, in all specimens, a 362 bp fragment of VP6 gene segment was 100% identical to the WC3 cognate gene of RotaTeq. Neither RT-PCR nor sequencing detected any other rotavirus types in the stool specimens of these 3 patients.

The stool specimen from patient 3 was ELISA-positive, with an optical density of 0.910 (Rotaclone-positive optical density > 0.15). For patients 1 and 2, ELISA tests were negative. The ELISA-positive stool specimen from patient 3 could be propagated in MA104 cells. Upon 5 passages, the virus remained stable as a double reassortant, and the sequencing of VP7, VP4 and VP6 were identical to the original isolate. Other viral agents that were studied were norovirus, sapovirus, bocavirus and coronavirus. In the 3 cases, all tests for these viruses were negative.

DISCUSSION

We describe 3 patients with symptomatic gastroenteritis associated with vaccine-derived new G1P[8] human-bovine double reassortant rotavirus. This is a small number compared with the estimated 8000 infants that had received RotaTeq vaccine in the 2-year period, 2009 to 2011, in the coverage area of Tampere University Hospital. This was 3.8% of the 79 rotavirus-positive cases of AGE detected during the 2-year period. On the other hand, it is possible that the true rate of symptomatic gastroenteritis, and, probably, asymptomatic shedding, due to vaccine-derived double reassortant may be higher, as mild cases of gastroenteritis associated with vaccination, and much less asymptomatic shedding, would not be detected in a hospital-based survey.

Wild-type rotavirus infects the mature enterocytes, and rotavirus may replicate for up to 2 weeks in the cells.[14,15] For a double reassortant to develop, G1P[5] and G6P[8] reassortants would have to infect the same cell. Apparently, this can happen in rare cases.[7,8,16] It is of interest that all our cases, as well as those reported previously, are reassortants with G1 and not with other human G-types. G1 appears to be shed more commonly after vaccination than other G-types,[17] and may therefore be more fit for multiplication. Although the formation of a double reassortant between G1 and P[8] is more likely than other combinations, it is not clear why a double reassortant G1P[8] should have greater virulence, resulting in clinical symptoms.

Two cases presented after the administration of the first dose of RotaTeq. It is likely that multiplication of vaccine virus in the intestinal enterocytes is more effective after the first dose, possibly giving a greater chance of formation of new reassortants. Patient 2 had mild symptoms of gastroenteritis after the second dose. In this case, it is possible that the first dose of RotaTeq had not taken, perhaps due to the presence of maternally acquired antibodies, and the second dose was effectively the first successful vaccination for this infant.[18,19]

Our studies of viral culture showed that G1P[8] double reassortant appears stable in vitro. In the United States, sibling transmission of vaccine-derived G1P[8] virus between an immunocompetent vaccinated sibling and a healthy immunocompetent unvaccinated sibling has already been reported.[8] It is not known, but plausible, that the double reassortant could remain in circulation even longer than 1 transmission cycle. As immunocompromised people may continue to shed the wild-type rotavirus and the vaccine-acquired rotavirus in their stools for several months, the prolonged replication increases the probability of adverse events and the possibility of infecting contacts.[7]

Altogether, shedding of RotaTeq vaccine virus in immunocompetent children appears to be more common than initially reported. In the RotaTeq Efficacy and Safety Trial study, shedding was evaluated by viral culture with use of a plaque assay and RNA

electropherotyping,[1] which are relatively insensitive methods for detection of the vaccine virus. Other studies using ELISA, have revealed a shedding rate of 21.4% (22/103) in full-term infants and 53% (8/15) in premature infants.[20,21] None of these studies detected any double reassortant viruses.

Apart from the 3 cases with the double reassortant, we also saw 1 infant with symptomatic gastroenteritis shedding the original G6P[8] single reassortant vaccine virus. This raises the possibility that P[8] alone may be associated with symptoms and might also be the component resulting in increased virulence of the double reassortant. When RotaTeq was being developed, a study was conducted to compare the pentavalent composition, quadrivalent (G1, G2, G3 and G4) composition and monovalent (P1A[8]) composition.[22] The results showed that the frequency of fever was higher after the first vaccination with pentavalent vaccine than with the quadrivalent composition group, suggesting a role in virulence of P[8].

Taken together, we propose that to induce diarrhea, the VP4 protein from human origin has to be present in the vaccine virus, either as the new double reassortant G1P[8] or, perhaps, only as the original vaccine virus G6P[8].

CONCLUSION

Formation of G1P[8] double reassortants may explain diarrheal symptoms in a small percentage of RotaTeq recipients. The reassortant between 2 vaccine strains may occur during intestinal replication even in immunocompetent infants. Such a double reassortant virus appears stable and might also be transmitted to contacts in the environment.

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SYSTEMIC AMYLOIDOSIS COMPLICATING MULTIDRUG-RESISTANT TUBERCULOSIS IN CHILDHOOD

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Abstract: Multidrug-resistant tuberculosis is increasingly common and is associated with long diagnostic delay and high morbidity. We present a 7-year-old child who developed steroid-resistant nephrotic syndrome while receiving treatment for tuberculosis. Renal biopsy results showed systemic amyloidosis; culture of peritoneal tissue confirmed disseminated multidrug-resistant tuberculosis.

Key Words: multidrug-resistant tuberculosis, amyloidosis, child, South Africa

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Multidrug-resistant tuberculosis (MDR-TB) is caused by strains of *Mycobacterium tuberculosis*, which are resistant to at least rifampin and isoniazid. MDR-TB is an important emerging disease, with an estimated 440,000 new cases reported