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Rotavirus detection and typing in the rotavirus vaccine era

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Abstract

Diarrhoeal disease is a leading cause of morbidity and mortality worldwide. Rotaviruses are the most common cause of severe childhood diarrhoea globally. In Australia, before rotavirus vaccination, rotaviruses caused approximately 10,000 hospitalisations, 22,000 emergency department visits, and 115,000 general practice consultations annually in children aged less than 5 years. On 1 July 2007 Australia became one of the first countries to include rotavirus vaccine into their national immunisation programme. Rotarix was initially used in the Australian Capital Territory, New South Wales, Northern Territory, Tasmania, and Western Australia (Western Australia changed to using RotaTeq in 2009). RotaTeq was initially used in Queensland, South Australia, and Victoria. As of 1 July 2017, all states and territories now use Rotarix.

While active hospital surveillance demonstrated the vaccine's success against severe rotavirus infections, an intensive community-based cohort is required to further understand the full impact upon post-vaccine rotavirus epidemiology. Furthermore, rotavirus gastroenteritis requires laboratory confirmation, and rotavirus detection data can be biased by changes in testing methods. My PhD thesis comprised four sub-studies focusing on both rotavirus detection and rotavirus-related disease in the vaccine era.

In Queensland, rotavirus infections are notifiable and notification data are used to examine the effect of rotavirus vaccine programmes. The accuracy of notification data is dependent upon the accuracy of laboratory methods used to confirm infection. In 2012, concerns were raised in Queensland about the specificity of the VIKIA Rota-Adeno assay (BioMérieux, France), an immunochromatographic (ICT) assay, following an unexplained increase in positive results and feedback from clinicians. By re-examining samples initially testing positive in the VIKIA Rota-Adeno assay with other commercially available ELISA rotavirus assays and, for a subset of specimens, by reverse transcription polymerase chain reaction (RT-PCR) assays, I discovered the specificity of this commercially available rotavirus ICT assay was seriously compromised. These studies translated into changes in routine rotavirus testing in Queensland and the reformulation of the VIKIA commercial method.

My next study was nested in a broader community-based project, the Observational Research in Childhood Infectious Diseases (ORChID) study, and aimed to document the

community-based epidemiology of rotavirus infections in the first 2 years of childhood. To gather initial information about the most prevalent enteric virus infections in the community in the rotavirus vaccine era, quantitative PCR (qPCR) assays were used to assess enteric viruses in the weekly stool samples collected from 5 healthy, fully rotavirus-vaccinated infants from the ORChID study over a 2 year period. Overall, 511 samples from the 5 patients were tested by 6 qPCR methods and the results compared. Rotavirus was not the most prevalent pathogen amongst these infants (only 7 positive samples from 3 subjects identified), and most (5/7) were vaccine strains. Other viruses, particularly adenovirus (131/511 samples, 25.6%, including types 1, 2, 5, 12, 31 and 41), were more commonly found. Frequent, silent shedding of up to 3 months by one or more of the other viruses was observed. These data highlight the complexity of gastroenteritis diagnosis, and show that a positive PCR result for enteric viruses may not always indicate the cause of gastroenteritis.

The main component of my thesis involved comprehensively investigating rotavirus genotypes and shedding in an unselected community-based birth cohort of Australian infants. This involved all available samples from infants (n = 158) enrolled in the ORChID cohort. Newborn infants were progressively enrolled between 2010 and 2012, and were followed until their second birthday. Parents recorded symptoms daily and collected weekly nappy swabs from their children and mailed these to the laboratory. The samples were tested for rotavirus by RT-qPCR, and rotavirus-positive samples were subjected to P and G-genotyping. Viral shedding, genotype, load and associations with symptoms were investigated. Rotavirus was frequently detected in the stool samples of infants from the cohort (1068/11,139 samples; 9.6%); but when genotyped these were mainly vaccine viruses (95.7%), which across each of the 3 doses were shed for a median (interquartile range) 2 (1-3) weeks. Symptomatic wild-type rotavirus detections, but not vaccine virus, were associated with higher viral loads. However, the predictive value of these load data were insufficient to be useful clinically.

In conclusion, a problem related to an unexplained increase in rotavirus notifications was addressed and led to changes in pathology testing practices. Post-implementation of the rotavirus vaccine programme, my community-based cohort studies found the virus was no longer a common gastrointestinal pathogen. In contrast, rotavirus vaccine strain shedding occurred frequently and was more prolonged than previously documented in clinical trials and post-licensure studies. Prolonged shedding of vaccine virus and increases in asymptomatic detections may be a potential problem for RT-qPCR diagnostics, requiring

assays that specifically distinguish vaccine from wild-type infection. Future work should focus on addressing changes in laboratory methods to improve specificity in detecting wild-type disease, and expanding the use of routine genotyping, an important tool to understand rotavirus prevalence and epidemiology in the vaccine era.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publication 1:

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List of Abbreviations

AGE	Acute gastroenteritis
ACIR	Australian Childhood Immunisation Register
ACT	Australian Capital Territory
AdV	Adenovirus
CI	Confidence interval
Ct	Cycle Threshold
DNA	Deoxyribonucleic acid
EHV	Equine herpes virus
ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immunoassay assay
EM	Electron microscopy
ETEC	Enterotoxigenic Escherichia coli
ERV3	Endogenous retroviral-3
GEMS	Global Enteric Multicenter Study
FN	False Negative
FP	False positive
G	Gram
HBoV	Human bocaviruses
HCoV	Human coronaviruses
ICT	Immunochromatographic test
IID	The Infectious Intestinal Disease Study
MD	Mean difference
µl	Microliter
µM	Micromolar

MERS-CoV	Middle East respiratory syndrome coronavirus
ND	Not detected
NSP	Non-structural proteins
ORChID	The Observational Research in Childhood Infectious Diseases project
ORFs	Open reading frames
PCR	Polymerase chain reaction
QC	Quality control
qPCR	Quantitative polymerase chain reaction (also known as real-time polymerase chain reaction)
REST	Rotavirus Efficacy and Safety Trial
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RV	Rotavirus
SARS-CoV	Severe acute respiratory syndrome coronavirus
SD	Standard deviation
S.T.A.R buffer	Stool Transport and Recovery buffer
TN	True negative
TP	True positive
VLPs	Virus like particles
VP	Viral protein
WHO	The World Health Organization

CHAPTER 1

Thesis Structure

This Thesis has five chapters: Chapter 1 provides a literature review on rotavirus, including epidemiology, vaccines and immunisation, diagnosis, public health significance, and gaps in current knowledge. Chapters 2 to 4 comprise research studies conducted as part of this PhD study; these are now either published in scientific journals (chapters 2 and 3; three articles in total) and or submitted for publication (chapter 4). Chapter 5 comprises further discussion of research outcomes and findings, and proposes areas worthy of additional study.

CHAPTER 1

Chapter 1 – Literature Review

1.1 Background - Diarrhoea in early childhood

Diarrhoeal disease is one of the most common illnesses affecting children and has long been recognised as a leading cause of morbidity and mortality worldwide. In 2015, it was estimated that diarrhoeal disease was responsible for almost 500,000 deaths globally in children younger than 5 years of age, which represents 8.6% of the 5.82 million deaths that year in this age group (Collaborators GDD., 2017). Overall, diarrhoea was the fourth leading cause of death in young children, ranked behind complications of preterm birth, neonatal hypoxic ischaemic encephalopathy and lower respiratory infections. Most deaths from diarrhoea are in low and low-middle income countries. In the past decade, from 2005 to 2015, due to improvements in access to safe water, sanitation, and the introduction of rotavirus vaccine, the mortality associated with diarrhoeal disease has reduced by an estimated 20.8% (Collaborators GDD., 2017).

Despite this decline in mortality, an estimated 958 million episodes of diarrhoea occurred in children less than 5 years of age in 2015, which represented a decrease of only 10.4% in incidence over the same 10-year period (Collaborators GDD., 2017). This suggests the reduction in mortality is from improved case management, including access to healthcare and oral rehydration solutions. While the average attack rate of diarrhoea is estimated at 3.2 episodes per child per year, this can be as high as 12 episodes per child per year in some low-income countries (Kosek et al., 2003). Nevertheless, improvements in nutrition, safe water supply, sanitation and hygiene, and the introduction of rotavirus vaccine programmes have also had a positive impact on rates of diarrhoeal disease. Of children aged less than 5 years in Australia, for the period 1998 to 2003 which was prior to the introduction of rotavirus vaccines, there were typically about 10,000 hospital admissions (at an average cost of $1,890 each), 22,000 visits to emergency departments (each at a cost of $320), and 115,000 general practice consultations (at a cost of $36.60 each) annually for rotavirus infection alone, with an estimated direct health cost of $A30m according to the health cost data for 2005/6 (Galati et al., 2006). A more recent evaluation conducted by Reyes and team estimated that approximately 77,000 hospitalisations (at a cost of $ 2,350 each for 2007/8) were prevented by implementation of the rotavirus vaccine program (Reyes et al., 2017).

With several years of vaccine implementation having now passed it is likely the cost savings are even greater in 2017.

There is a wide diversity of aetiological agents, including non-infectious (e.g. toxins) and infectious agents (bacterial, protozoal, and viral), that can cause diarrhoeal illness (Elliott, 2007; Nawaz et al., 2012). Globally, the most common bacterial pathogens of gastroenteritis are *Campylobacter* spp (especially *Campylobacter jejuni*), *Vibrio cholerae* (the cause for cholera), *Salmonella* spp, including *Salmonella typhi* (typhoid fever), *Shigella* spp, *Yersinia enterocolitica*, and a variety of enteropathotypic *Escherichia coli* strains, including the enterotoxigenic *E.coli* (ETEC) strain (Elliott, 2007). The intestinal protozoa *Giardia intestinalis*, *Cryptosporidium* and *Entamoeba histolytica* are also important causes of diarrhoeal illness (Elliott, 2007; Einarsson et al., 2016; Squire and Ryan, 2017). In high-income countries, viruses account for the majority of gastrointestinal infections, with about 70% of disease reported to be of viral origin (Elliott, 2007; Kotloff et al., 2013). Among the viral agents, rotaviruses and noroviruses, together with adenoviruses, astroviruses, and sapoviruses are the major aetiological agents of viral gastroenteritis in children (Iturriza-Gomara et al., 2008). Whereas, in low and middle-income countries, bacterial and protozoal pathogens also play an important role (Elliott, 2007). The Global Enteric Multicenter Study (GEMS), which was conducted in sub-Saharan Africa and south Asia, found most cases of moderate-to-severe diarrhoea were associated with one of four pathogens: rotavirus, *Cryptosporidium*, ETEC producing heat stable toxin (ST-ETEC; with or without co-expression of heat-labile enterotoxin), and *Shigella* (Kotloff et al., 2013). Cryptosporidium was second only to rotavirus as a contributor to moderate-to-severe diarrhoeal disease in their particular study.

Rotaviruses remain the most common cause of severe diarrhoea globally (Walker et al., 2013), whereas noroviruses cause most gastroenteritis outbreaks in developed countries (Clark & McKendrick, 2004). Prior to the introduction of rotavirus vaccines, rotaviruses infected every child at least once before their fifth birthday (Collaborators GDD, 2017). They remain the leading cause of diarrhoeal mortality in children worldwide (Collaborators GDD, 2017; Tate et al.,2016). In 2015 there were 199,000 deaths attributable to rotaviruses, including 147,000 in children younger than 5 years of age. Nevertheless, between 2005 and 2015, death from rotavirus in young children declined by 44%, faster than the all-cause diarrhoea mortality, and is likely due to the introduction of rotavirus vaccine programmes

into some low-income countries supported by the Gavi Vaccine Alliance. In addition, rotavirus infections result in considerable morbidity, being implicated a decade ago with an estimated 114 million episodes of diarrhoea, 24 million clinic visits, and 2.4 million hospitalisations (Dennehy, 2015). In contrast, noroviruses affect all age groups, particularly older children, adults and the elderly (Ahmed et al., 2014b). It is more prevalent in the community than in hospital-based studies and the World Health Organisation (WHO) estimates across all age groups there are 685 million cases and more than 200,000 deaths annually (Hall et al., 2016). In children aged less than 5 years there were an estimated almost 15,000 deaths in 2015 from noroviruses, which is a fraction of the deaths attributed to rotavirus-associated diarrhoeal disease (Collaborators GDD, 2017)..

During the last ten years, due to advances in metagenomics and other molecular biological approaches, several novel viruses have been discovered, including cardiovirus (saffold virus), cosaviruses, aichi viruses (kobuvirus), and salivirus/klasseviruses. Likewise, many studies have been undertaken aiming to conclusively determine the association of these new viruses with clinical disease. However, for many of these viruses, their clinical importance remains unclear.

1.2 The enteric viruses: an overview

The following section comprises a summary of the current literature as it stands for some of these viruses found in the gastrointestinal tract. These include examples of the ‘established viruses’, ‘novel viruses’, and ‘respiratory viruses detected in stool samples’.

1.2.1 Rotaviruses

Rotaviruses were discovered originally in the intestinal tissue of mice in 1963. However, it was not until 1973 that they were first identified by Ruth Bishop and her colleagues in Melbourne, who using transmission electron microscopy (EM) described virus-like particles in duodenal mucosal biopsy specimens obtained from hospitalised infants with gastroenteritis (Anderson & Weber, 2004).

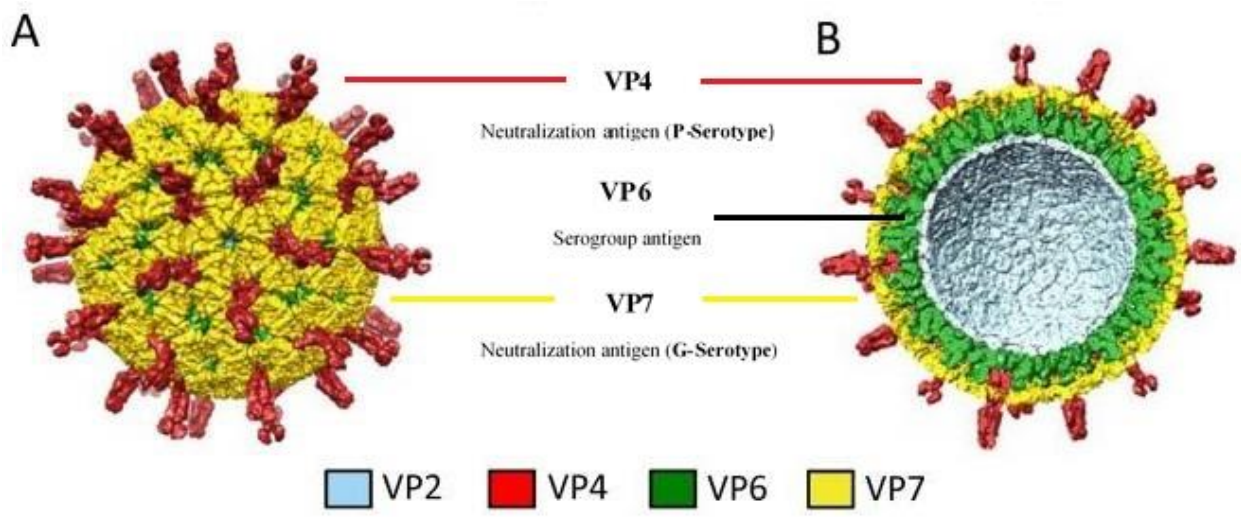
CHAPTER 1

1.2.1.1 Characterization of rotavirus

Morphology and classification

The Rotavirus genus belongs to the family Reoviridae. The virus particles are 70 nm in diameter, have non-enveloped icosahedral structures, and a triple-layered capsid. When visualised by EM they have the appearance of a 'wheel' (Figure 1). The viral genome is composed of 11 segments of double-stranded RNA, which codes for six structural (VP1, 2, 3, 4, 6, and 7) and six non-structural proteins (NSP1–6) (Wilhelmi et al., 2003). The virus is stable in the environment, meaning that rotavirus can survive and remain infectious on non-porous materials (glass, stainless steel, a smooth or rough plastic) at least 10 days at 4 and 22 °C, and at least 6 days at 36°C (Sattar et al., 1986). Rotaviruses are classified into genus, serogroups (groups), subgroups, serotypes, and genotypes according to differences in the antigenic properties, gene sequences, and genomic pattern. VP6 is an inner capsid protein for which there are group-specific antigenic determinants; there are eight major rotavirus groups (A-H) based on the VP6, and four of which (A, B, C, and H) are human pathogens. Rotavirus A is the most common and causes the majority of rotavirus infections in humans (Esona & Gautam, 2015). The outer capsid proteins, VP4 (protease-activated) and VP7 (glycoprotein), are targets for neutralizing antibodies and are used to determine the serotypes (P-type and G-type, respectively) (Hoshino & Kapikian, 2000; Chen et al., 1989).

Figure 1.1 Gene coding assignments and three-dimensional structure of rotavirus particles. (A) Intact triple-layered virus particle. (B) Cut-away of capsid revealing the three protein layers of the virus particle. (C) Double-stranded RNA segments of the RV genome separated by gel electrophoresis. The 11 segments and their protein products, and associated functions or properties (Genotype name) are listed. The underlined letter identifies the segment in the gene constellation acronym: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx. (Figure modified from Parashar and Patton.) (Parashar et al., 1998; Patton, 2012)



C

Gene	Product	Genotype Name
g1	VP1	RNA polymerase
g2	VP2	Core shell
g3	VP3	RNA-capping Methyltransferase
g4	VP4	Protease-activated spike
g5	NSP1	interferon Antagonist
g6	VP6	Intermediate capsid shell
g7	NSP3	Translation regulation
g8	NSP2	octameric NTPase
g9	VP7	outer-shell Glycoprotein
g10	NSP4	Enterotoxin
g11	NSP5/NSP6	pHosphoprotein

Due to the segmented nature of the rotavirus genome, reassortment events can occur. Thus, in 2008, rotavirus A was classified further based on the nucleotide sequence for each of the 11 rotavirus A genome segments. For example, a genome of individual rotavirus strains may be represented as follows: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, which are based on the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 sequences (x = Arabic

numbers starting from 1) (Matthijnssens et al., 2011). So far, there are 166 genotypes that have been established, including 27 G serotypes and 35 P serotypes (Matthijnssens et al., 2011).

1.2.1.2 Clinical Features and Epidemiology

Rotavirus is highly contagious, with as few as 100 viral particles required to initiate infection (Graham et al., 1987). Transmission is mainly by the faecal-oral route, and the incubation period is less than 2 days. The clinical spectrum of rotavirus illness in children ranges from subclinical illness to mild, watery diarrhoea of limited duration, and to severe diarrhoea with vomiting, fever, dehydration with shock, acid-base disturbance, electrolyte imbalance, and death. Gastrointestinal symptoms generally resolve in 3–7 days (Cortese & Parashar, 2009).

Rotavirus is the most common cause of severe and fatal childhood diarrhoea worldwide, and is associated with 28% of severe cases and 28% of fatal cases (Walker et al., 2013). It accounted for more than 200,000 deaths globally in 2013, most of which occurred in low and low-middle income countries (Tate et al., 2013). Rotavirus disease is most common and severe in children following their initial infection between 3 and 36 months of age (Grimwood & Lambert, 2009). Natural immunity acquired after a first rotavirus infection is incomplete, thus multiple infections can still occur. However, with each subsequent infection symptoms become less severe (Velázquez et al, 1996). In contrast, clinical illness is uncommon during the neonatal period in full-term babies. Factors such as immaturity of the neonatal gut, maternal antibodies and the reduced virulence properties of unique rotavirus strains capable of replicating in the neonatal gut are possible explanations for subclinical infection found in neonates (Grimwood & Lambert, 2009).

There is no specific therapy for treating rotavirus disease. The primary treatment, including replacing fluids and electrolytes, is to protect against dehydration caused by vomiting and diarrhoea (Guarino et al., 2014). Therefore, rotavirus vaccination remains the key strategy to prevent rotavirus gastroenteritis.

1.2.1.3 Rotavirus Vaccines

The WHO recommends routine rotavirus vaccination of all infants against this vaccine-preventable infectious disease. There are only a few licenced rotavirus vaccines, and vaccine candidates, which are under development worldwide. These rotavirus vaccines aim

to mimic natural rotavirus infection, and to provide partial immunity for protecting against moderate to severe disease (Grimwood, Lamber, & Milne, 2010).

Human-Animal Reassortant Rotavirus Vaccine

Human-rhesus reassortant Vaccine

RotaShield® (Wyeth Laboratories, Marietta, PA, USA), the first licensed rotavirus vaccine, was developed by coinfecting cell cultures with rhesus rotavirus strain (MMU18006) (G3) and human rotavirus strains D (G1), DS-1(G2), and ST3 (G4) (Estes and Cohen, 1989). It contains three reassortant viruses expressing human serotypes G1, G2, and G4 combined with rhesus rotavirus serotype G3, which is immunologically similar to human G3 (Bines, Patel, & Parashar, 2009). In a vaccine trial conducted in Venezuela, the efficacy of RotaShield was 48% against a first episode of rotavirus diarrhoea. However, it gave 88% protection against severe diarrhoea, 75% protection against dehydration, and produced a 70% reduction in hospital admissions Unfortunately, RotaShield was withdrawn 9 months into the national vaccination program in the United States because of an unexpected strong association with intussusception (about 1 in 12,000 vaccine recipients) in 1999 (Bines, Patel, & Parashar, 2009). Intussusception is a medical emergency involving the invagination of one segment of the intestine into a more distal segment, and can be fatal if not treated. It occurs most often in children around the age of 6 to 12 months (Tate et al., 2008). Although the mechanism of the relationship between intussusception and rotavirus vaccine remain unclear (Vazquez, 2014), the risk for intussusception was found elevated more than 20-fold in the 3- to 14-day period after the first dose of RotaShield (Murphy et al., 2001). In light of the RotaShield experience, the WHO recommends that countries implementing rotavirus vaccination should conduct post-marketing surveillance to assess the risk for vaccine-attributed intussusception (Carlin et al., 2013).

Human-bovine reassortant vaccine

RotaTeq® (Merck and Co, Whitehouse Station, NJ, USA) is a live, oral multivalent human-bovine reassortant vaccine that contains five live-attenuated reassortant rotaviruses. Four reassortant rotaviruses express the most common human rotavirus A G-types, G1, G2, G3, and G4, and the fifth reassortant virus expresses the single most common human rotavirus A P-type, P[8], on a parental bovine-WC3 (G6P7[5]) core backbone (Dennehy, 2008). First licensed in the United States in 2006, it consists of three doses, which are administered beginning at 6-12 weeks of ages, followed by the subsequent doses at a 4-10 weeks interval,

and with the last dose administered before 32 weeks of age. During 2001-2004, over 70,000 healthy infants were randomised in a Rotavirus Efficacy and Safety Trial conducted in 11 countries. The data demonstrated the efficacy of RotaTeq in preventing severe rotavirus disease was 98% (95%CI 88-100), and 74% (95%CI 67-79) against rotavirus disease of any severity (Vesikari et al., 2006). This vaccine was licenced in the U.S. in 2006.

Live Attenuated Human Vaccine

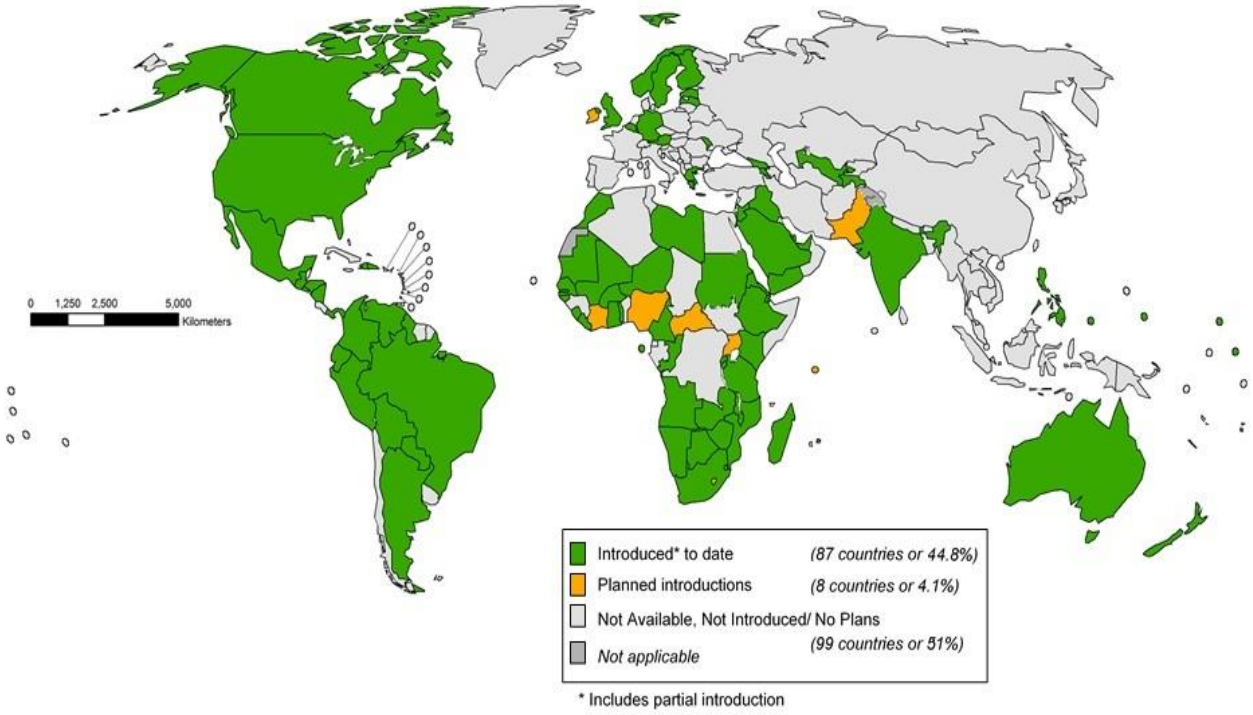
Rotarix® (Glaxo-SmithKline Biologicals, Rixensart, Belgium) is a single-strain live, attenuated human G1P[8] vaccine. This vaccine was first licensed globally in Mexico in 2005 and in the United States in 2008. The Rotarix vaccine is administrated as a two-dose oral series, which is given to infants beginning at 6-weeks of age, followed by the second dose administered after a minimum 4 weeks interval and before 24-weeks of age. An efficacy trial of approximately 4,000 infants in six European countries showed a vaccine efficacy of 87.1% (95% confidence interval (CI) 80–92) against rotavirus disease of any severity, and 90% (95% CI 85–94) against severe disease (Vesikari et al., 2007).

Effectiveness of Live rotavirus vaccine, RotaTeq and Rotarix

Both RotaTeq and Rotarix are available internationally and recommended by the WHO for all infants worldwide to decrease the global burden of rotavirus disease (WHO, 2013). By the end of 2017, rotavirus vaccination is expected to be implemented in national vaccination programs in 87 (44.8%) countries (Figure 2). It has been reported that Rotarix and RotaTeq exhibit similar effectiveness against homotypic and heterotypic rotavirus strains (Leshem et al., 2014). The pooled vaccine effectiveness of Rotarix against homotypic strains and fully heterotypic strains was 94% (95% CI 80-98) and 87% (95% CI 76-93), respectively As for RotaTeq the pooled vaccine effectiveness ranged from 83% (95% CI 78–87) against homotypic strains to 75% (95% CI 47–88) against single-antigen non-vaccine type strains (Leshem et al., 2014). Since the introduction of rotavirus vaccines into national immunization programs from 2006, substantial reductions have been observed in the number of children with severe and fatal diarrhoea in low-middle to high income countries (Patel et al., 2011). The rotavirus hospitalization rates in children aged younger than 5-years have declined by 63-94% in the United States (Leshem et al., 2015), 65-84% in Europe (Karafillakis et al., 2015), and by 73% in Latin America (Santos et al., 2016). While much is now known about the impact of rotavirus vaccines on moderate to severe rotavirus diarrhoea and healthcare utilisation, much less is known about their impact on mild disease managed in the

community where clinical trial data suggest they may be less effective (Hungerford et al., 2017; Gentsch et al., 2005).

Figure 1.2 Countries with rotavirus vaccine in the national immunization program to date; and planned introductions by end 2017. *(Data source: WHO/IVB Database, as of 16 December 2016. Map production Immunization Vaccines and Biologicals (IVB), World Health Organization.)*



In Australia, rotavirus vaccines were included in the publically funded national immunisation programme in July 2007, with an earlier introduction in the Northern Territory in October 2006. RotaTeq was administered in the states of Victoria, South Australia, Western Australia (since May 2009) and Queensland, while Rotarix was used in New South Wales, Western Australia (until April 2009), Tasmania, the Northern Territory, and the Australian Capital Territory. Nationally, the introduction of rotavirus vaccines was associated with a 71% decline in rotavirus-coded hospitalisations of children younger than 5 years of age (from 261 per 100,000 pre-vaccine to 75 per 100,000) and a 38% decrease in non-rotavirus coded hospitalisations for acute gastroenteritis (from 1419 per 100,000 pre-vaccine to 880 per 100,000) in the 2009-2010 financial year (Dey et al., 2012). Similar declines were observed in children in this age group presenting to hospital Emergency Departments where for example in New South Wales there was a 77% decrease in rotavirus attributable non-admitted presentations in this age group (Davey et al., 2015). Meanwhile in Queensland,

the introduction of RotaTeq was followed within 18 months by a 65% fall in rotavirus notifications across all age groups, indicating the presence of both direct and indirect vaccine effects (Lambert et al., 2009). A data linkage study involving the first eligible annual birth cohort from Queensland found the three dose vaccine effectiveness for RotaTeq was up to 64% for preventing non-rotavirus gastroenteritis hospitalisations and as much as 94% for rotavirus coded hospitalisations (Field et al.,2010). Additional protective effects were also observed in older age groups. In contrast, mixed results were found for Rotarix effectiveness in the Northern Territory. Shortly after its introduction in the Northern Territory, a G9P[8] outbreak amongst Indigenous Australian children in Central Australia showed Rotarix was 85% protective against hospitalisation in infants who had recently received the vaccine (Snelling et al., 2009). However, in a subsequent outbreak in the same region involving a non-vaccine related G2P[4] strain, a protective effect against severe disease was found only in a subset of infants less than 12 months of age, suggesting waning immunity in this high-risk population (Snelling et al., 2011). Nevertheless, a recent economic analysis found rotavirus vaccine was cost-saving for Australia, and over a 6-year period post-implementation from 2007-2012, an estimated 77,000 hospitalisations and 3 deaths were averted, compared with an estimated excess of 78 cases of intussusception associated with vaccination (5.6 cases per 100,000 vaccinated infants) (Reyes et al., 2017). The vaccine attributable risk of intussusception was estimated to be 4.3 cases per 100,000 infants for Rotarix recipients, and 7.0 cases per 100, 000 for RotaTeq (Carlin et al., 2013). Both rotavirus vaccines are associated with a similar increase in the incidence of intussusception in the 21 days after the first vaccine dose, estimated at 6- to 10- fold in the first 7 days and 3- to 6-fold in the 8–21 days after vaccination. Despite the increased risk of intussusception in the 3 week period following administration of either vaccine, rotavirus vaccination in Australia has shown that the benefits of vaccination in preventing rotavirus gastroenteritis outweigh the overall very small risk of vaccine attributable-Intussusception (Carlin et al., 2013). The most recent development in Australia's rotavirus vaccine program occurred recently when all states and territories agreed to use Rotarix from July 2017.

Progress for new vaccines

Indigenous human-bovine reassortant vaccine

ROTAVAC (116E, Bharat Biotech International Ltd., Hyderabad, India) is derived from a natural bovine-human reassortant strain (G9P[11]), which was isolated from Indian neonates. This vaccine is licenced in India after showing 55% efficacy against severe

rotavirus diarrhoea, and was included in the Indian universal immunisation programme in 2015 as a 3 dose schedule (Kirkwood et al., 2017; Bhandari et al.,2014).

Lamb vaccine

Lanzhou lamb rotavirus vaccine (LLR-85, Lanzhou Institute of Biological Products) is an attenuated monovalent lamb rotavirus (G10P[12] strain) vaccine (Bai et al., 1994). This vaccine is licensed in China, and available in the Chinese private market.

Human neonatal rotavirus vaccine

A monovalent vaccine (BRV3-BB, neonatal G3P[6] strain) was developed by Bishop and colleagues (Dennehy et al., 2008). This strain was isolated originally from naturally infected neonates in Melbourne neonatal nurseries. It appears to be naturally attenuated, and provides protection for asymptomatically infected infants against subsequent rotavirus disease (Bishop et a l.,1983). The intention is for BRV3 vaccine to be administrated at birth. A recent phase II study of this vaccine in New Zealand infants demonstrated that it was both immunogenic and well-tolerated when administered as 3 dose schedule, beginning within the first 5 days of life (Bines et al., 2015). Recently, this vaccine underwent further field trials in Indonesia where it was found to have a vaccine efficacy of 75% (95% CI 44, 91) when administered in the first 5 days of life (Bines et al., 2018; Kirkwood et al., 2017).

Other vaccines

Non-replicating rotavirus vaccines have been considered to avoid the possible rare, but severe adverse events, such as intussusception, of live attenuated oral vaccines. For example, monovalent P2-P8 (non-replicating G1P[8] VP8 subunit parenteral rotavirus, PATH) vaccine was shown recently to be well-tolerated and immunogenic against homologous strains in South African infants, although it induced only modest or no neutralising antibodies against heterologous P[4] and P[6] bearing strains respectively (Groome et al., 2017). Importantly, it did not interfere with subsequent immune responses to Rotarix, implying there may be a role for mixed parenteral and oral schedules. Further phase I/II trials of a candidate trivalent P2-VP8 (P[4], P[6], and P[8] are now underway in South Africa. Other approaches such as a heat inactivated human rotavirus (G1P[8] strain) vaccine (Centers for Disease Control and Prevention, USA) and virus-like particles (VLPs, Baylor college of Medicine, USA) are under development (Velasquez et al., 2015; Kirkwood et al., 2017).

1.2.1.4 Rotavirus epidemiology in the pre-vaccine and post-vaccine era

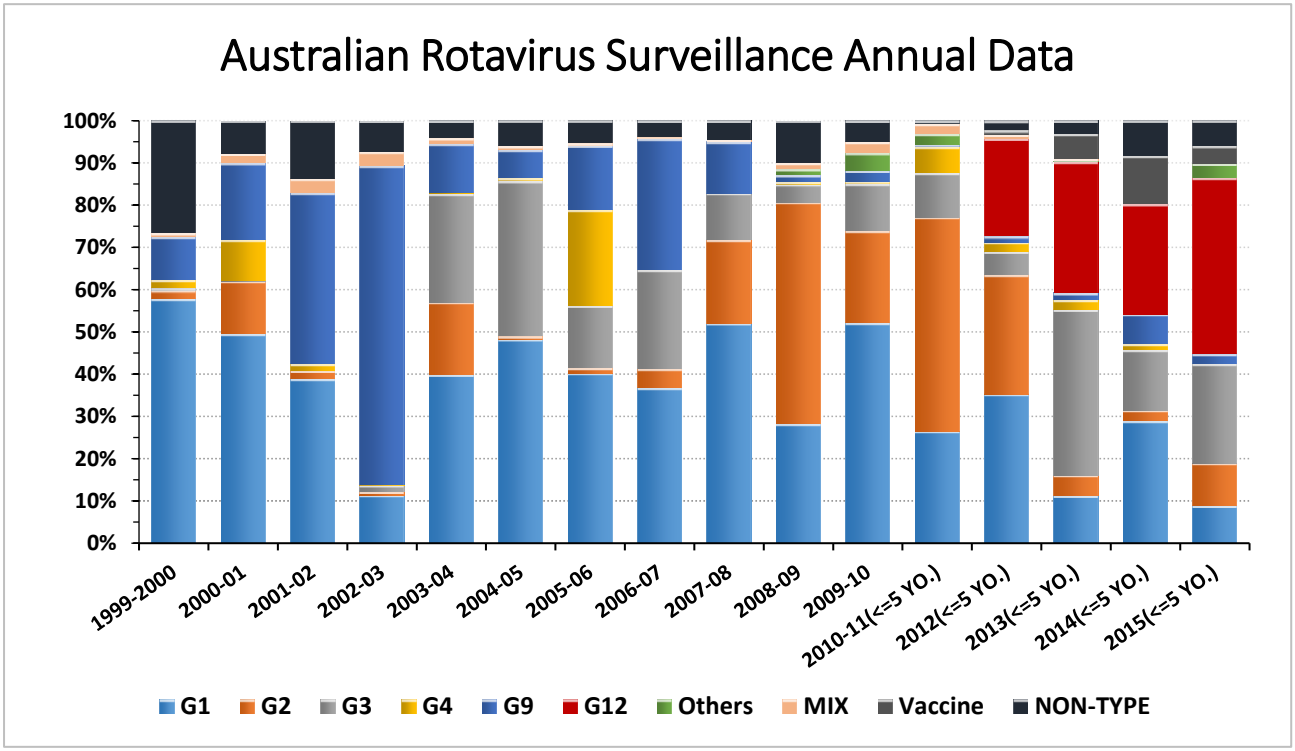
Diarrhoeal illness imposes an enormous disease burden globally. Whilst the most severe disease occurs in low and middle-income countries, in high-income countries the impact of the illness is seen with its high morbidity and in the high incidence of hospitalisation. Between 1973 and 2003, four common rotavirus strains, G1P[8], G2P[4], G3P[8], and G4P[8], accounted for 88.5% of the rotavirus diarrhoea among children worldwide. In Australia, these four strains represented over 90% of rotavirus infections between 1973-2003 (Santos and Hoshino, 2005). Of note, in the same time period, the G1P[8] strain alone accounted for over 70% of rotavirus infections (Santos and Hoshino, 2005). Since 2006, Rotarix and RotaTeq vaccines have been available globally, and post-licensure vaccine surveillance has been closely monitored worldwide to determine whether changes in strain ecology have occurred that may affect rotavirus vaccine effectiveness.

The Australian Rotavirus Surveillance Program began in 1999, and comprises collaborating laboratories from throughout Australia in the laboratory based rotavirus surveillance program. Annual Australian rotavirus surveillance reports are published describing the genotypes of rotavirus strains identified via screening of hospitalised children with acute gastroenteritis (examples can be found at http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-pubs-annlrpt-rotavar.htm). In Queensland, this program involves collaborating laboratories such as the Forensic and Scientific Services (FSS) and Pathology Queensland (Central Laboratory plus Townsville, Cairns, and Gold Coast laboratories). Methods used by these laboratories include both polymerase chain reaction (PCR) and immune-based assays.

Based on the Australian rotavirus surveillance reports, the circulating rotavirus strains have indeed fluctuated from year to year (Figure 3). Also, geographic differences in genotypes have been observed since rotavirus vaccines were introduced in July 2007 depending on the vaccine used (Kirkwood et al., 2011). In summary: the proportion of G2 infections increased during the 1st and 2nd year post-vaccine introduction in states where Rotarix were in used. In the 3rd year, this pattern was also observed in RotaTeq states with the G2P[4] strain becoming more common in the RotaTeq states. The G12 strain emerged in 2012, and the proportion significantly increased in the following years. G12P[8] strains subsequently became the dominant genotype in children less than 5 years of age in the RotaTeq states for the following three years, comprising 33%, 29.6%, and 54% of all strains in 2013, 2014,

and 2015, respectively (Roczo-Farkas et al., 2016). A novel equine-like G3P[8] emerged in 2013, and was commonly observed in locations using Rotarix. By 2015, G3P[8] were now dominant, and represented 50% of all strains in Rotarix states; whereas in RotaTeq states, the wild type G3P[8] strain (11%) was more commonly found than equine-like G3P[8] (4.3%) (Roczo-Farkas et al., 2016). Overall these data highlight the propensity for dominant rotavirus strains to change over time. However, as these strains were not collected systematically and strains from some smaller laboratories in isolated geographic regions were over-represented, no definite conclusions can be drawn over vaccine-related selective pressure occurring. Indeed, to date globally there is little evidence of vaccine introduction leading to any significant strain shifts or escape mutants (Dóró et al., 2014). Nevertheless, continued surveillance is required to monitor for any changes in circulating strains and their potential impact upon vaccine effectiveness.

Figure 1.3 Yearly variation in the distribution of rotavirus G-types between 1999 and 2014 in Australia, (7-years pre-vaccine and post-vaccine era) (Masendycz et al., 2000; Masendycz et al., 2001; Kirkwood et al., 2002; Kirkwood et al., 2003; Kirkwood et al., 2004; Kirkwood et al., 2006; Kirkwood et al., 2007; Kirkwood et al., 2009a; Kirkwood et al., 2011; Kirkwood et al., 2014; Kirkwood & Roczo-Farkas, 2014, 2015; Roczo-Farkas et al., 2016)



1.2.2 Other established viruses that cause gastroenteritis

1.2.2.1 *Caliciviruses* (including Noroviruses and Sapoviruses)

The viruses in the *Caliciviridae* family are single-stranded, positive-sense RNA viruses. There are five genera described in this family, in which two (Norovirus and Sapoviruses) infect humans (karst, 2015). Both can cause acute gastroenteritis with varying combinations of diarrhoea and vomiting, however Norovirus causes more disease than Sapoviruses. (Wilhelmi et al., 2003)

Noroviruses

There are two sizes of viral particles of norovirus observed, with diameters of 30 to 38 nm and 20 to 23 nm were found under EM (Someya, 2011; Lou, 2012). Their genome contains three open reading frames (ORFs 1-3). Norovirus can be separated into six genogroups (I–VI) based on the sequence of the major capsid protein VP1 (which is encoded by ORF-2 and -3) (Robilotti et al., 2015). Within these genogroups, genogroup I, II, and IV (GI, GII and GIV) cause gastroenteritis in humans (Karst et al., 2015). Norovirus was first described by Kapikian et al. in 1972, after an outbreak of gastroenteritis in a school in Norwalk, Ohio (Kapikian et al., 1972).

Norovirus is highly infectious, as exposure to minute amounts of virus carries a high risk of infection (Approximately 50% for a single infectious virus particle) (de Graaf et al., 2016). Transmission is primarily through the faecal–oral route, by both direct and indirect contact, while airborne spread following vomiting has been implicated in some outbreaks. Transmission can also occur following ingestion of contaminated food and water (de Graaf et al., 2016; Patel et al., 2009). For example, in the United States, norovirus accounted for 25.7% (3,444/13,405) of reported foodborne disease outbreaks and 43.1 % of outbreaks with known aetiology during 1998-2008 (Kirkwood et al., 2003). In one birth cohort study, almost 71% of Peruvian infants experienced at least one episode of norovirus-associated diarrhoea by 2 years of age (Saito et al., 2014). A systematic review of the literature published between January, 2008, and March, 2014, has estimated that 18% of acute gastroenteritis cases in all age groups were associated with norovirus (Ahmed et al., 2014b).

Clinical symptoms include nausea, vomiting, abdominal cramps, myalgia, fever, and non-bloody diarrhoea (Patel et al., 2009). Norovirus infections are general self-limiting; however, it can cause severe complications in immunocompromised individuals, the elderly and young

children (de Graaf et al., 2016). Norovirus laboratory diagnosis can be made by EM detection, enzyme immunoassay, immunochromatographic (ICT) and reverse transcription-polymerase chain reaction (RT-PCR) assays (Vinje, 2015). Currently, there is no licensed norovirus vaccine. With the considerable burden of norovirus disease, there have been several attempts at vaccine development. However, given the lack of an *in vitro* culture system for human noroviruses, live attenuated and inactivated norovirus particles vaccine have not yet been created (Cortes-Penfield et al., 2017; Duizer et al., 2004). Recombinant expression of norovirus capsid protein has paved the way for the development of promising vaccine candidates based on VLPs and P particles (Cortes-Penfield et al., 2017); these include a GI.1/GII.4 VLPs-based vaccine, an adenovirus vector-based GI.1 VP1 vaccine, a 'trivalent' vaccine containing a rotavirus VP6 protein and norovirus GI.3 and GII.4 VLPs, and an *E. coli*-produced P particle vaccine (Cortes-Penfield et al., 2017; Ballard et al., 2015).

Sapoviruses

Sapovirus was named after Sapporo, Japan where it was first identified by EM in an outbreak of gastroenteritis in a home for infants in 1977 (Chiba et al., 1979). The viral particles are about 30 to 38 nm in diameter, and have icosahedral structures and cup-shaped depressions on the surface (Oka et al., 2015). There are eight sapovirus genogroups (GI-GVIII) reported, and four (GI, GII, GIV and GV) genogroups have been detected in humans (Oka et al., 2015). Sapoviruses was found associated with acute gastroenteritis in both children and adults worldwide (Oka et al., 2015; Chiba et al., 2000). Transmission occurs through the fecal-oral route. Clinical symptoms of Sapoviruses infection frequently include diarrhoea and vomiting; other symptoms, such as nausea, stomach/abdominal cramps, chills, headache, myalgia, or malaise, are also not uncommon (Oka et al., 2015). Sapoviruses may be diagnosed by several detection methods, including EM, ELISAs and reverse transcription-PCR. A review article by Oka and colleagues has highlighted that sapovriuses account for 1.3% to 22.6% of gastroenteritis outbreaks, and the prevalence of sapoviruses ranges from 2.2 to 12.7% in sporadic cases (Oka et al., 2015).

1.2.2.2 Adenoviruses

Adenoviruses were first identified from adenoid issue cell culture in 1953 by Wallace Rowe and his colleagues (Rowe et al., 1953). They belong to the family *Adenoviridae*. Adenoviruses are non-enveloped DNA viruses, about 70 nm in diameter in size and have an icosahedral symmetrical structure (Wilhelmi et al., 2003; Okitsu-Negishi et al., 2004).

Novel adenoviruses continue to be described, and there are now over 60 different adenovirus types in six species (A-F) described in humans (Matsushima et al., 2013). The two types that are most frequently associated with gastroenteritis are types 40 and 41 (also recognised as the 'enteric adenoviruses') in the AdV-F adenovirus group. Beyond 40 and 41, there are other types adenoviruses that have been found in diarrhoeal specimens, and these include types 12, 18, and 31 of AdV-A and types 1, 2, 5, and 6 of AdV-C (Clark and McKendrick, 2004; Wilhelmi et al., 2003). It is stated adenoviruses count for up to 15% of diarrhoea cases (Clark and McKendrick, 2004). However, questions remain over the clinical significance of the non-40/41 types in gastrointestinal disease.

Other non-F adenoviruses are often found in diarrhoeal specimens. These non-F adenoviruses cause a range of clinical symptoms, including conjunctivitis, gastroenteritis, hepatitis, myocarditis, and pneumonia (Ghebremedhin et al., 2014). It remains controversial to whether non-F adenoviruses are associated with gastroenteritis. Of the seven adenoviruses species, besides AdV-E (AdV-4), detection of all the others has been associated with gastrointestinal symptoms: AdV-52 (AdV-G) was discovered by Jones et al (2007) (Jones et al., 2007), and was found to be responsible for an outbreak of gastroenteritis (Jones et al., 2007). The adenovirus AdV-A (including AdV-31) has been linked to infections of gastrointestinal tract as well as to respiratory and urinary infections. While AdV-B (e.g., type 7) and AdV-C (e.g., type 2) are recognised to be frequent causes of respiratory infection, they are also thought to cause gastrointestinal and urinary tract infections. AdV-D AdV has been associated with conjunctivitis and may also cause gastroenteritis (Ghebremedhin, 2014). Thus, the importance of non-F adenoviruses as causal pathogens in paediatric gastroenteritis remains controversial.

1.2.2.3 Astroviruses

Human Astroviruses are within the genera of *Mamastrovirus*, which belong to the family of *Astroviridae*. They were initially identified in 1975 in the faeces of children with acute diarrhoea (Wilhelmi et al., 2003; Clark and Mckendrick 2004). The viral particles were described as 28-41nm in diameter, non-enveloped, and have a five or six-pointed star structure (Bosch, 2014). The genome of Astroviruses is a single-stranded positive sense RNA molecule containing three open reading frames (ORFs): ORF1a, ORF1b, and ORF2 (Clark and Mckendrick 2004). Astroviruses are transmitted via the fecal oral route (Dennehy, 2011). Symptoms associated with astroviruses infections include a mild, watery diarrhoea

that lasts 2 to 3 days, associated with vomiting, fever, anorexia, and abdominal pain. While astroviruses may not cause as severe disease as other viruses (such as rotavirus, noroviruses), they are still well recognized as endemic pathogens worldwide. Astrovirus infection frequently occurs during early childhood; 60% of Mexican Mayan children were detected positive for astrovirus by ELISA within a 3-year study period in a longitudinal birth cohort (Maldonado et al. 1998). Astrovirus diarrhoea is usually self-limited in an immunocompetent host, and asymptomatic infections are common (Dennehy, 2011).

1.2.3 Other viruses, including respiratory viruses found in faeces

1.2.3.1 Enteroviruses

Enteroviruses are a genus of the *Picornaviridae* family. They are positive-sense ssRNA viruses and comprise a large group of immunologically distinct serotypes belonging to four species; polioviruses, coxsackie viruses, echoviruses and the enteroviruses (Christensen et al., 2003; Hsiung and Wang., 2000; Murray and Baron, 2003). It has been reported that viruses within the enterovirus genus have a vast distribution globally and with expansive diversity (Apostol et al., 2012). Enterovirus infections can range from subclinical disease, febrile illnesses with a rash or mild upper respiratory symptoms through to meningitis, encephalitis, hepatitis, myocarditis and severe sepsis-like illness with multi-organ failure (Christensen et al., 2003). The bowel is the primary site of enterovirus replication, and protracted detection of enterovirus in stool for several weeks is well recognised (Cinek et al., 2006). However, although enteroviruses may persist in the bowel, it is considered that they do not produce significant amounts of diarrhoeal illness in humans (Cukor and Blacklow, 1984). Enteroviruses are also commonly detected in other specimens such as nasopharyngeal secretions, blood and cerebrospinal fluid (Cinek et al., 2006).

1.2.3.2 Bocaviruses

The recently discovered human bocaviruses (HBoV) have been classified into the bocavirus genus (family *parvoviridae*, subfamily *Parvovirinae*). HBoV was first discovered by Allander in 2005 from pooled nasopharyngeal aspirate samples from children with acute respiratory tract infections. In 2009-2010, there were three other species of human bocaviruses (HBoV2, 3, 4) identified, expanding this genus to four species (Jartti et al., 2012). Human bocaviruses have been detected from a wide range of specimens. Contrary to HBoV1, which is typically found in respiratory samples, other species (HBoV2-4) have been mainly detected in human stool (Nawaz et al., 2012; Arthur et al., 2009; Kapoor et al., 2010).

However, these viruses are usually co-detected with other potential pathogens (more than 80% of samples) and whether they are genuine enteric pathogens, facilitate infection by other viruses or simply are shed asymptomatically remains to be determined (Melamed et al., 2017).

1.2.3.3 Coronaviruses

Coronaviruses are medium-sized, lipid-containing, enveloped RNA viruses (Kahn & McIntosh, 2005). The traditional thinking was that the coronavirus family only caused the common cold (up to a third of all coryzal illness in humans), with HCoV-OC43 and 229E only occasionally associated with gastroenteritis in young children from low and middle-income countries and the immunocompromised. In the year 2002-2003, this changed when the severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in the Guangdong province of China (Walker et al., 2013); in addition to severe respiratory disease, this new coronavirus also caused significant gastrointestinal symptoms. In the initial Hong Kong outbreak, a total of 38.4% (53/138) of patient with SARS-CoV infection developed diarrhoea during the first 3 weeks of their illness, and some patients presented only with gastrointestinal symptoms (Leung et al., 2003). Two years later 2 novel coronaviruses were identified, NL63 and HKU1 (van der Hoek et al., 2004; Woo et al., 2005). These viruses are associated with mainly mild respiratory symptoms and like HCoV-OC43 and 229E are found worldwide. Then in 2012 a new coronavirus emerged in the Middle East associated with camel exposure and capable of causing severe pneumonia (Zaki et al., 2012). This new virus was labelled the Middle East respiratory syndrome coronavirus (MERS-CoV) and its clinical features overlapped with those of SARS-CoV. Patients with MERS-CoV are older than SARS-CoV, and may progress to respiratory failure much more rapidly than SARS-CoV (Hui et al., 2014). Gastrointestinal symptoms have also been frequently found in MERS-CoV patients, including vomiting (21%) and diarrhoea (26%) (Assiri et al., 2013).

The above section has described the various viral aetiological agents found in the gastrointestinal tract, and these viruses may be co-present with other potential pathogens. The major causes of diarrhoeal disease include rotavirus, noroviruses, AdV-F, astrovirus and sapovirus. However, other viruses (eg., enterovirus) and respiratory viruses are also found in faeces (eg., HBoV and HCoV). These viruses may also be responsible for acute gastroenteritis. Consequently, a comprehensive study needs to be conducted to determine

whether they are genuine causative agents of gastroenteritis or are being shed asymptomatically, and this highlights the need for a community based study.

1.3 Community based studies:

1.3.1 The need for community-based studies

A major problem in terms of understanding gastroenteritis infections in childhood, including the viruses listed above, is that most of our current knowledge is based on cross-sectional hospital-based studies. The main limitation of such studies, when considered alone, is that such data may lead to an underestimate of the overall disease burden. This can be overcome via community-based studies. The advantage of community-based investigations is that they provide more geographically representative information on the disease burden, strain prevalence, and incidence rates of enteric infections in the community, whereas the hospital-based surveillance systems only provide detailed information on severity and strain prevalence in sick children with diarrhoea illness presenting to a hospital (Banerjee et al., 2006). Thus, in order to gain a clear view of the spectrum of rotavirus infection, a combination of community- and hospital-based studies, which investigate both mild and severe disease, is required.

1.3.2 Studies conducted to date

Since 1964, there have been 24 birth cohorts conducted in 18 countries to investigate rotavirus infections in children, and these are summarised in Tables 1 and 2. In brief, these have taken place in five continents (America, Europe, Africa, Asia, Australia), and have included low and middle income (Argentina, Bangladesh, Costa Rica, Central African Republic, Egypt, Guatemala, India, Mexico, Nicaragua, Nigeria, Republic of Guinea-Bissau, and Vietnam), and high-income countries/region (Australia, Canada, Chile, Finland, Hong Kong, and Israel) (Table 1).

The key findings and associated limitations from these studies are summarised as follows:

- *Rotavirus positivity has varied between studies.* Rotavirus was detected in 30% (395/1309) of diarrhoeal episodes in the Vietnam study, 26% (65/248) in Finland, 23% (39/167) in Canada, 17% (324/1856) in the Indian and 15% (21/145) in the Chilean cohorts, and 10% in Bangladesh (121/1,181), Costa Rica (5/51), and in one of the studies undertaken

in Mexico(37/372) (Anders et al., 2015; Ruuska & Vesikari, 1991; Gurwith et al., 1981; Paul et al., 2014; O'Ryan et al., 2009; Simhon et al., 1985; Qadri et al., 2007; Cravioto et al., 1998). The lowest percentage of rotavirus related diarrhoea episode was recorded in Mexico, with rotavirus accounting for less than 1% (2/305) of diarrhoeal episodes in 1998 (Maldonado et al., 1998)).

• *The incident rate of rotavirus and symptomatic rotavirus infection varies between studies.* The incidence and symptomatic rotavirus incidence rate were reported or could be calculated from 37.5% (9/24) and 83.3% (20/24) of studies respectively. Of the 8 studies where both rates were reported, all but one were from a low or middle-income region. Among these studies from low and middle-income counties, about 2 in 3 rotavirus infections were symptomatic in Guatemala and Bangladesh, about 1 in 3 in the Republic of Guinea-Bissau and Mexico, 1 in 5 in Costa Rica, and 1 in 7 in Nicaragua; whereas in Hong Kong (China), less than 8% were symptomatic. The symptomatic rotavirus infection rate among high-income countries/regions were similar (ranging from 0.1-0.2 episodes per child-year of observation), whereas the rate varied greatly between studies from low and middle-income countries (ranging from 0.1-0.8 episodes). The highest incidence rate was detected in Guatemala (0.8 episodes per child-year of observation), and followed by Nigeria (0.3), India (0.3), Mexico (0.3), Egypt (0.2-0.3), Bangladesh (0.2), Canada (0.2), and the Republic of Guinea-Bissau (0.2). The symptomatic rotavirus incidence rates were the same at 0.1 episodes per child-year of observation for four high-income countries/regions and five low and middle-income countries (including Vietnam) where rotavirus testing was available (Velázquez et al., 1996; Anders et al., 2015; Gurwith et al., 1981; Paul et al., 2014; Qadri et al., 2007; Cravioto et al., 1998; Hasan et al., 2006; Mata et al., 1983; Naficy et al., 1999; Reves et al., 1989; Grinstein et al., 1989; Ahmed et al., 2014a; Simhon et al., 1990; Oyejide et al., 1988; Georges-Courbot et al., 1988; Ruuska & Vesikari, 1991; Zheng et al., 1989).

• *Viral agents examined are different between studies.* Overall only eight of the forementioned 24 studies tested for more than one virus, and similar to the above findings, there was considerable variation in proportions of gastroenteritis cases attributable to each virus, including rotavirus. Rotavirus was the most commonly detected pathogen in the Vietnamese study, and accounted for 53% (395/748) of the pathogens detected in their samples (Anders et al., 2015). In Canada, rotavirus was associated with 60% (39/65) of pathogens detected during a gastrointestinal illness (Gurwith et al., 1981). Norovirus was

the second common pathogen (24%, 176/748) in the Vietnamese study (Anders et al., 2015). However, norovirus was more commonly detected (18%, 26/145) than rotavirus (15%, 21/145) in acute diarrhoea episodes in Chile (O'Ryan et al., 2009). Astrovirus was associated with 26% (78/305) of diarrhoeal episodes in Mexico. Among the Mayan children, the prevalence of astrovirus (61%, 164/271) was much higher than that of adenovirus 40/41 (13%, 35/271) and rotavirus (4%, 10/271) (Maldonado et al., 1998). Astrovirus was also tested for in a Bangladeshi study, and was identified in 9% (34/389) of diarrhoeal samples tested, whereas rotavirus was only identified in 5% (89/1,748) of diarrhoeal stools from the same study (Hasan et al., 2006).

- *Rotavirus detection methods vary between studies.* A major problem in terms of comparing the above data is that between these studies there were considerable methodological differences (Table 1.4). The Hong Kong study, where cord blood and serum specimens were obtained from 38 infants at 4-month intervals for 2 years, rotavirus infections were monitored by serological methods. The Guatemala birth cohort, which was performed 4 decades ago, followed 45 children for 3 years, collected stool samples daily during the first week after birth, and then weekly; and targeted rotavirus and common bacterial agents. Again, a limitation of this study was that low sensitivity laboratory methods (that were common place at the time) were used for viral detection (EM, ELISA) {further discussion of detection methods is provided in the next section}. In contrast, the most recent study that was completed in Bangladesh, and which followed 147 infants for 1 year, used molecular methods (that offer enhanced sensitivity over traditional methods) to test for an extensive range of bacterial, protozoal, fungal and viral pathogens; a total of 32 qPCR assays were applied.

CHAPTER 1

Table 1.1: Previous birth cohort studies investigating gastrointestinal infections

	Countries and region	Location	Year of study	Reference
	American region			
1	Guatemala	Santa Maria Cauque, Guatemala	1964-1969	(Mata et al., 1983)
2	Canada	Winnipeg, Manitoba, Canada	1976-1979	(Gurwith et al., 1981)
3	Costa Rica	Puriscal, Costa Rica	1981-1984	(Simhon et al., 1985)
4	Mexico	Mexico city, Mexico	1982-1983	(Cravioto et al., 1988)
		Mexico city, Mexico	1987-1990	(Velázquez et al., 1996)
		Navenchauc, Mexico	1992-1995	(Maldonado et al., 1998)
5	Argentina	Avellaneda, Argentina	1983-1986	(Grinstein et al., 1989)
6	Nicaragua	Leon, Nicaragua	1992-1995	(Espinoza et al., 1997)
7	Chile	Santiago, Chile	2006-2008	(O'Ryan et al., 2009)
	Eastern Mediterranean Region			
8	Egypt	Bilbeis, Egypt	1981-1983	(Reves et al., 1989)
		Abu Homos, Egypt	1995-1996	(Naficy et al., 1999)
		Abu Homos, Egypt	2004-2007	(Ahmed et al., 2014a)
	African region			
9	Nigeria	Ibadan, Nigeria	Not provided	(Oyejide & Fagbami, 1988)
10	Central African Republic	Bangui, CA	1983-1985	(Georges-Courbot et al., 1988)
11	Republic of Guinea-Bissau	Capital of Guinea-Bissau, Guinea-Bissau	1996-1998	(Fischer et al., 2002)
	Europe region			
12	Israel	Gaza, Israel	1984-1987	(Simhon et al., 1990)
13	Finland	Tampere, Finland	1984-1987	(Ruuska & Vesikari, 1991)
	South east Asia region			
14	Bangladesh	Mirzapur, Bangladesh	1993-1996	(Hasan et al., 2006)
		Mirpur, Dhaka, Bangladesh	2002-2004	(Qadri et al., 2007)
		Dhaka, Bangladesh	2008-2009	(Taniuchi et al., 2013)
15	India	Vellore, South India	2002-2006	(Gladstone et al., 2011)[
	West Pacific region			
16	Australia	Melbourne, Australia	1977-1981	(Bishop et al., 1983)
17	China	Hong Kong, China	Not provided	(Zheng et al., 1989)
18	Viet Nam	HCMC and DongThap, Vietnam	2009-2013	(Anders et al., 2015)

• *Definitions of symptoms vary between studies.* How an episode of gastroenteritis/diarrhoea was defined may also have influenced the outcomes for some of these previous community cohort studies. For example, for the Canadian birth cohort, acute gastrointestinal illness was defined as occurrence of signs of vomiting, or a change in character of the stool, or a significant increase in the number of stools. However, in the India study, it was defined as 3 or more loose or watery stools in a 24-hour period or a change in consistency and number of stools in children less than 6 months of age. There were also differences in rotavirus vaccine coverage in these studies. For example, the Finnish study was conducted as part of a double-blind placebo-controlled vaccine efficacy trial. In the Vietnam study, less than 20% of infants were vaccinated. Moreover, the majority of the studies were conducted before the rotavirus vaccines were introduced.

Overall there have only been a few birth cohort studies conducted to date, especially in the vaccine era. Furthermore, no recent birth cohort studies have so far been conducted in Australia. These limitations and related questions being address by my PhD thesis are further detailed in section 1.5 below.

Table 1.2 Rotavirus infections result from Previous Birth Cohort studies

Countries	No. Infants	Age (years)	Specimen	Collection Method	Samples numbers	Detection Method	Other viral agent	RV Inc. (infections/child-year)	Symptomatic RV Inc. (infections/child-year)	Reference
American region										
Guatemala	45	0-3	Stool	Daily till 7 days, then weekly	5891	ELISA	NIL	1.2	0.8	(Mata et al., 1983)
Canada	104, and 62 siblings	0-1	Stool	At least every three months	167	EM	norovirus adenovirus	-	0.2	(Gurwith et al., 1981)
Costa Rica	51	0-2	Serum Stool	Weekly, and from diarrhoea episodes	4,317	ELISA, PAGE Blocking ELISA,	NIL	0.5	0.1	(Simhon et al., 1985)
Mexico	56	0-2	Stool	Fortnightly, and when diarrhoea occurred	372	EM	NIL	-	0.3	(Cravioto et al., 1988)
	200	0-2	Stool, blood	Weekly, and when diarrhoea occurred	15,503	ELISA,	NIL	1	0.3	(Velázquez et al., 1996)
	271, siblings	6wk- 0.5	Stool	From the infant at weeks 0, 1, 2, 4, 6, 8, 9, 10, 12, 14, 17	2,254	RT-PCR ELISA	astrovirus adenovirus 40/41	-	-	(Maldonado et al., 1998)
Argentina	49, and family members	0-2	Stool, blood	Every 6 month, and when any family member has diarrhoeal occurred	204	ELISA, Blocking	NIL	-	0.1	(Grinstein et al., 1989)
Nicaragua	235	0-2	Stool	At 1, 3, 6, 9, 12, 18 and 24 months	1,322	ELISA, Electrophoresis ELISA	NIL	0.7	0.1	(Espinoza et al., 1997)
Chile	198	0-1.5	Stool	Monthly, and when diarrhoea occurred	2,278	ELISA, RT-PCR	norovirus	-	-	(O'Ryan et al., 2009)

Countries	No. Infants	Age (years)	Specimen	Collection Method	Samples numbers	Detection Method	Other viral agent	RV Inc. (infections/child-year)	Symptomatic RV Inc. (infections/child-year)	Reference
Eastern Mediterranean Region										
Egypt	363	0-1	Stool	When diarrhoea occurred and control stool (1 after every diarrhoeal stool)	2,549	ELISA	NIL	-	0.2	(Reves et al., 1989)
	178	0-1	Rectal swab	Fortnightly, and when diarrhoea occurred	2,293	EM	NIL	-	0.3	(Naficy et al., 1999)
	348	0-2	Rectal swab	When diarrhoea occurred	4,001	ELISA, RT-PCR	NIL	-	0.3	(Ahmed et al., 2014a)
African region										
Nigeria	131	0-1.3/ 2	Stool/ Rectal swab	When diarrhoea occurred	280	ELISA	NIL	-	0.3	(Oyejide & Fagbami, 1988)
Central African Republic	111	0-2	Stool, blood	Biweekly, until 6 month, and when diarrhoeal occurred	1,237	ELISA, Electrophoresis, Blocking ELISA	NIL	-	0.1	(Georges-Courbot et al., 1988)
Republic of Guinea-Bissau	200	0-2	Stool/ Rectal swab	Weekly	11,987	ELISA	NIL	0.6	0.2	(Fischer et al., 2002)
Europe region										
Israel	104	0-1	Stool/ Blood	4-6 visits during first 2 months, and when diarrhoeal occurred	880	ELISA, EM	NIL	-	0.1	(Simhon et al., 1990)
Finland	336	0-1	Stool	4-5 times scheduled visit, and when diarrhoea or gastrointestinal upset occurred	248	ELISA	NIL	-	0.1	(Ruuska & Vesikari, 1991)

Countries	No. Infants	Age (years)	Specimen	Collection Method	Samples numbers	Detection Method	Other viral agent	RV Inc. (infections/child-year)	Symptomatic RV Inc. (infections/child-year)	Reference
South east Asia region										
Bangladesh	252	0-2	Stool/ Rectal swab	Monthly, and when diarrhoea occurred	7,460	ELISA	astrovirus	0.3	0.2	(Hasan et al., 2006)
	321	0-2	Stool/ Rectal swab	Monthly, and when diarrhoea occurred	7,617	Not provided	NIL	-	0.2	(Qadri et al., 2007)
	147	0-1	Stool	Biweekly, and when diarrhoea occurred	1,805	RT-PCR	norovirus GI and GII, sapovirus, adenovirus	-	-	(Taniuchi et al., 2013)
India	373	0-3	Stool	Fortnightly, and when diarrhoea occurred	31,661	ELISA, RT-qPCR	NIL	1.0	0.3	(Gladstone et al., 2011)
West Pacific region										
Australia	81	0-3	Stool, blood	Daily during the first 14 days, or symptom of acute enteritis developed	Not provided	EM, ELISA	NIL	0.2	-	(Bishop et al., 1983)
China	371	0-2	Serum	4-month intervals	Not provided	ELISA, Electrophoresis	NIL	1.3		(Zheng et al., 1989)
Vietnam	6706	0-1	Stool	From diarrhoea episodes	1,309	RT-qPCR	norovirus	-	0.1	(Anders et al., 2015)

Table 1.3 Key finding from Previous Birth Cohort studies

Region	Country	Vaccination	Rotavirus positive rate, % (no. positive/total)	Other viral pathogens, % (no. positive/total)	Reference
American					
1	Guatemala	N/A	2.4% (142/5,891) specimen	N/A	(Mata et al., 1983)
2	Canada	N/A	24.9% (59/237) diarrhoeal episodes	adenovirus 3.0% (7/237 episodes); norovirus 0.4%(1/237)	(Gurwith et al., 1981)
3	Costa Rica	N/A	1.0% (45/4,317) stools, 9.8% (5/51) diarrhoeal episodes	N/A	(Simhon et al., 1985)
4	Mexico	N/A	9.9% (37/372) diarrhoeal cases	N/A	(Cravioto et al., 1988)
		N/A	2.0% (316/15,503) stools, 28.3% (89/315) infections	N/A	(Velázquez et al., 1996)
		N/A	0.5% (12/2,254) stools, 16.7% (2/12) associated with diarrhoea; 3.7% (10/271) infants	astrovirus: 20.1%(452/2,254) stools,60.5% (164/271) infants: adenovirus 40/41: 1.7% (39/2,254) stools, 12.9% (35/271) infants	(Maldonado et al., 1998)
5	Argentina	N/A	5.0% (10/204) diarrhoeal episodes studied	N/A	(Grinstein et al., 1989)
6	Nicaragua	N/A		N/A	(Espinoza et al., 1997)
7	Chile	N/A	2.5% (56/2,278) stools; 15% of 145 acute diarrhoeal episodes evaluated	Norovirus 9.6% (219/2,278) stools; 18% ADE evaluated	(O'Ryan et al., 2009)
Eastern Mediterranean					
8	Egypt	N/A	3.8% (71/1,870) diarrhoeal cases; 23.7% (86/363) children	N/A	(Reves et al., 1989)
		N/A	3.8% (48/1270) diarrhoeal episodes	N/A	(Naficy et al., 1999)
		N/A	40.2% (140/348) of children	N/A	(Ahmed et al., 2014a)

Region	Country	Vaccination	Rotavirus positive rate, % (no. positive/total)	Other viral pathogens, % (no. positive/total)	Reference
African					
9	Nigeria	N/A	7.7% (22/280) diarrhoeal episodes	N/A	(Oyejide & Fagbami, 1988)
10	Central African Republic	N/A	0-6 months:34.2% (38/111) children; 0-2 years: 27% (30/111) of children with diarrhoeal; 8.0% (40/502) diarrhoeal stools	N/A	(Georges-Courbot et al., 1988)
11	Republic of Guinea-Bissau	N/A	1.1% (132/11,987) stools, symptom associated with 40% (46/116) infections	N/A	(Fischer et al., 2002)
Europe					
12	Israel	N/A	6.9% (9/130) diarrhoeal episodes	N/A	(Simhon et al., 1990)
13	Finland	N/A	26.2% (65/248) diarrhoeal episodes	N/A	(Ruuska & Vesikari, 1991)
South east Asia					
14	Bangladesh	N/A	5.1% (89/1,748) diarrhoeal stools, 44.0% (111/252) infants	astrovirus 8.7% (34/389) diearrhoeal stool tested	(Hasan et al., 2006)
		N/A	38.5% (109/283) of children, 10.2% (121/1,181) diarrhoeal stool tested	N/A	(Qadri et al., 2007)
		N/A	7.9% of probable detection	norovirus GI & GII, sapovirus, and adenovirus: N/A	(Taniuchi et al., 2013)
15	India	N/A	5.5% of 1857 diarrhoeal episodes	norovirus:3.1% diarrhoeal episodes	(Gladstone et al., 2011)
West Pacific					
16	Australia	N/A	54% (44/81) children,	N/A	(Bishop et al., 1983)
17	China	N/A	10.8% (40/371) children	N/A	(Zheng et al., 1989)
18	Viet Nam	available	30.2% (395/1309) diarrhoeal episodes	norovirus: 13.4% (176/1,309) diarrhoeal episodes	(Anders et al., 2015)

CHAPTER 1

1.4 Established laboratory diagnostic technologies used in enteric virus detection

As shown in Table 1.2, the previous studies go back over 50 years and laboratory diagnostic technologies have dramatically changed and improved since the initial studies took place. In particular, real-time PCR (qPCR) and more recently genomic methods have revolutionised the way clinical microbiology laboratories diagnose and characterise many human viral infections. Such techniques are now commonplace in routine diagnostic laboratories. The relative insensitive and labour intensive traditional methods, such as EM and cell culture, have largely been replaced. It is for these reasons that many of the earlier studies may have missed significant numbers of infections. The Infectious Intestinal Disease Study (IID Study) in the United Kingdom (UK) (1993-1996) provides a clear example of these issues. The purpose of this large case-control study was to determine the burden and causative agents of sporadic cases of gastroenteritis in the UK population (Note that this was not a community-based study, but the results in term of methodology remain important). Initially, traditional diagnostic methods (e.g. EM and ELISA) were used for diagnosis and by using these methods a potential aetiological agent or toxin failed to be identified in 49% of cases. However, by later retesting the archived samples from this study with molecular methods, the diagnostic gap for gastroenteritis was reduced from 49% to 25%. Of further relevance to this study was that the later results reaffirmed that viruses were the most common aetiological agents of gastroenteritis (Nawaz et al., 2012).

The following provides an overview of the common methods that have been used for diagnosis of gastrointestinal pathogens, and includes key advantages and disadvantages.

1.4.1 Classic microbiological methods for virus detection

1.4.1.1 Electron microscopy

EM involves direct visualisation of viral particles. There are several advantages of using EM to identify the cause of viral gastrointestinal infections. Firstly, viruses are generally easily recognisable due to their distinct morphology. Second, the methods require a minimum degree of processing for examination of viruses in stool specimens. Furthermore, the fact that detection by EM requires high quantities of virus to facilitate detection (e.g. $>10^6$ viral particles per mL of sample) means that it typically corresponds to active infection and not asymptomatic shedding (Murray & Baron, 2003). Disadvantages of EM include the low

sensitivity of the method (particularly for epidemiological studies examining asymptomatic infections, co-infections, and active infections), the requirement for technical expertise, the need for expensive EM instrumentation, training and labour cost, and that it is a low throughput technology (i.e. it is not ideal for the simultaneous testing of high numbers of stools samples as would typically occur in a clinical laboratory) (Murray & Baron, 2003; Pang et al., 2004).

1.4.1.2 Enzyme-linked immunosorbent assay (ELISA) and other rapid immunoassays

ELISAs are plate-based assays designed for detecting antibodies, antigens, and other substances. In an ELISA, an antigen is coated to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is through a highly specific antibody-antigen interaction and by assessing measureable product produced by the conjugated enzyme activity via incubation with a substrate (Gautam et al., 2014). While ELISA assays are 10-100 times more sensitive than EM, their sensitivity and specificity are still lower than RT-PCR assays. Also, there can often be considerable variability (i.e. a lack of correlation) between different ELISA assays (Murray & Baron, 2003).

There are several commercially available ELISA assays that are well-recognised and used for gastrointestinal agents; these include Premier Rotaclone (Meridian Bioscience, the United States) for rotavirus, Premier Adenoclone (Meridian Bioscience, the United States,) for enteric adenoviruses and ProSpecT Astrovirus test (Oxoid, the United Kingdom) for astroviruses. However, ELISA methods are lacking for the novel agents, and this is likely due to a lack of commercial incentive to develop such methods. Similar to the ELISA methods, there are a range of other antigen-based assays, such as rapid membrane-based enzyme immunoassays and latex agglutination tests, which are available for the established viruses. These rapid antigen-based assays, including ICT, are generally less sensitive than ELISA methods but are advantageous in terms of providing rapid results and being relatively simple to perform (Murray & Baron, 2003).

1.4.1.3 Viral culture

Viral cell culture methods date back to the early 1960s, after the discovery that human cells could be cultivated *in vitro* in early 1900s. However, it was not until the early 1970s, as the result of the availability of highly purified reagents and commercially prepared cell lines, that

employment of viral cell culture methods by diagnostic services expanded dramatically (Leland & Ginocchio, 2007).

Specimen collection, processing, and cell culture inoculation guidelines and procedures may differ from laboratory to laboratory, however the general methods include a specimen clarification step according to sample type prior to inoculation into cell cultures. Inoculated cell culture tubes are then incubated at 35°C to 37°C in stationary slanted racks or, alternatively, in rotating/rolling racks. Daily microscopic examination of cell monolayers is required to maximise the detection of viral growth. The detection of virus is made by visualising cytopathogenic changes or cytopathic effect (caused by viral proliferation) in the cells. These degenerative changes can range from swelling, shrinking, and rounding of cells to clustering, syncytium formation, and, in some cases, complete destruction of the monolayer (Leland & Ginocchio, 2007).

The advantages of traditional cell culture method are, first, it is suitable for isolation of a wide variety of viruses (including mixed cultures); secondly, it provides an isolate for additional studies (including antiviral susceptibility testing, serotyping, and epidemiological studies) and thirdly, it has increased sensitivity over rapid antigen testing. Disadvantages include: not all viruses are able to be cultured (e.g. anelloviruses); long incubation periods are required for some viruses, the need for purchasing or maintaining a variety of cell types, the requirement for specific technical expertise, and appropriate sample transport techniques must be used to maintain viral infectivity (Leland & Ginocchio, 2007).

1.4.2 Molecular approaches

1.4.2.1 Conventional PCR and real-time PCR

The application of PCR assays in molecular diagnostics has increased to a point that is now the routine for many infectious agents. It is also now accepted as the gold standard for detecting many organisms, and has become an essential tool in the research laboratory (Malek et al., 2006). The principle of the PCR involves a pair of synthetic oligonucleotides or primers, each hybridising to one strand of a double-stranded DNA (dsDNA) target (for the case of DNA virus detection). The region spanned by the primer pair will be exponentially amplified by the PCR reaction; each newly extended PCR primer then serves as a template for the DNA polymerase to sequentially add deoxynucleotides and create a complementary strand (Cukor et al., 1984).

Conventional PCR (whereby PCR and detection are performed separately) requires laborious post-PCR handling steps. Such detection steps may involve; (a) Evaluation of the PCR products (amplicon) upon electrophoresis of the nucleic acids in the presence of ethidium bromide and visual analysis of the bands after irradiation by ultraviolet light; or (b) by Southern blot hybridisation with a labelled oligonucleotide probe. A key limitation is that these steps are time consuming, and pose a risk of contamination via the multiple PCR product handling steps (Cukor et al., 1984).

In contrast to conventional PCR methods, qPCR enables the detection of amplicons during the amplification steps i.e. in real-time. The key feature of qPCR is that it combines PCR with fluorescent detection chemistry in the same reaction vessel. As such, it has improved rapidity, and dramatically reduces the risk of carry-over contamination. It is because of qPCR that PCR methodology has gained broad acceptance in clinical microbiology (Mackay et al., 2002).

The main advantages of PCR methods are that they are typically more sensitive than traditional methods, such as ELISA. For example, for rotavirus detection this approach has reported to be several orders of magnitude more sensitive than ELISA (Cukor et al., 1984). Other advantages include short turnaround time, high through put, and it is useful for viruses that cannot be readily cultured. Disadvantages include the requirement for technical expertise, expensive instrumentation, the high cost of certain reagents (e.g., reaction mix and fluorophore labelled probes), and that the technology is highly targeted i.e. probes and primers are extremely specific and may miss mutated virus or viruses not being targeted (Chieochansin et al., 2016).

In order to outweigh the disadvantages outlined above, multiplex qPCR assays (incorporating multiple tests into a single test reaction) have been adopted to increase sample throughput and reduce the expense of sample screening. Multiplex PCR assays were first introduced by Chamberlain et al. in 1988 to screen for prenatal and postnatal diagnosis of Duchenne muscular dystrophy lesions (Chamberlain et al., 1988). In recent years, with improved technologies for sample preparation and nucleic acid extraction/purification, qPCR (including simplex and multiplex qPCR) has gradually made its way into diagnostic laboratories for comprehensive gastrointestinal pathogen detection, such as norovirus, rotavirus, and bacterial and protozoal pathogens (Zhang et al., 2015).

1.5 The PhD Project

My PhD thesis includes analysis of data from an Australian National Health and Medical Research Council funded birth cohort study (APP615700) that is investigating gastrointestinal and respiratory viruses in the first 2 years of life (Lambert et al., 2012). The overall objective of this project was to study rotavirus infection in the rotavirus vaccine era. This study will be able to provide insights into the shedding of both vaccine and wild-type rotavirus genotypes.

1.5.1 The key issues being address in this work

Since universal vaccination against rotavirus was included in the Australian national immunisation program in 2007, substantial reductions in the number of rotavirus infections have been observed in young children. It is estimated that approximately 77,000 hospitalisations (17,000 coded rotavirus and 60,000 unspecified gastroenteritis) and 3 deaths were prevented (Reyes et al., 2017). However, these continued successes are contingent on vaccines providing adequate coverage against current circulating strains. While there are national surveillance programmes in place to address this for patients seeking healthcare, there is no active surveillance in the community and, as noted above, limited research in this area regarding the impact of vaccines upon mild disease and asymptomatic infection. Although in comparison with symptomatic infections asymptomatic infections do not directly pose a burden in terms of healthcare costs, GP visits, or family disruption, asymptomatic infections nevertheless still present a risk of rotavirus transmission to others and may act as a silent reservoir for emergent pathogens in outbreaks. For these reasons, the significance of asymptomatic infections should not be overlooked, and in fact need to be better understood so as to enhance disease control.

In light of the successful rotavirus vaccination program, a shift in the aetiological role of rotaviruses and other enteric pathogens causing acute gastroenteritis is being unmasked. Norovirus has overtaken rotavirus as the most common cause of paediatric gastroenteritis in the United States, and recently accounted for 21% of gastroenteritis, compared with 12% for rotavirus (Payne et al., 2013). Such studies also highlight that adenovirus, astrovirus and sapovirus (together accounting for 22.1% of acute gastroenteritis cases) remain important causes of gastroenteritis in hospitalised children less than 5 years of age in the US (Chhabra et al., 2013). In an attempt to provide insights into viral gastroenteritis in the rotavirus vaccine

era, and the impact of rotavirus vaccines on rotavirus epidemiology, a community-based birth cohort study should be conducted to focus not only on the symptomatic and asymptomatic rotavirus infections, but also on the prevalence of other established viral agents that are associated with gastroenteritis.

In the past decade, molecular techniques have been emerged as an important method for rotavirus surveillance. Notably, they enable genotyping of the virus and provide insight into their epidemiological profile; as mentioned above, such methods are used routinely by the Australian Rotavirus Surveillance Programme to assess and report on the molecular epidemiology of rotavirus in the Australian population each year. However, until recently, molecular methods have not been used extensively as the first line method for diagnosing rotavirus infection. Rather, diagnosis has remained reliant on ELISA and other rapid antigen-based methods. For instance, immunoassay-based methods (antigen testing) was originally the predominant test used in Queensland prior to this PhD project. With the increasing trend towards using qPCR for the routine diagnosis of rotavirus, there is now a need to better understand the predictive value of these tests. In Australia, rotavirus notification data are used to examine the effect of rotavirus vaccine programmes. The accuracy of notification data is dependent on the accuracy of laboratory methods used to diagnose infection.

Laboratory testing issues for rotavirus can impact upon the pattern of notifications. For example, in Queensland, rotavirus become a notifiable condition in 2005, and pathology providers were required to notify any positive test results for rotavirus. Since the vaccination program began in mid-2007, there has been a rapid decline in notifications. The number of notifications of rotavirus reduced from 2,495 cases in 2006 to 1,186 in 2007 (State of Queensland, 2017). However, in 2011, an abnormal peak in rotavirus notifications in Queensland was observed after years of decline in rotavirus notifications rates following introduction of the rotavirus vaccine. And in late 2015, a similar increase was observed in the number of notifications in children younger than one year old, coinciding with the introduction of PCR testing for rotavirus infection (State of Queensland, 2017).

Key questions include whether immunoassay-based methods are indeed suitable for routine diagnostics; are they too insensitive and, based on recent feedback from local clinicians, are they also non-specific? Alternatively, does the increased sensitivity of qPCR lead to the

detection of clinically meaningless low-load infections and, in the vaccination era, is detection of vaccine virus confounding the aetiological diagnosis of acute gastroenteritis?

1.5.2 Aims and hypotheses

Specific Aim 1: To investigate an unexplained increase in positive results in Queensland that was associated with a particular immunoassay.

Hypothesis #1: The VIKIA Rota-Adeno assay (BioMérieux, France), a widely used ICT assay for rotavirus diagnosis, is not specific for rotavirus.

Overview: Assay-specific issues should be considered in the event of unexplained increases of rotavirus disease in the vaccine era. Here, test specificity was compared to other commercially available rotavirus ICT Assays and qPCR. Selection of an unsuitable diagnostic laboratory method can confound rotavirus-related disease surveillance and control.

Specific Aim 2: To identify an appropriate method for routine diagnosis of rotavirus infection by comparing both molecular and immunoassay-based approaches.

Hypothesis #2: PCR assays provide sensitive and specific detection of rotavirus in patients presenting with symptoms of gastrointestinal infection.

Overview: Diagnostic improvements have provided various choices for rotavirus detection. Molecular methods such as PCR have several benefits over Immunoassays, including enhanced test sensitivity, when applied to samples submitted for routine rotavirus screening.

Specific Aim 3: To conduct a pilot study examining and comparing the presence enteric viral pathogens in the first two years of life.

This study as well as Aim 4 below were conducted as part of the Observational Research in Childhood Infectious Diseases (ORChID) Study (Lambert et al., 2012). The ORChID project is a community-based, prospective birth cohort study examining both acute respiratory and gastrointestinal infections in infants up until age 2-years in Brisbane, Australia (Lambert et al., 2012). Several studies have been published in relation to the respiratory aspects of the study (Alsaleh et al., 2014b; Rockett et al.,2015; Sarna et al., 2016; Sarna et al., 2018), whereas here the focus in on gastrointestinal infections in these children. Further details of

the ORChID study, including study design, sample and data collection, and qPCR testing, are provided in chapter 3.

Hypothesis #3: We predict that, in the vaccine era, rotavirus will still be responsible for causing diarrhoea in a small proportion of children. However, we predict it will no longer be the most common cause of viral gastroenteritis in the community and that other viral pathogens, such as norovirus and adenovirus, will be more common.

Overview: Acute gastroenteritis remains an important cause of hospitalisation in children. However little data are available regarding the aetiological role of rotavirus and other viral pathogens, and their presence in young children in the community. This pilot study investigated the frequency of different viral pathogens, and provided valuable information including the nature of viral shedding from the gastrointestinal tract and determining disease-pathogen associations in healthy infants and young children.

Specific Aim 4: In the final key investigation of this PhD study, we aim to comprehensively determine the strains of rotaviruses circulating in the community, and their association with diarrhoea.

Hypothesis #4: Wild-type rotavirus remains an important cause of diarrhoea in infants in the Australian community.

Hypothesis #5: Rotavirus vaccine strains are shed asymptomatically for short periods following each vaccine dose.

Overview: In this community-based, prospective birth cohort study, qPCR based methods were used to test for rotavirus infection in weekly collected stool samples from children in the first two years of life. Rotavirus infections were detected in both symptomatic and asymptomatic individuals. Frequent shedding of vaccine virus was observed. The findings have important implications for both diagnosis and surveillance of rotavirus infection, including that the enhanced sensitivity of qPCR may not necessarily be advantageous when testing children younger than 1 year of age.

CHAPTER 2

Chapter 2 – Resolving a rotavirus diagnostic issue

This chapter is presented as two published original research articles:

2.1. A problem with rotavirus detection using the VIKIA Rota-Adeno kit in Queensland

Ye, S., Roczo-Farkas, S., Whiley, D., Lambert, S., Robson, J., Heney, C., Nimmo, G., Grimwood, K., Sloots, T., & Kirkwood, C. (2013). Evidence of false-positive results in a commercially available rotavirus assay in the vaccine era, Australia, 2011 to 2012. Eurosurveillance, 18(21).

2.2. Comparison of different commercial available Rotavirus detection kits - further assessment of the VIKIA Rota-Adeno investigation

Ye, S., Lambert, S. B., Grimwood, K., Roczo-Farkas, S., Nimmo, G. R., Sloots, T. P., Kirkwood, C. D., & Whiley, D. M. (2015). Comparison of test specificities of commercial antigen-based assays and in-house PCR methods for detection of rotavirus in stool specimens. Journal of Clinical Microbiology, 53(1), 295-297. doi:10.1128/JCM.02251-14

These papers are as published, except for the following modifications: table numbers have been modified to fit with the thesis format, the abstracts have been removed, abbreviations have been adjusted for consistency throughout the thesis, the introduction has been shortened to minimise replication of text from chapter 1, and all references have now been added to a single reference section at the end of the thesis

CHAPTER 2

2.1 A problem with rotavirus detection using the VIKIA Rota-Adeno kit in Queensland

Evidence of false-positive results in a commercially available rotavirus assay in the vaccine era, Australia, 2011 to 2012.

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CHAPTER 2

2.1.1 Introduction

We recently became concerned about the specificity of the VIKIA Rota-Adeno assay (BioMérieux, France) following an unexplained increase in positive results and feedback from clinicians; As noted in chapter 1, in 2011 there was an abnormal peak in rotavirus notifications in Queensland observed after years of decline in rotavirus notifications rates following introduction of the rotavirus vaccine (State of Queensland, 2017).

Accurate detection of rotavirus is essential for prevention and control of rotavirus outbreaks and disease monitoring. There are two common methods used for routine diagnosis: ICT assays and ELISA. ICT assays are relatively inexpensive, easy to use, rapid (results within 20 min) and with reportedly good sensitivity (96.6%) and specificity (92.9%) (de Rougemont et al., 2009). Many diagnostic laboratories in Australia use the VIKIA Rota-Adeno assay for detection of rotavirus in faecal specimens.

We therefore re-examined samples initially testing positive in the VIKIA Rota-Adeno ICT with other commercially available ELISA rotavirus assays and, for a subset of specimens, by RT-PCR.

2.1.2 Methods

Ethics approval for this study was provided by the Children's Health Queensland Human Research Ethics Committee.

Clinical specimens

We obtained a convenience sample set of 133 faecal specimens submitted for diagnostic rotavirus testing and collected between July 2011 and August 2012 from patients with symptoms of acute gastroenteritis. Specimens were from two laboratories in Queensland (n=113: Pathology Queensland, a publically funded laboratory, and Sullivan Nicolaides Pathology, a private laboratory) and from a private laboratory network in Victoria (n=20: Melbourne Pathology). The latter were submitted to the National Rotavirus Reference Centre (NRRC) in Melbourne, Victoria, for genotyping. All samples had been tested initially for rotavirus using the VIKIA ICT method according to the manufacturer's instructions (Queensland: 81 positive, 32 negative; Victoria: 20 positive).

RT-qPCR, Queensland samples only

All 113 Queensland specimens were tested initially in Queensland employing two RT-qPCR assays, using primers and TaqMan probe sequences described previously (Table 2.1). RNA extraction was performed by homogenising ca. 25 µl of stool specimen with 225 µl of phosphate buffered saline to provide a concentration of ca. 10%. Then 200 µl of this suspension were extracted into a volume of 50 µl using the Roche High Pure Nucleic Acid extraction kit as per kit instructions (Roche Diagnostics, Australia). As described previously, specimens were spiked before extraction with 5 µl of equine herpes virus (EHV, with an expected Ct value of 33 cycles) as an extraction and inhibition control (Bialasiewicz et al., 2009).

All RT-qPCR reactions were performed using a Qiagen one-step RT-PCR kit. Each reaction mix contained in a total volume of 25.0 µl in RNase-free water: 0.4 µM of forward and reverse primers, 0.16 µM of Taqman probe, 1.0 µl of Qiagen one-step RT-PCR dNTP mix, 5.0 µl of Qiagen one-step RT-PCR buffer (5x), 1.0 µl of RT-enzyme and 2.0 µl of RNA extract or control. Cycling was performed on a Rotor-Gene instrument (Qiagen, Australia) or Applied Biosystems 7500 real-time PCR system (Life Technologies, United States) with the following cycling conditions: initial hold steps at 50 °C for 20 min and 95 °C for 15 min, followed by 45 cycles at 95 °C for 15 sec and 60 °C for 30 sec, with fluorescence signal read on green at 60 °C.

ELISA testing

There were 103 samples available at the NRRC for further testing: 83 from Queensland (51 VIKIA-positive, 32 VIKIA-negative specimens) and 20 from Victoria. Thirty VIKIA-positive specimens from Queensland were not sent to the NRRC due to insufficient sample volume. Available specimens were retested using three commercial rotavirus ELISA assays: ProSpecT (Oxoid, United Kingdom), Premier Rotaclone (Bioline, United Kingdom) and Ridascreen (R-Biopharm AG, Germany). All three methods were performed as per the manufacturer's instructions.

VIKIA retesting

To confirm initial VIKIA assay results, Queensland specimens with sufficient remaining sample (positive: n=35; negative: n=26) after PCR and ELISA testing, were retested using the VIKIA assay.

In-house VP6 RT-PCR

At NRRC, any samples that gave a discordant result for the ELISA methods or appeared to be falsely positive in the VIKIA assay (n=55), were further tested using a rotavirus VP6-specific RT-PCR with primers (Table 2.1).

Table 2.1 Oligonucleotide primers and probes used in RT-PCR assays

Primer/probe	Nucleotide sequence (5'– 3')	Reference
NVP3 assay		
Rota NVP3-F1	ACCATCTACACATGACCCTC	(Pang et al., 2004; Pang et al., 2011)
Rota NVP3-F2	ACCATCTTCACGTAACCCTC	
Rota NVP3-R	GGTCACATAACGCCC	
NVP3 Probe	ATGAGCACAATAGTTAAAAGCTAACACTGTCAA	
JVK assay		
Rota-JVK-F	CAGTGGTTGATGCTCAAGATGGA	(Jothikumar et al., 2009)
Rota-JVK-R	TCATTGTAATCATATTGAATACCCA	
Rota-JVK Probe	ACAACTGCAGCTTCAAAAGAAGWGT	
VP6 assay		
ROT3	AAAGATGCTAGGGACAAAATTG	(Elschner et al., 2002; Donato et al., 2012)
ROT5	TTCAGATTGTGGAGCTATTCCA	

2.1.3 Results

Samples from Queensland retested in a second VIKIA assay

Of the 81 VIKIA-positive and 32 VIKIA-negative Queensland samples, there was sufficient remaining specimen for VIKIA retesting on 35 and 26 specimens, respectively. Thirty of 35 initially VIKIA-positive and one of 26 initially VIKIA-negative Queensland specimens were positive on retesting (Table 2.2).

Twenty-seven of the 30 VIKIA twice-positive samples were negative in every other assay applied (Table 2.3). Of the 10 VIKIA retest-positive specimens with sufficient sample volume available for testing at the NRRC, seven were negative by all three ELISA assays (Table 2.3). The initially negative, but retest-positive specimen was negative by the NVP3 and JVK PCR assays, and all three ELISA tests.

Other specimens from Queensland and Victoria available for testing in other assays

There were further specimens from Queensland (n=52; 46 positive, six negative) and Victoria (n=20; all positive) which were not retested using the VIKIA assay, but for which PCR and ELISA results were available.

Of 20 VIKIA-positive specimens from Victoria, 13 were negative in all three ELISA assays and the VP6 PCR assay (Table 2.4). The six negative specimens from Queensland were negative in both NVP3 and JVK PCR assays, and all three ELISA assays. Four of the 46 positive specimens did not have sufficient specimen volume remaining for ELISA testing; two of these positive in both the NVP3 and JVK assays, and two negative in both (Table 2.4). Of the remaining 42 specimens, 14 were positive in both Queensland PCR assays and all three ELISA assays, and 23 were negative in each of these assays as well as the VP6 PCR assay. The remaining five specimens provided mixed results.

Table 2.2 Comparison of original VIKIA test results and repeat VIKIA test results for specimens with sufficient volume for re-testing, Queensland, July 2011–August 2012 (n=61)

		Repeat VIKIA test		
		Positive	**Negative**	**Total**
Original VIKIA test	**Positive**	**30**	**5**	**35**
	Negative	**1**	**25**	**26**
	Total	**31**	**30**	**61**

Table 2.3 Test results for specimens with sufficient volume for VIKIA re-testing, in PCR and ELISA assays, Queensland, July 2011–August 2012 (n=61)

		Queensland PCR (NVP3 and JVK)			Melbourne ELISA assays (ProSpect, Rotaclone, Ridascreen)			Victorian PCR			
		Positive	**Negative**	**Total**	**Positive**	**Negative**	**Total**	**Positive**	**Negative**	**NP**	**Total**
Repeat VIKIA test	**Positive**	2	29	31	3[a]	7	10	0	7	3	10
	Negative	1[b]	29	30	0	25	25	0	0	25	25
	Total	3	58	61	3	32	35	0	7	28	35

ELISA: enzyme-linked immunosorbent assay; Negative: negative in all assays; NP: specimen not tested in this assay; PCR: polymerase chain reaction; positive: positive in any assay.

[a] Two specimens positive in all three assays, one specimen positive in Rotaclone only.

[b] One sample positive in single RT-qPCR assay, NVP3, only (cycle threshold: ca. 37 cycles).

Table 2.4 Retesting with different diagnostic assays of faecal specimens positive in the VIKIA assay, Queensland and Victoria, July 2011-August 2012 (n=133)

VIKIA initial[a]	Queensland PCR assays		ELISA assays			VIKIA retest[b]	Melbourne PCR assay	Number
	NVP3-PCR[c]	JVK-PCR[c]	ProSpecT[d]	Rotaclone[d]	Ridascreen[d]		VP6-PCR[e]	
Queensland specimens from Pathology Queensland and Sullivan Nicolaides Pathology, original diagnostic test: VIKIA								**113**
Queensland specimens with insufficient material for sending to the National Rotavirus Reference Centre								**30**
POS	**POS**	**POS**	NP	NP	NP	NP	NP	2
POS	NEG	NEG	NP	NP	NP	**POS**	NP	21
POS	NEG	NEG	NP	NP	NP	NEG	NP	5
POS	NEG	NEG	NP	NP	NP	NP	NP	2
Queensland specimens with sufficient material for sending to the National Rotavirus Reference Centre								**83**
POS	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	NP	2
POS	**POS**	**POS**	**POS**	**POS**	**POS**	NP	NP	14
POS	**POS**	**POS**	**POS**	**POS**	NEG	NP	**POS**	1
POS	**POS**	**POS**	NEG	**POS**	**POS**	NP	NEG	1
POS	NEG	**POS**	NEG	NEG	NEG	NP	NEG	1
POS	NEG	**POS**	NEG	NEG	**POS**	NP	NEG	1
POS	**POS**	NEG	NEG	NEG	NEG	NP	**POS**	1

VIKIA initial[a]	Queensland PCR assays		ELISA assays			VIKIA retest[b]	Melbourne PCR assay	Number
	NVP3-PCR[c]	JVK-PCR[c]	ProSpecT[d]	Rotaclone[d]	Ridascreen[d]		VP6-PCR[e]	
POS	NEG	NEG	NEG	**POS**	NEG	**POS**	NEG	1
POS	NEG	NEG	NEG	NEG	NEG	**POS**	NEG	6
POS	NEG	NEG	NEG	NEG	NEG	NP	NEG	23
NEG	**POS**	NEG	NEG	NEG	NEG	NEG	NP	1
NEG	NEG	NEG	NEG	NEG	NEG	**POS**	NP	1
NEG	NEG	NEG	NEG	NEG	NEG	NP	NP	6
NEG	NEG	NEG	NEG	NEG	NEG	NEG	NP	24
Specimens from Melbourne provided to the NRRC for genotyping, original diagnostic test: VIKIA								**20**
POS	NP	NP	NEG	NEG	NEG	NP	NEG	13
POS	NP	NP	NEG	**POS**	NEG	NP	NEG	1
POS	NP	NP	**POS**	**POS**	**POS**	NP	**POS**	6

ELISA: enzyme-linked immunosorbent assay; NEG: specimens negative in this assay; NP: test not performed on this specimen; NRRC: National Rotavirus Reference Centre; PCR: polymerase chain reaction; POS: specimens positive in this assay.

[a] Initial diagnostic test.

[b] VIKIA retest performed on specimens with remaining adequate volume after PCR/ELISA testing.

[c] Queensland PCR, not performed on Victorian samples.

[d] ELISA tests performed at the National Rotavirus Reference Centre, Melbourne.

[e] PCR performed at the National Rotavirus Reference Centre, Melbourne, on specimens discordant for any of the ELISA tests.

2.1.4 Discussion

The results of our study highlight the need to review the validity of diagnostic assays when disease incidence changes unexpectedly. Australia implemented a nationwide rotavirus vaccination programme in July 2007, and since that time notifications of laboratory-confirmed rotavirus infections and hospitalisations have fallen quickly in targeted and older age-groups (Lambert et al., 2009; Field et al., 2010; Buttery et al., 2011). Anecdotal feedback from clinicians and an unexplained increase in disease notifications prompted this investigation, which has identified a problem with false positivity in an ICT assay used widely in Australia and elsewhere.

Even though ours is a convenience sample, the results point towards inability to confirm by a variety of PCR and ELISA methods a substantial proportion of specimens twice positive using the VIKIA kit. These findings were reinforced by specimens from Queensland and Victoria which were tested only once using the VIKIA assay. As a sensitivity analysis, if we assume the remaining 46 initially VIKIA positive specimens with insufficient volume for retesting had all retested negative, there would still remain 27 of 81 Queensland specimens that were twice positive by the VIKIA assay, but were negative in two PCR assays (n=21) or three PCR assays and three ELISA assays (n=6) (Table 2.4).

Given the consistency of other methods it is unlikely that the ICT assay is detecting true positive results. Notably, our data suggest that between one and two thirds of VIKIA-positive samples may be actually false-positive results. Furthermore, of initially positive samples from Queensland with sufficient volume for repeat testing using the VIKIA kit, 86% remained positive on retest, with only three of these 30 specimens positive in one or more other assay, by PCR or ELISA. Given the consistency of the VIKIA retest values and our PCR and ELISA assay findings, conducted at different times in different locations, with all assays performed according to the manufacturer's instructions, we do not believe specimen degradation or test conditions are a logical or sustainable explanation for the apparent specificity issue. There were six Queensland specimens for which the VIKIA retest value differed from the original result, with five of these initially positive and negative in the repeat test. Possible reasons for these discrepancies include sample stability, human error in result interpretation, and specimens with low virus load.

The VIKIA ICT kit insert states that the method has 100% sensitivity and 100% specificity for rotavirus detection, based on testing of 103 positive and 290 negative stools (VIKIA® Rota-Adeno package insert). In a prospective study of 57 samples from children younger than 36 months in Lyon in childcare centres during 2004-05, the reported sensitivity and specificity of this kit, compared to a PCR-based method, was 96.6% and 96.4% respectively (PPV: 96.5%, NPV: 92.9%) (de Rougemont et al., 2009). Similar high specificity (100%) was reported by Bon et al. in 2006 (Bon et al., 2007). Given this, it is difficult to know if our findings are due to recent changes in the assay or to specificity problems exposed by reduced disease incidence in a high vaccine coverage setting. We are therefore investigating further the specificity of this and other assays in a prospective study.

2.1.5 Conclusion

We have shown a suboptimal test specificity using a commercially available rotavirus ICT Assay. Assay-specific issues should be considered in the event of unexplained increases of rotavirus disease in the vaccine era.

CHAPTER 2

2.2 Comparison of different commercial available Rotavirus detection kits - further assessment of the VIKIA Rota-Adeno investigation

As highlighted above, assay-specific issues should be considered in the event of unexplained increases of rotavirus disease in the vaccine era. In above study, we observed poor specificity using a commercially available rotavirus ICT assay. Questions now remain over selection of a suitable detection method to be used in our local diagnostic laboratory, as well as for further screening to be conducted as part of our ORChID study investigations in subsequent chapters. Here, we further assessed the performance of a wider range of ICT and ELISA rotavirus detection methods.

Comparison of test specificities of commercial antigen-based assays and in-house PCR methods for detection of rotavirus in stool specimens.

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CHAPTER 2

2.2.1 Introduction

Our recent findings (Ye et al., 2013) raised concerns regarding the specificity of the VIKIA Rota-Adeno assay (BioMérieux, France) for detecting rotavirus in stool samples and followed an unexplained increase in positive results in a highly vaccinated population in which surveillance had previously shown rotavirus vaccine to have been highly effective in significantly reducing rotavirus notifications and rotavirus-related hospitalizations (Lambert et al., 2009; Field et al., 2010).

In that study (Ye et al., 2013), we found that, of 81 available stool specimens submitted for diagnostic testing (collected between July 2011 and August 2012) and reported as positive using the VIKIA kit, only 28% to 37% could be confirmed as positive using additional RT-PCR and ELISA-based testing. The results were highly suggestive of an unacceptably low specificity in the VIKIA rotavirus ICT assay. In this follow-up study, we sought to examine whether false positivity in the VIKIA kit is an ongoing problem and to assess the performance of a wider range of ICT and ELISA rotavirus detection methods.

2.2.2 Materials and Methods

Convenience sampling of stool specimens submitted from patients with acute gastroenteritis to the publicly funded Central Microbiology Laboratory of Pathology in Queensland, Brisbane, Australia, for rotavirus testing occurred between July 2012 and June 2013. Samples were tested initially for rotavirus using the VIKIA ICT method. Only samples with sufficient volumes for subsequent testing were included in the study. These were stored at -20°C until they underwent further testing by the additional assays.

Overall, 182 stool samples from patients up to 94 years of age (median, 11 years; mean, 28 years) were included; the samples were from 101 males and 81 females. There were VIKIA rotavirus positive (n=92) and VIKIA rotavirus-negative (n=90) specimens in this sample. We tested these specimens with six additional commercial rotavirus tests (three ICT kits and three ELISAs) and three in-house RT-PCR assays (Tables 2.5 and 2.6). All the ICT assays and ELISAs were performed according to their manufacturer's manufacturer's instructions. Performance characteristics according to the kit inserts are listed in Table 2.7. The RT-PCR methods comprised two TaqMan-based RT-qPCR assays (NVP3-PCR and JVK-PCR) and a conventional PCR (VP6 RT-PCR) and were performed as described previously (Ye et al.,

2013). The oligonucleotide primers and probes used in the RT-PCR assays are provided in Table 2.1.

In order to confirm the initial VIKIA assay results, we retested all specimens with the VIKIA assay according to the manufacturer's instructions. The test performance characteristics from this study for each assay are reported as their sensitivity, specificity, and true-positive and true-negative proportions. The 95% CI for each of these values were calculated using Stata version 12 (Stata Corp, College Station, TX). The Children's Health Queensland Human Research Ethics Committee approved the study.

2.2.3 Results

The results for the 182 specimens with each kit and the associated performance characteristics are summarized in Table 2.5 and 2.6, respectively. Overall, there was close agreement between all methods with the exception of the VIKIA assay. Specifically, 67 of the 90 (74%) samples that were positive in the VIKIA test were negative in every other assay evaluated upon retesting (Table 2.5). When the VIKIA results were excluded, only 12 (6.6%) of the 182 samples provided discrepant results among the remaining ICT, ELISA, and PCR methods. The performance characteristics (Table 2.6) were determined on the basis of a reference standard whereby samples that provided positive results in three or more methods were considered true positives; all other samples were considered true negatives. On the basis of this standard, the sensitivities of the kits ranged from 80% to 100%. Specificity was lowest for the VIKIA kit at 54.3%, whereas observed specificities for the remaining methods were 99.4% to 100%.

Table 2.5 Summary of results for all 182 samples.

No. of specimens with indicated result (total no) at initial testing using the VIKIA kit	VIKIA		ICT assay			ELISA			Rota PCR			Reference Result[a]
	Initial	Repeat	SD Bioline	CerTest	Quick Stripe	Rota-clone	Rida-screen	Pro-spect	NVP3 (CT value)[b]	JVK (CT value)[b]	VP6	
Positive for rotavirus (92)												
2	Detected	ND[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
67	Detected	Detected	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1	Detected	Detected	Detected	ND	ND	ND	ND	ND	ND	ND	ND	ND
1	Detected	Detected	ND	ND	ND	Detected	ND	ND	ND	ND	ND	ND
1	Detected	Detected	ND	ND	ND	ND	ND	ND	ND	Detected (41.1)	ND	ND
2	Detected	Detected	ND	ND	ND	ND	ND	ND	ND	ND	Detected	ND
1	Detected	Detected	ND	ND	ND	ND	ND	Detected	Detected (38.5)	ND	Detected	Detected
1	Detected	Detected	Detected	Detected	Detected	Detected	ND	Detected	Detected (33.2)	Detected (34.1)	Detected	Detected
16	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected (26.8–34.5)	Detected (25.9–34.3)	Detected	Detected

No. of specimens with indicated result (total no) at initial testing using the VIKIA kit	VIKIA		ICT assay			ELISA			Rota PCR			Reference Result[a]
	Initial	Repeat	SD Bioline	CerTest	Quick Stripe	Rota-clone	Rida-screen	Pro-spect	NVP3 (CT value)[b]	JVK (CT value)[b]	VP6	
Negative for rotavirus (90)												
2	ND	Detected	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1	ND	ND	ND	ND	ND	ND	ND	ND	Detected (40.8)	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	ND	ND	Detected (36.0–38.2)	Detected (37.2–37.4)	Detected[d]	Detected
2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Detected	ND
83	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Classified as a true positive (TP) if rotavirus was detected in three or more tests, otherwise classified as a true negative (TN).

[b] Cycle threshold (Ct) values; ranges obtained for positive results in the RT-qPCR methods (NVP3 and JVK) are provided in parentheses.

[c] ND, not detected.

[d] A VP6 amplicon from one of these two samples where there were discrepant positive results between PCR- and antigen-based assays was also sequenced and confirmed as being consistent with rotavirus.

Table 2.6 Performance characteristics of each rotavirus assay applied to 182 samples from Queensland between July 2012 and June 2013[a]

Assay type	TP (n)	TN (n)	FP (n)	FN (n)	TP/RefP (sensitivity [%] [95% CI])[b]	TN/RefN (specificity [%] [95% CI])[b]	TP/TP + FP (% TP [95% CI])[b]	TN/TN + FN (% TN [95% CI])[b]
VIKIA initial	18	88	74	2	90.0% (68.3 - 98.8)	54.3% (46.3 - 62.2)	19.6% (12.0- 29.1)	97.8% (92.2 - 99.7)
VIKIA repeat	18	88	74	2	90.0% (68.3 - 98.8)	54.3% (46.3 - 62.2)	19.6% (12.0- 29.1)	97.8% (92.2 - 99.7)
SD Bioline	17	161	1	3	85.0% (62.1 - 96.8)	99.4% (96.6)	94.4% (72.7 - 99.9)	98.2% (94.7 - 99.6)
CerTest	17	162	0	3	85.0% (62.1 - 96.8)	100.0% (97.7)	100.0% (80.5)	98.2% (94.8 - 99.6)
Quickstripe	17	162	0	3	85.0% (62.1 - 96.8)	100.0% (97.7)	100.0% (80.5)	98.2% (94.8 - 99.6)
Rotaclone	17	161	1	3	85.0% (62.1 - 96.8)	99.4% (96.6)	94.4% (72.7 - 99.9)	98.2% (94.7 - 99.6)
Ridascreen	16	162	0	4	80.0% (56.3 - 94.3)	100.0% (97.7)	100.0% (79.4)	97.6% (93.9 - 99.3)
ProSpecT	18	162	0	2	90.0% (68.3 - 98.8)	100.0% (97.7)	100.0% (81.5)	98.8% (95.7 - 99.9)

Assay type	TP (n)	TN (n)	FP (n)	FN (n)	TP/RefP (sensitivity [%] [95% CI])[b]	TN/RefN (specificity [%] [95% CI])[b]	TP/TP + FP (% TP [95% CI])[b]	TN/TN + FN (% TN [95% CI])[b]
ROTA NVP3	20	161	1	0	100.0% (83.2)	99.4% (96.6)	95.2% (76.2 - 99.9)	100.0% (97.7)
ROTA JVK	19	161	1	1	95.0% (75.1 - 99.9)	99.4% (96.6)	95.0% (75.1 - 99.9)	99.4% (96.6)
ROTA VP6	20	158	4	0	100.0% (83.2)	99.4% (93.8 -99.3)	83.3% (62.6 - 95.3)	100.0% (97.7)

[a] RefP, positive by the reference standard criteria; RefN, negative by the reference standard criteria; CI, confidence interval (considered a true positive if rotavirus was detected in three or more tests [RefP], otherwise considered a true negative [RefN]); TP, true positive; TN, true negative; FP, false positive; FN, false negative.

[b] Where the percentage point estimate is 100%, the lower 97.5% confidence limit is provided.

Table 2.7 Comparison of performance characteristicS of each commercial tests according to kit inserts.

Name	Detection	Method	Sensitivity / Specificity
VIKIA Rota/Adeno*	Rotavirus and Adenovirus	A rapid immunochromatographic assay (ICT)	100% / 100% (96.1% agreement 4 specimens not detected with the comparative technique but positive using an EIA and/or a PCR technique) Comparison with commercial ICT test
SD Bioline Rota/Adeno Rapid*	Group A Rotavirus and Adenovirus	A rapid immunochromatographic assay (ICT)	100% / 99.7% Comparison with commercial ELISA
QuickStripe Adeno/Rota*	Rotavirus and Adenovirus	A rapid immunochromatographic assay (ICT)	Not provided (99.0% agreement) Comparison with other commercially available rapid adenovirus/rotavirus kits
CerTest Rotavirus-Adenovirus*	Rotavirus and Adenovirus	A rapid immunochromatographic assay (ICT)	>99% / 98%
ProSpecT Rotavirus	Group A Rotavirus	A direct enzyme immunoassay (EIA)	98.7% / 99.0% (99.0% agreement) Comparison with EIA
Premier Rotaclone	Rotavirus	A Qualitative enzyme immunoassay (EIA)	100% / 97% (99.0% agreement) Comparison with EM/RNA
RIDASCREEN	Rotavirus	A Qualitative enzyme immunoassay (EIA)	98.4% /98.9% Comparison with another certified test

* It should be noted that the VIKIA method and the three additional ICT methods also simultaneously test for adenovirus, but that the adenovirus data were not assessed in this study.

2.2.4 Discussion

None of the assays achieved the sensitivities described in their kit inserts (Table 2.7). However, this is most likely due to the use of PCR assays in this study, whereas most antigen-based assays would have been validated using other antigen-based methods. In fact, including PCR test results within the reference standard may be viewed as having negatively biased the sensitivity values for other the assays, as PCR can detect low-level virus shedding from infection weeks earlier and unrelated to the current illness (Richardson et al., 1998). This is further reflected in those samples providing negative results by one or more antigen-based methods but being positive by RT-qPCR, with their cycle threshold (Ct) values exceeding 33 cycles (Table 2.5). These particular Ct values were among the highest observed and indicated a low viral load. Another limitation of our study was that there were only 20 true-positive samples, and this may have influenced the certainty around sensitivity calculations, as shown by the broad 95% confidence intervals associated with these data. In contrast, except for the VIKIA method, the specificities for all the other commercial methods were comparable with those reported by the manufacturers.

2.2.5 Conclusion

These data show that the specificity problems observed previously with the VIKIA assay (Ye et al., 2013) remain and that the same problems are not evident with the other ICT or ELISA methods we studied. Based on our results, PCR provided the best overall sensitivity and specificity. While the antigen tests were not as sensitive as PCR, they, excluding the VIKIA, were highly specific. We therefore agree with a recent international study of childhood diarrhea evaluating molecular-based detection techniques, which found that antigen testing remained suitable for rapidly diagnosing rotavirus infection in clinical samples (Liu et al., 2014).

CHAPTER 3

Chapter 3 – A pilot study of enteric viruses in the community

This following section is presented as a published original research article:

Detection of viruses in weekly stool specimens collected during the first 2-years of life: a pilot study of five healthy Australian infants in the rotavirus vaccine era

Ye, S., Whiley, D. M., Ware, R. S., Sloots, T. P., Kirkwood, C. D., Grimwood, K., & Lambert, S. B. (2017). Detection of viruses in weekly stool specimens collected during the first 2 years of life: A pilot study of five healthy Australian infants in the rotavirus vaccine era. Journal of Medical Virology, 89(5), 917-921. doi:10.1002/jmv.24716

The paper is as published, except for the following modifications: table numbers have been modified to fit with the thesis format, the abstract has been removed, abbreviations have been adjusted for consistency throughout the thesis, the introduction has been shortened to miminise replication, and all references have now been added to a single reference section at the end of the thesis

CHAPTER 3

Detection of viruses in weekly stool specimens collected during the first 2-years of life: a pilot study of five healthy Australian infants in the rotavirus vaccine era

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CHAPTER 3

3.1 Introduction

As outlined in chapter 1, sections 1.2.1.4, successful national rotavirus vaccine programs and recent discoveries of novel viruses have raised important questions over the relative contributions of existing and emerging enteric viruses to childhood diarrhea. So far, most studies have been cross-sectional, lacking suitable controls, and based in healthcare settings, increasing the likelihood of selection bias (Scallan et al., 2005; Lambert et al., 2012). The aim of this community-based pilot study was to assess the prevalence of enteric virus infections and their association with gastrointestinal symptoms in the first 2 years of life in five otherwise healthy infants recruited prospectively from birth. This pilot study investigated not only the frequency of different viral pathogens, and provided valuable information including the viral shedding from the gastrointestinal tract, determining disease-pathogen associations in healthy infants and young children, and determining whether a PCR Ct value cut-off can be used to improve diagnostic accuracy of viral gastroenteritis.

3.2 Materials and Methods

Study design and cohort The ORChID project (clinical trials.gov: NCT01304914) is a community-based, prospective birth cohort study examining the nature and frequency of acute respiratory and gastrointestinal infections in infants up until age 2-years in the subtropical city of Brisbane, Australia (Lambert et al., 2012). Parents recorded in a daily diary (Figure 3.1) any fever, respiratory or gastrointestinal symptoms in their infant and collected nose and stool swab samples weekly from birth until the infant's second birthday. The nose and stool swab samples were submitted each week to the research laboratory by regular surface mail. The QPID laboratory clinical trials staff managed the study recruitment, cohort maintenance, specimen collection and transport, and recording of socio-demographic and clinical data.

Recruitment of parents and infants was conducted at the antenatal clinics in either the Royal Brisbane and Women's or the North Western Private Hospitals. The recruitment was planed to enrol infants progressively throughout two years, so as to delineate the seasonal and year to-year variation in virus activity. From September 2010 to October 2012, 165 healthy babies were recruited antenatally. Exclusion criteria for enrolment and ongoing participation included gestational age at birth of less than 36 weeks, infants with major congenital abnormalities or underlying chronic disorders {such as chronic heart, respiratory (except for

asthma), gastrointestinal, neurological or immunological disorders}, parents unable to converse in English, and family living outside the Brisbane metropolitan region or planning to move from the area within the next 2 years.

Following recruitment, sociodemographic and health history, pregnancy and birth details were collected from the parants. Telephone interviews were conducted every 3 months to update immunisation, feeding and childcare attendance details. The characteristics of infants enrolled in the ORChID study are shown in Table 3.1, and the proportion of infants remaining in the cohort by child age (months) and swab returned from birth until their second birthday illustrated in Figure 3.2.

Figure 3.1. An example of Symptom Diary Card (Figure received from Alsaleh A.)

ORChID daily symptom diary **April 2011**

SUBJECT NAME :

Subject ID number 065	No symptoms	Fever OR temperature greater than 37.5°C	Wheezing	Shortness of breath	Pulmonary congestion (moist cough)	Pneumonia	Ear infection	Runny nose (nasal congestion)	Sore throat	Cough	Muscle aches	Chills	Headache	Irritability	Decreased activity (lethargy / weakness)	Number of vomits	Number of loose stools	*(office use only)*
Fri 01 Apr																		
Sat 02 Apr																		
Sun 03 Apr																		
Mon 04 Apr																		
Tues 05 Apr																		
Wed 06 Apr																		
Thur 07 Apr																		
Fri 08 Apr																		
Sat 09 Apr																		
Sun 10 Apr																		
Mon 11 Apr																		
Tues 12 Apr																		
Wed 13 Apr																		
Thur 14 Apr																		
Fri 15 Apr																		

* If you have taken your child's temperature on any day, please record the highest temperature for that day in degrees. Remember, only record your child's temperature using the underarm digital thermometer.

ORChID

ORChID daily symptom diary card: version 1, 01 March 2010

Qpid Queensland Paediatric Infectious Diseases Laboratory

Sample processing, screening and storing were all located at the QPID laboratory. Nucleic acid extractions were performed as described previously (Ye et al., 2015). Each stool sample was homogenised in approximately 2.5mL S.T.A.R buffer (Roche Diagnostics, Castle Hill, Australia) to make up a 10% stool suspension. The suspension was centrifuged

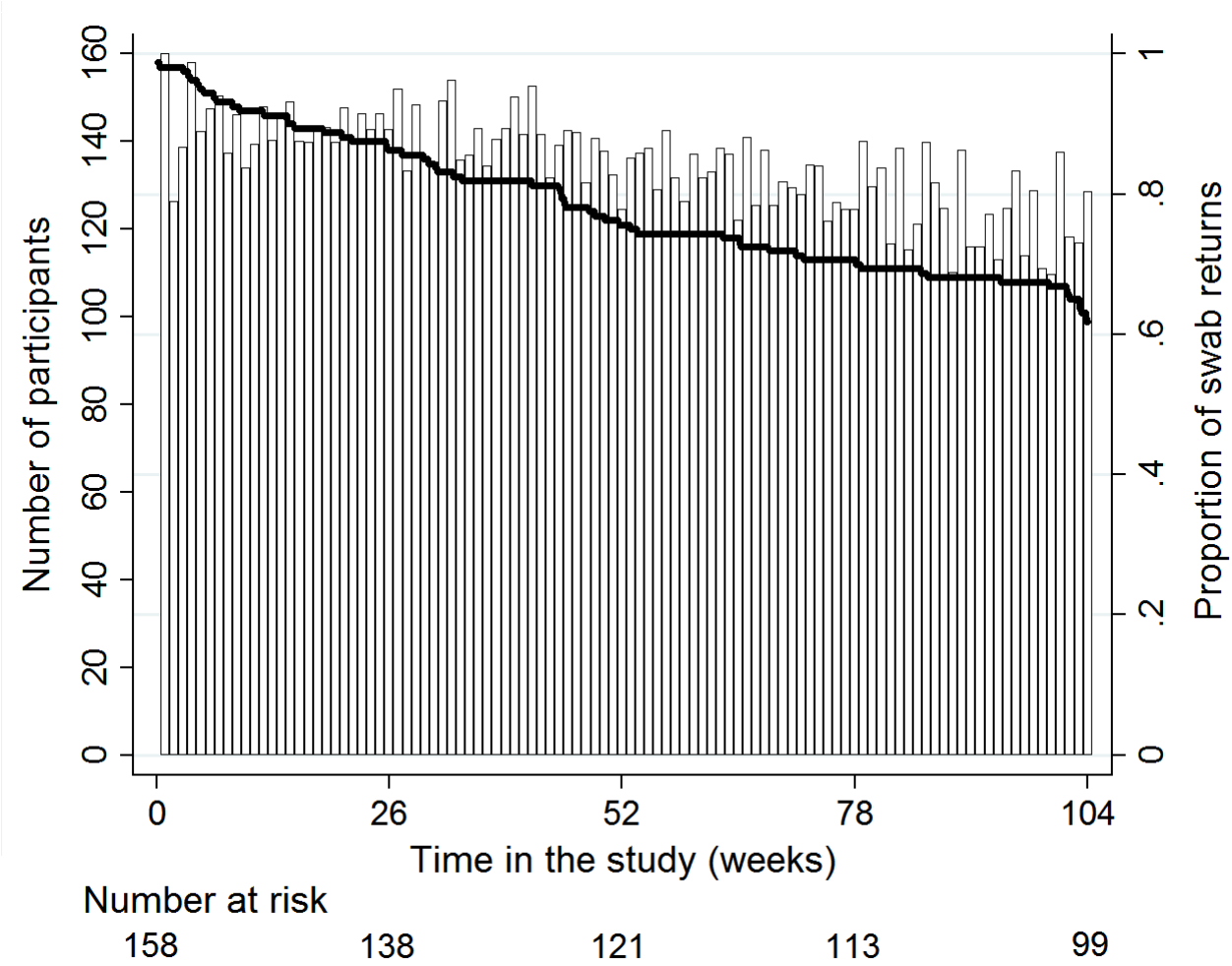
to remove large particles before the supernatant (200uL) underwent nucleic acid extraction. Extraction efficiency and inhibition were monitored using an EHV splike (Ye et al., 2013) and qPCR. Endogenous retroviral-3 (ERV3), which is a marker of human genomic DNA and epithelial cells, has also been tested to assess the quality of sample collection.

Table 3.1. Characteristics of infants enrolled in the Observational Research in Childhood Infectious Diseases Study.

Characteristic (n=158 unless otherwise stated)	Frequency (%) or Mean (±SD)
Sex (male)	75 (47.5%)
Gestational age (weeks)	39.8 (±1.3)
Birth weight (g)	3530.8 (± 430.4)
Birth order (maternal)	
First born	106 (67.1%)
Infant rotavirus immunization status to 8-months (RotaTeq doses, n=149)	
0	3 (2.0%)
1	10 (6.7%)
2	7 (4.7%)
3	129 (86.6%)
Exclusive breastfeeding	
At birth (n=149)	142 (95.3%)
At 3-months (n=142)	97 (68.3%)
At 6-months (n=133)	5 (3.8%)
Childcare	
At 6-months (n=133)	33 (24.8%)
At 9-months (n=123)	48 (39.0%)
At 12-months (n=115)	72 (62.6%)
At 15-months (n=110)	87 (79.1%)

Abbreviations: g, grams; RotaTeq, multivalent human-bovine reassortant rotavirus vaccine; SD, standard deviation.

Figure 3.2. Proportion of infants remaining in the cohort by child age (months) from birth until their second birthday.



Patients selected for this pilot study A convenience sample of the first five ORChID participants to complete the study with a full set of stool samples were included in this pilot. At 2, 4 and 6-months of age, each had received three doses of oral human-bovine reassortant rotavirus vaccine (RotaTeq®; Merck & Inc., Co., New Jersey, USA) as part of Queensland's publically funded immunisation program (actual immunisation dates confirmed by the Australian Childhood Immunisation Register). The Human Research Ethics Committees of the Children's Health Queensland Hospital and Health Service, the Royal Brisbane and Women's Hospital and The University of Queensland approved the study.

Laboratory testing Six enteric viruses were tested using qPCR and RT-qPCR assays. The choice of viruses was determined primarily by availability of PCR templates within the laboratory and included established enteric virus pathogens (rotavirus, norovirus), recognised and emerging respiratory viruses that can be associated with gastrointestinal

symptoms (adenovirus – subgroup F, human coronavirus, human bocavirus-1), and systemic viruses shed from the gastrointestinal tract (enterovirus).

qPCR and RT-qPCR testing of specimens for DNA and RNA viruses respectively was performed using primers and probes for adenovirus (both Pan AdV and AdV type 40/41) (Alsaleh et al.,2014a), enterovirus (Maunula et al., 2008), norovirus group II (Kageyama et al., 2003), rotavirus (Pang et al., 2011), human bocavirus-1 (Tozer et al., 2009), and human coronavirus OC43, 229E, NL63 (Gunson et al., 2005) and human coronavirus HKU (Dare et al., 2007).

Adenovirus positive samples were further characterised using an established adenovirus genotyping method(Sarantis et al., 2004). Each adenovirus-positive sample was re-amplified by PCR using primers spanning the hypervariable regions (HVRs-7) of the hexon gene. PCR products were sent for automated bidirectional sequencing at the Australian Genome Research Facility sequencing laboratory at The University of Queensland.

Rotavirus-positive samples were screened for vaccine strains by a RotaTeq VP6 gene-specific quantitative RT-PCR assay (Gautam et al., 2014). Rotavirus-positive samples negative for the RotaTeq VP6 gene were further genotyped using an established method based on the rotavirus G (VP7) and P (VP4) sequences (Kirkwood, 2010).

Daily symtom diary and associated analyses The symptom diary captured gastrointestinal symptoms daily. A symptomatic episode, was defined as >3 loose stools and/or vomiting within a 24-hour period. A symptomatic episode started on the first day of acute gastroenteritis symptoms and concluded with the last day of symptoms; a new episode required at least 3 asymptomatic days between it and the previous episode to commence.

To examine whether symptoms were associated with higher viral loads, linear regression models were constructed with episode type (symptomatic/asymptomatic) entered as the main effect and semi-quantitative Ct values from the qPCR assays as the outcome. All analyses were performed with Stata v11.0 (StataCorp, College Station, USA).

CHAPTER 3

3.3 Results

Overall, 511 stool samples (98.1% of maximum anticipated specimens) were collected from the five infants during the 2-year study period. The median (interquartile range) time for these specimens to reach the laboratory from the day of collection was 3 (2-4) days. At least one virus was detected in 208 (40.7%) samples. Adenovirus was detected most frequently (n=131; 25.6%), followed by enterovirus (63; 12.3%), norovirus (26; 5.1%), human bocavirus-1 (21; 4.1%), rotavirus (7; 1.4%), and human coronavirus (3 OC43, 1 NL63, 1 229E; 1.0%). A single virus was detected in 166 positive samples (79.8%), while two were co-detected in 39 samples (18.8%), and three in three samples (1.4%). The most frequent co-detections were for adenovirus and enterovirus (n=23, 54.7% of co-detections), followed by norovirus with either adenovirus (n=6, 14.2%) or enterovirus (n=7, 16.7%).

Of the 131 adenovirus positive samples, 62 (47.3%) were genotyped successfully. The observed genotypes were adenovirus types 31 (n=9) and 12 (n=2) of species A, types 1 (n=8), 2 (n=37), and 5 (n=2) of AdV-C, and type 41 of AdV-F (n=4). The failure to genotype the remaining 69 adenovirus-positive samples was attributed to viral load being below the detection limit of the genotyping protocol. The mean Ct value for successfully genotyped detections was 32.3 cycles (SD 7.0) and 39.3 cycles (SD 3.2) for the non-genotyped detections; with a mean difference of 7.0 cycles (95% CI 5.1 to 8.8).

Six of the seven rotavirus positive samples were observed between the ages of 8 and 15 weeks, corresponding to the timing of the first dose of the RotaTeq vaccine. The remaining rotavirus positive sample was collected at 50 weeks of age. Genotyping showed that five rotavirus positive samples were from the RotaTeq vaccine strain, while the sample at 50 weeks was a wild-type G1P[8] strain (subject 4). The remaining rotavirus positive sample (subject 1, week-9) could not be genotyped due to low viral load (Ct value = 39.1 cycles).

The distribution of virus detections among the five infants is summarised in the Table 3.2 and Figure 3.3 (with acute gastroenteritis episodes marked). Only 22 of the 208 (10.6%) virus-positive samples were associated with gastrointestinal symptoms and >1 virus was detected in seven (31.8%) of these episodes. Detection of >1 virus in asymptomatic virus detection episodes was 18.8% (35/186). Amongst individually-detected viruses, just 14/131 (10.7%) adenovirus detections (type 2, (2/37); type 5 (1/2); type 31 (1/9); type 41 (2/4); and non-typable (8/69)) were associated with symptoms. Similarly, only 9/63 (14.3%)

enterovirus, 7/26 (27.0%) norovirus and 1/21 (4.8%) human bocavirus-1 detections were accompanied by symptoms. However, no gastrointestinal symptoms were associated with the seven rotavirus positive or five human coronavirus positive samples. Symptomatic enterovirus detections had lower viral load than asymptomatic detections (mean Ct value difference=2.34 cycles; 95% CI= 0.1, 4.7) (Table 8). No similar association was observed for other viruses. In contrast, and unlike the other four participants, subject 2 had 15 symptomatic gastroenteritis episodes ranging from 1 to 5-days duration and where in 11 none of the six viruses were detected.

At various times, adenovirus, enterovirus, and norovirus each had periods of continuous virus shedding (Figure. 3.3), lasting as long as 11 weeks for adenovirus, 6 weeks for enterovirus, and 4 weeks for norovirus. Based on the adenovirus genotyping data, adenovirus type 2 was shed continuously for 11 weeks in subject 1 (weeks 37-47) and for 9 weeks in subject 3 (weeks 30-38). Sequential detections of different virus genotypes were also observed during periods of continuous adenovirus shedding. For example, subject 4 (the only infant not to have any recorded gastroenteritis episodes during their first 2 years of life) had serial detection of adenovirus types 2, 31, and 1, occurring between 80 and 90 weeks of age (Figure. 3.3).

3.4 Discussion

In this pilot study, weekly stool samples from five children from a community-based cohort during the first 2 years of life were tested. Frequent shedding of one or more of the targeted six viruses was identified in weekly swabs, but often in the absence of gastrointestinal symptoms. Of these six viruses, norovirus G II, was found to have the highest proportion of positive detections associated with gastrointestinal symptoms and is consistent with its relative importance as a cause of acute gastroenteritis in young children already immunized against rotavirus infections (Koo et al., 2013). These findings confirm and extend those of previous observations, including from two infants in the first year of life where there was almost constant shedding of various enteric viruses – adenovirus, anellovirus, bocavirus, enterovirus, parechovirus, and picobirnavirus – in weekly stool samples with only occasional minor symptoms being present (Kapusinszky et al., 2012).

Of note in the current study was the duration of shedding observed for certain adenovirus types. Whilst not all adenovirus-positive samples could be assigned a specific type (due to low viral load), adenovirus, especially adenovirus type 2 could be shed for almost 3-months without gastrointestinal symptoms being present. Unlike co-detection of different adenovirus types, sequential detection of various adenovirus types as observed in subject 4 over a 10-week period has received little attention in the literature. Two of the three types (types 2 [AdV-C] and 31 [AdV-A]) had been detected intermittently beforehand in this subject. It is plausible that the serial detections reported in this infant represent independent reactivations of these two types, whose adenoviruses speciesinfect lymphoid tissue at different sites of the gastrointestinal tract (Kosulin et al., 2015), followed by a new adenovirus type 1 [AdV-C] infection.

Only one of these five fully immunised children had a wild type rotavirus strain detected; a subclinical G1P[8] infection identified at 50-weeks of age. Of the remaining six rotavirus-positive samples, five were confirmed as a vaccine strain. The sixth sample was detected 10-days after the first dose of RotaTeq vaccine, could not be genotyped, but the next sample from this subject collected 1-week later was confirmed RotaTeq vaccine strain positive. These data support findings from a recent Australian study where RotaTeq vaccine viruses accounted for 72% of rotavirus RT-qPCR positives samples in children aged <32-weeks submitting stool samples for diagnostic testing (Schepetiuk et al., 2015).

This pilot study also identified issues with the predictive value of molecular diagnostic assays in stool samples from infants. While PCR is very sensitive, 186 virus-detected (89.4%) samples were not associated with gastrointestinal symptoms. Other studies have suggested that this limitation could be overcome in children with acute gastroenteritis by measuring viral loads using qPCR Ct values and developing cut-off values for detections that were meaningful clinically (Corcoran et al., 2014). However, with this small sample population, only the Ct values for enterovirus detections were associated significantly with symptoms. Given enterovirus is recognised as only a minor contributor to acute gastroenteritis (any gastrointestinal symptoms are likely part of a more systemic illness, including fever and/or rash), and that the observed difference in Ct values between symptomatic and asymptomatic virus detections was relatively small (2.34 cycles or <1-log difference in viral load), drawing further conclusions from these data must be done with caution. Moreover, unlike blood, where a defined volume/cell number can be quantified, it is difficult to

accurately assess a viral load from stool samples that may vary widely in their fluid content. Although qPCR Ct values from stool samples were used as a surrogate, semi-quantitative estimate of viral load, this is still a limitation where Ct values in liquid stool may underestimate viral load.

The current pilot study only involved five participants and some recognized viral agents associated with acute gastroenteritis were not tested, including sapovirus, astrovirus and norovirus GI viruses. Nevertheless, this study assisted in refining methods and workflow for the much larger task of stool testing for the entire ORChID cohort (Lambert et al.,2012). It also found that it is possible to detect multiple viruses in parent-collected stool specimens returned to the laboratory by surface mail. Additional questions for the ORChID cohort include further details on the nature and duration of virus shedding in the stools of healthy infants, if important biological differences exist between the major adenovirus and enterovirus genotypes shed in stools, including their associations with gastrointestinal (and other) symptoms, and whether a Ct value cut-off can be used to improve diagnostic accuracy of viral gastroenteritis in young children.

In summary, these data highlight the complexity of viral shedding from the gastrointestinal tract and determining disease-pathogen associations in healthy infants and young children. The results affirm and extend previous observations of frequent and subclinical shedding of multiple and diverse viruses in the stools of two siblings during their first year of life.

Figure 3.3. Weekly virus detections with corresponding cycle threshold (Ct) values and symptoms of acute gastroenteritis in five healthy infants during the first 2 years of life. (Please note that for subject 2 there are distinct AGE episodes at weeks 25 and 26, and also at weeks 39 and 44, but that these cannot be distinguished in the figure due to the small scale).

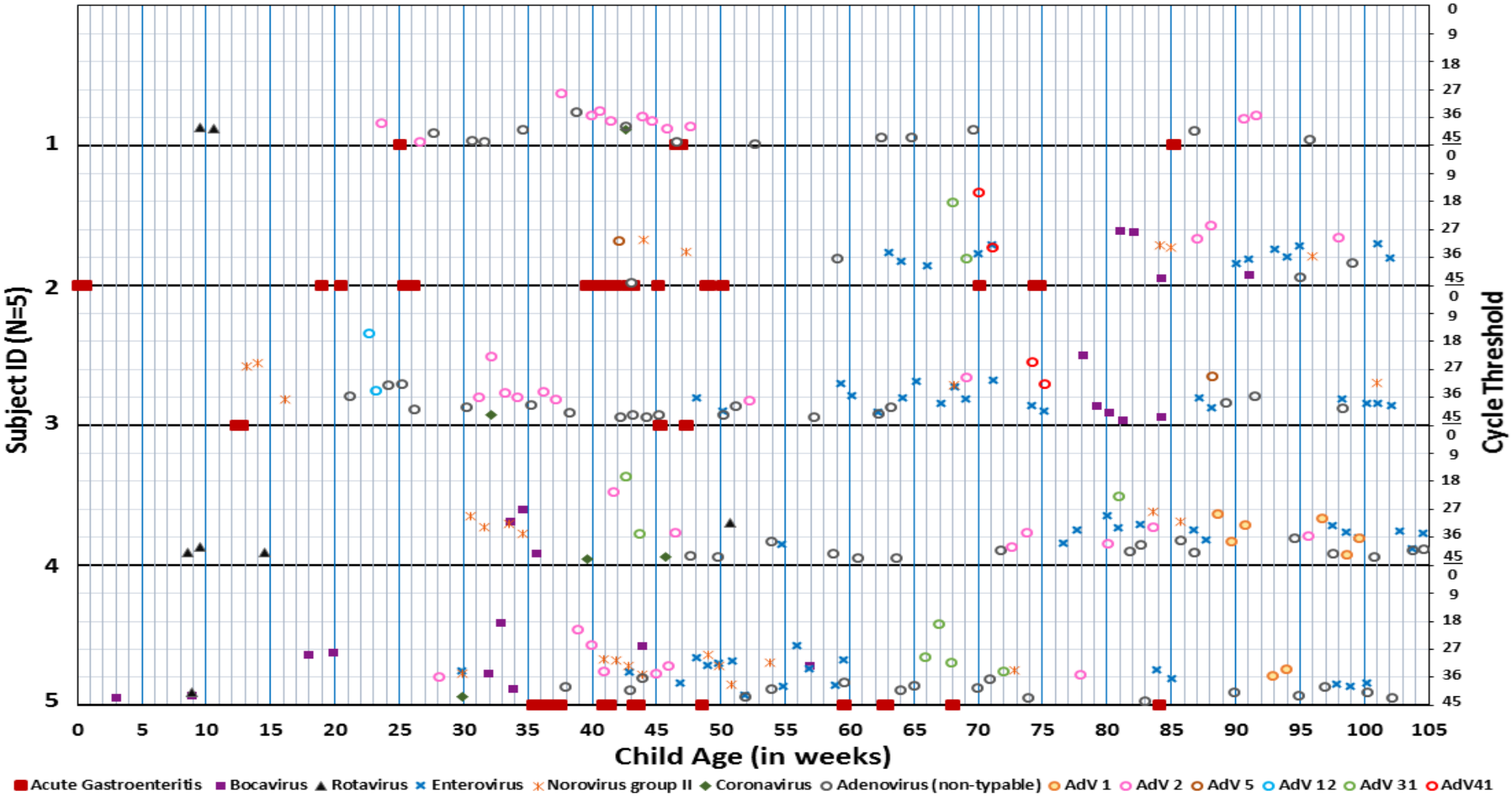


Table 3.2. Number of enteric virus detections and their association with acute gastroenteritis symptoms in weekly stool samples collected from five healthy infants during the first 2-years of life.

		Adenovirus	Enterovirus	Norovirus GII	Rotavirus	Human bocavirus-1	Human coronavirus
Asymptomatic detection	**No. of swabs**	**117**	**54**	**19**	**7**	**20**	**5**
	Ct count; mean(SD)	36.01 (6.26)	35.60 (3.35)	32.49 (3.13)	38.85 (3.45)	34.51 (7.76)	41.75 (1.09)
Symptomatic detection	**No. of swabs**	**14**	**9**	**7**	**0**	**1**	**0**
	Ct count; mean(SD)	35.95 (7.49)	33.26 (2.37)	30.54 (2.83)	—	25.64 (—)	—
Mean difference between asymptomatic and symptomatic detections; (95% Conf. Interval)		0.06 (-3.52, 3.64)	2.34 (0.01 to 4.67)	1.95 (-0.84 to 4.73)	—	8.87 (-7.78 to 25.52)	—
Subject number and qPCR positive swabs							
Infant 1	(n=102)	25	0	0	2	0	1 (229E)
Infant 2	(n=106)	12	12	5	0	4	0
Infant 3	(n=102)	32	19	5	0	5	1 (OC43)
Infant 4	(n=95)	32	14	6	4	3	2 (NL63; OC43)
Infant 5	(n=106)	30	18	10	1	9	1 (OC43)
Total	**(n=511)**	**131**	**63**	**26**	**7**	**21**	**5**

CHAPTER 4

Chapter 4 – Multivalent rotavirus vaccine and wild-type rotavirus strain shedding in Australian infants: A birth cohort study

This following section is presented as an original research article:

Ye, S., Whiley, D. M., Ware, R. S., Kirkwood, C. D., Lambert, S. B., & Grimwood, K. (2018). Multivalent Rotavirus Vaccine and Wild-type Rotavirus Strain Shedding in Australian Infants: A Birth Cohort Study. Clinical Infectious Diseases, 66(9), 1411-1418. doi:10.1093/cid/cix1022

The paper is as published, except for the following modifications. For example, table number been modified to fit with the thesis format, running title and summary removed, Keywords removed and abstract removed, abbreviations retained if already used etc, shorten introduction is already covered, conflict of interest and funding have been removed, and all references have now been added to a single reference section at the end of the thesis.

CHAPTER 4

Multivalent rotavirus vaccine and wild-type rotavirus strain shedding in Australian infants: a birth cohort study

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4.1 Introduction

Two live-attenuated oral rotavirus vaccines, RotaTeq® (Merck & Co.) and Rotarix® (GSK Biologicals) were licensed in 2006 following their demonstrated safety and efficacy in large field trials in Europe and the Americas (Vesikari et al., 2006; Ruiz-Palacios et al., 2006). As vaccine coverage increased, rotavirus hospitalizations in children aged <5-years declined by 71% in Australia (Dev et al., 2012), 63-94% in the United States (Leshem et al., 2015), 65-84% in Europe (Karafillakis et al., 2015), and by about 70% in Latin America (Santos et al., 2017).

Before Australia's rotavirus vaccine program began in mid-2007, rotavirus gastroenteritis led to 4% of children being hospitalized by age 5-years, 9% attending Emergency Departments (ED), and 45% consulting their family doctor, with approximately 60% of these healthcare contacts occurring in the first 2-years of life (Galati et al., 2006). In Queensland, Australia, RotaTeq was chosen for the state's publically funded rotavirus vaccine program. This multivalent vaccine is given as a three-dose schedule in the first 6-months of life and contains five live-attenuated human-bovine reassortant strains, four of which express one of the human virus VP7 outer capsid proteins, G1, G2, G3, or G4, while the fifth expresses the VP4 attachment protein, P[8], derived from human parent strains (Vesikari et al., 2015).

In contrast with hospital-based data, little is known about the impact of rotavirus vaccines on mild gastroenteritis episodes managed within the community where clinical trial data suggest vaccines are less efficacious (Hungerford et al., 2017). Changes in diagnostic testing practices have also occurred recently and may impinge upon monitoring vaccine effectiveness. In Queensland, and elsewhere, multiplexed qPCR assays are replacing conventional methods for identifying gastrointestinal pathogens (Siah et al., 2014). This poses a potential problem since RotaTeq as a live vaccine replicates within the gut and is shed in stools post-vaccination where highly-sensitive assays may detect, but not discriminate between, vaccine and wild-type rotavirus strains.

The pivotal RotaTeq trial observed vaccine shedding in 13% of subjects following their first vaccine dose and none after the second and third doses using relatively insensitive viral culture techniques (Vesikari et al., 2006). In contrast, a Taiwanese study adopting a vaccine strain-specific RT-qPCR identified 17/18 (94%) infants shed RotaTeq strains for 2-14 days after the first vaccine dose, and two infants were still shedding vaccine virus at 25-days

(Hsieh et al., 2014). Shedding was however, less frequent following the second (29/43; 67%) and third (23/36; 62%) doses, and confined to the first 2-weeks post-vaccination. Nonetheless, there is some evidence that shedding may occur for longer periods. Studies of infants hospitalized with respiratory infections report vaccine virus shedding up to 8-months of age and12-weeks after the third RotaTeq dose (Markkula et al., 2015).

Our primary objective was therefore to investigate the prevalence and duration of rotavirus vaccine shedding in an unselected community-based birth cohort of Australian infants. Secondary objectives were to record the incidence of wild-type rotavirus infections and to assess if semi-quantitative estimates of viral load, determined by RT-qPCR Ct values, were positively associated with diarrheal symptoms and could differentiate between vaccine and wild-type strains.

4.2 Methods

Study design and sample collection

The ORChID Study (clinical trials.gov: NCT01304914) is a community-based birth cohort study of infections in the first 2-years of life (Lambert et al., 2012). Women were approached antenatally and their infants enrolled at birth progressively from September 2010 to October 2012 at two hospitals (one public and one private hospital) in Brisbane, a subtropical city in Queensland, Australia. Eligible infants were born at term (36-42 weeks) and needed to be healthy, without underlying chronic disorders or congenital abnormalities. Parents collected weekly stool swabs from their infants, beginning in the first-week of life and ceasing at their second birthday. Swabs were mailed to our research laboratory, taking a median of 3 (IQR 2-4) days for delivery, where they were processed and stored at -80°C. We and others have successfully used mailed swab specimens in community-based studies to detect rotavirus and other enteric agents (Ye et al., 2017; Simonen-Tikka et al., 2013). The infant's parents maintained a daily symptom diary, which included diarrhea as a data field, and this was returned by mail at the end of each month.

In Queensland, RotaTeq vaccine is given at 6-8 weeks, and at 4 and 6-months of age. RotaTeq vaccination status of study infants were reported by parents, and confirmed on the national, population-based Australian Immunisation Register. The Children's Health Queensland, Royal Brisbane and Women's Hospital, and The University of Queensland

Human Research Ethics Committees approved the study. Parents of each child provided written, informed consent at enrolment.

Rotavirus detection by RT-PCR and genotyping assays

Stool samples were made up to 10% suspensions by homogenizing in 2.5mL S.T.A.R buffer (Roche Diagnostics, Castle Hill, Australia). The CAS1820 Xtractor Gene automated system (Qiagen, Australia) or MagNA pure 96 System (Roche Life Science, Australia) extracted viral RNA as per the manufacturer's instructions. Extraction efficiency and inhibition were assessed using an equine herpes virus spike (Ye et al., 2013). RT-qPCR testing was run for 45 cycles using previously published primers and probes targeting the rotavirus NSP3 region (Pang et al., 2011), and was defined as positive if any virus was detected (see Supplementary methods). Ct values from RT-qPCR for rotavirus-positive samples are inversely proportional to the amplified nucleic acid in the sample and were used as semi-quantitative markers of viral load. Rotavirus positive samples were genotyped using a rotavirus vaccine strain-specific RT-qPCR assay (RotaTeq VP6 RT-PCR) (Gautam et al., 2014) and a genotyping method described previously targeting the rotavirus VP4 and VP7 regions (Kirkwood et al, 2010).

Definitions

A priori definitions for diarrhea, rotavirus detection, rotavirus episode, and symptomatic and asymptomatic episodes are provided in Table 4.1.

Statistical Analysis

Summary statistics are reported as either mean (standard deviation) or median (IQR) values for continuous variables and frequency (percentage) for categorical variables. Linear regression models were used to investigate the following associations; (a) Ct values from successfully genotyped detections were compared to non-genotyped rotavirus detections, (b) shedding duration was compared between asymptomatic and symptomatic episodes, and (c) Ct values from the first rotavirus detection in asymptomatic episodes were compared with the first rotavirus detection from symptomatic episodes. Effect estimates are presented as mean differences and 95% confidence intervals (95%CI). All analyses were performed with Stata v11.0 (StataCorp, College Station, USA).

Table 4.1. Definitions.

Term	Definition
Diarrhea	≥3 stools (above normal baseline) of liquid consistency within a 24-hour period (Gidudu et al., 2011).
Rotavirus detection	Rotavirus was detected by reverse transcription polymerase chain reaction assay in a single sample.
Rotavirus episode	The period from the first to the last positive rotavirus samples. New episodes commenced from the first identification of a different rotavirus genotype, or identification of the same genotype >21-days from the last identification of the same genotype.
Symptomatic episode	A rotavirus episode where diarrhea occurred within ± 7-days of the first rotavirus detection.
Asymptomatic episode	A rotavirus episode where diarrhea was not observed within ± 7-days of the first rotavirus detection.

4.3 Results

Of 165 infants from 163 families enrolled in the study, one was excluded due to pre-term birth and six failed to provide any swabs (Figure 4.1). The remaining 158 (75 males) infants provided 11,139 swabs (Figure 4.1, 66.5% of maximum expected swabs; median 85 swabs per child, range 1-106 from birth until their second birthday) with 67.7% followed until at least age 23-months (see Figure 3.2). Their sociodemographic characteristics are presented in the Table 3.1.

Symptom diaries were submitted for 154 infants (Figure 4.1), who provided 88,811 child-days of recorded observation from birth until their second birthday (79.4% of maximum expected; median 730, range 1-730 days). Nine infants withdrew before the recommended age of the first RotaTeq dose, three did not receive the vaccine, while 10, 7, and 129 infants received one, two, and three doses, respectively (See Table 3.1).

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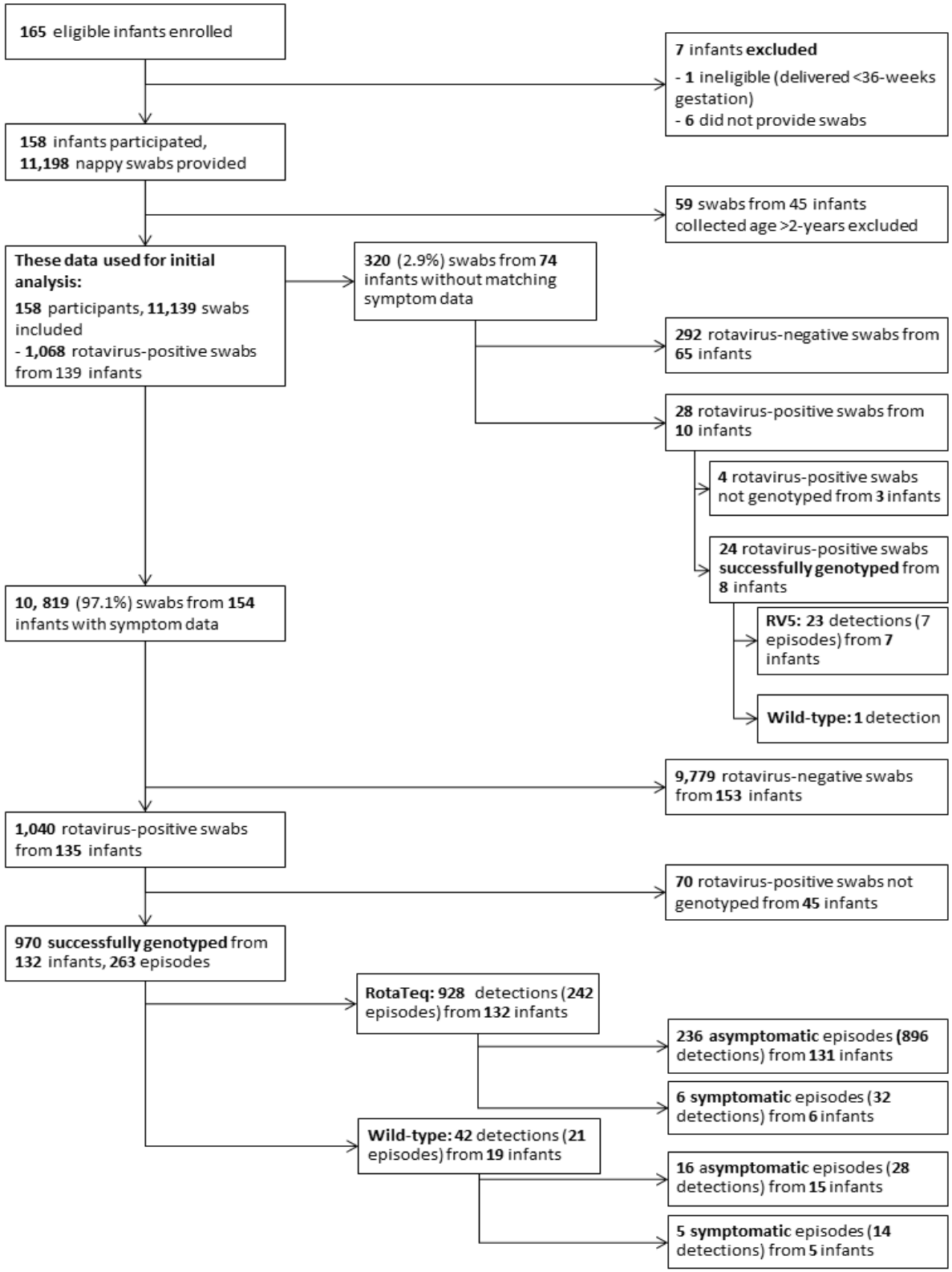
Figure 4.1. Submission of stool swabs, symptom diaries and positive rotavirus detections in the Observational Research in Childhood Infectious Diseases birth cohort.



Footnote: As infants can have both symptomatic and asymptomatic rotavirus detection episodes, the total number of infants with symptomatic and asymptomatic episodes exceeds the total number of infants with rotavirus detections.

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Rotavirus detections

Overall, rotaviruses were detected in 1,068/11,139 (9.6%) stool swabs from 139 infants. Genotyping was successful for 994/1,068 (93.1%) specimens, with RotaTeq strains accounting for 951/994 (95.7%) of all rotavirus detections genotyped successfully. Of the 1,068 rotavirus-positive detections, 1,033 (96.7%) were in the first-year of life.

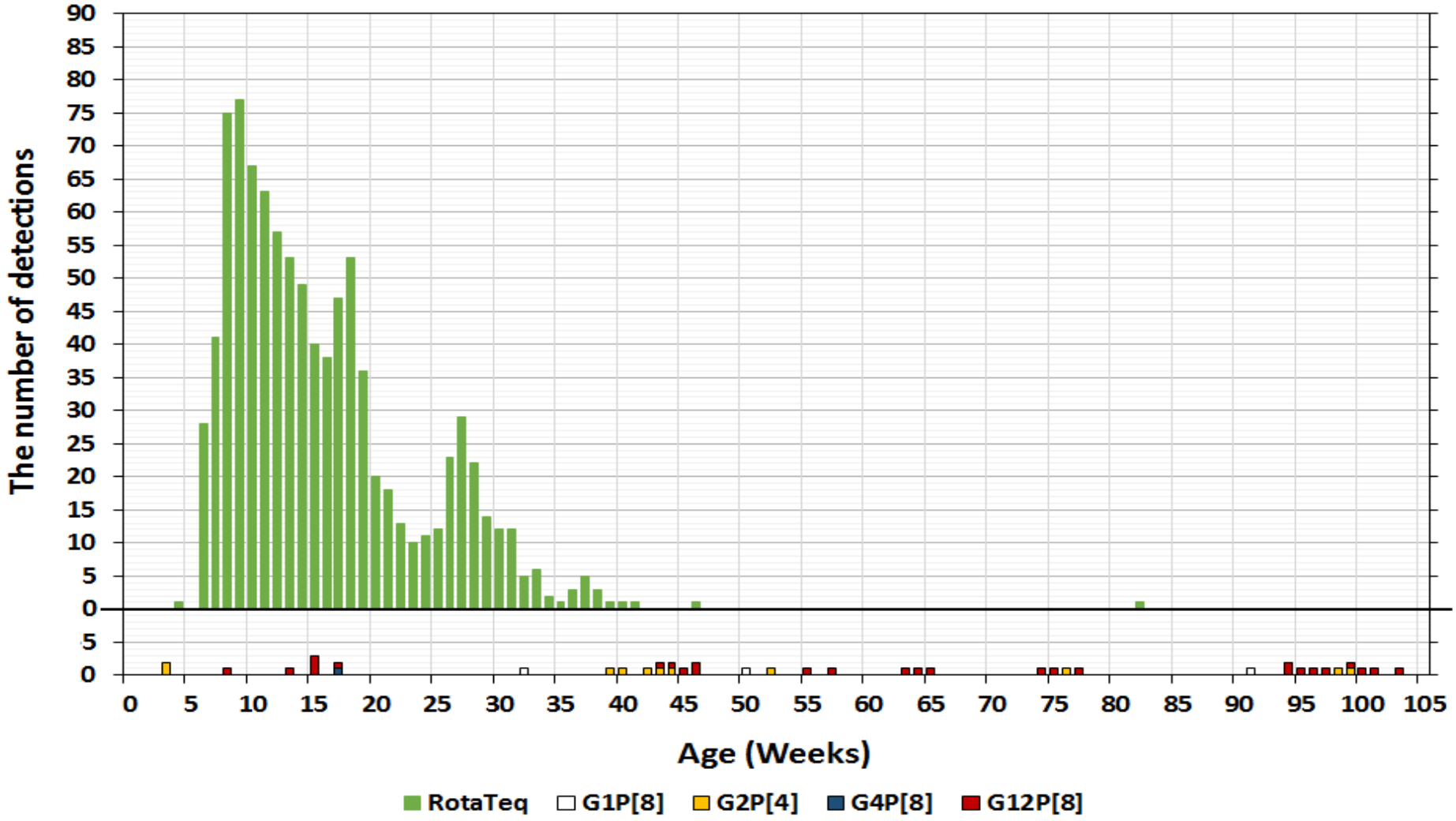
Amongst the 951 typed as the RotaTeq vaccine strain, all but three were detected between 6 and 42-weeks of age in 136/146 (93.2%) infants who had received at least one vaccine dose (Figure 4.2). In contrast, 43 (4.0%) wild-type strains from 20 infants were found throughout the first 2-years of life (Figure 4.2 and Figure 4.3). These included: G12P[8] (n=28), G2P[4] (n=11), G1P[8] (n=3), and G4P[8] (n=1). Except for one child who shed wild-type rotavirus before reaching the age of vaccination, all the other children with wild-type detections were vaccinated.

Table 4.2a shows that mean Ct values for RotaTeq strains were higher (ie. lower viral loads) than for wild-type strains: (33.5 vs 31.1; mean difference 2.4 cycles (95%CI: 1.1-3.7). We could not genotype 74/1,068 (6.9%) rotavirus-detections and their mean Ct values were significantly higher than those able to be genotyped (39.8 vs 33.4; mean difference 6.4, 95%CI: 5.5-7.4), suggesting non-typeable positive swabs had much lower viral loads.

Rotavirus shedding

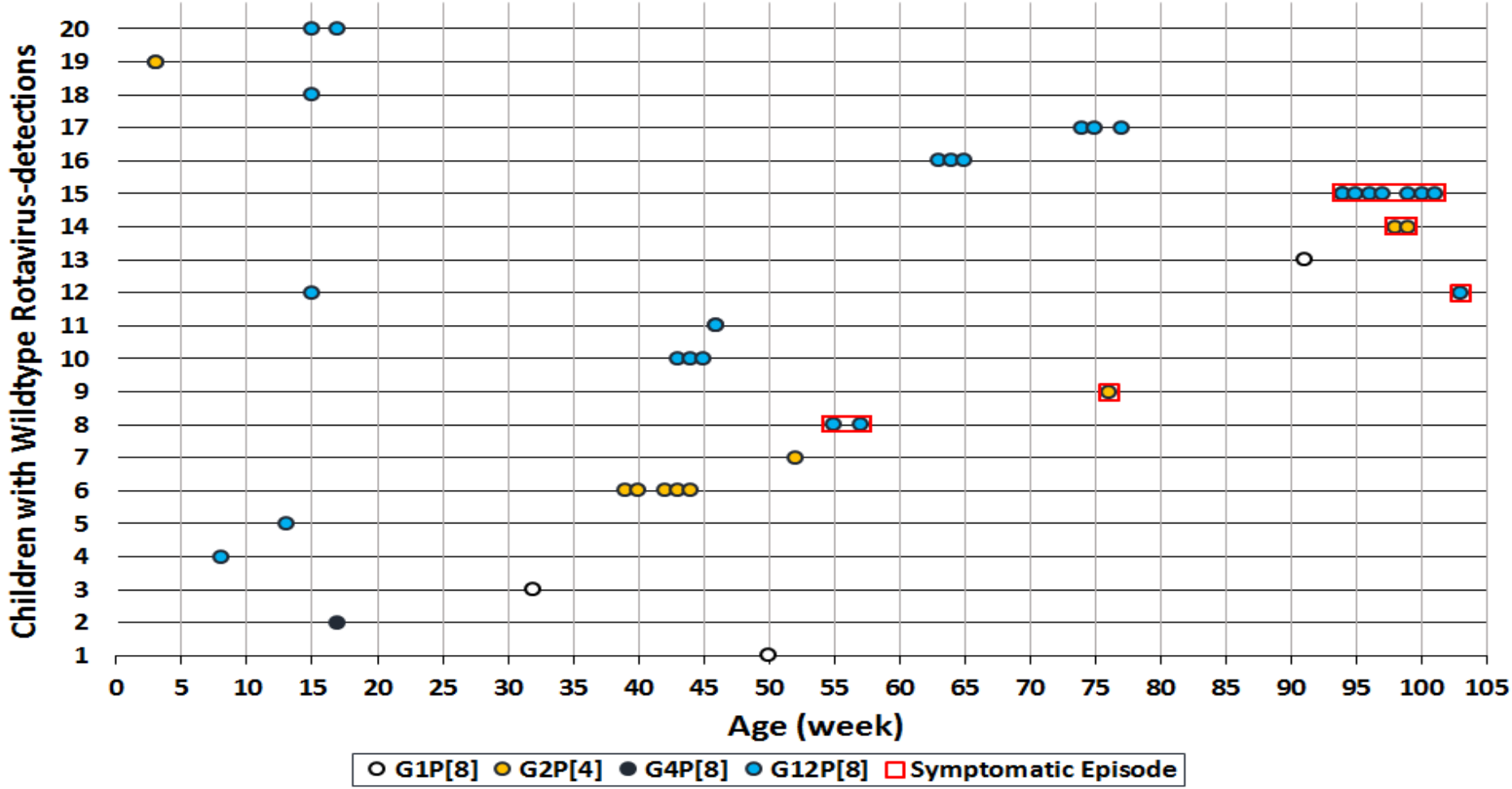
The cumulative proportions of infants shedding RotaTeq detected at least once after the first, second, and third doses were 87.0% (127/146), 57.4% (78/136), and 47.3% (61/129). The week-by-week proportion of vaccine-strain shedding in the 10-weeks following each dose is displayed in Figure 4.4. The median duration of RotaTeq shedding was 3-weeks (IQR 1-8; range 1-13), 1.5 weeks (IQR 1-3; range 1-9) and 1-week (IQR 1-2; range 1-14) after the first, second, and third doses, respectively. In contrast, the median duration of rotavirus shedding after a wild-type infection was 1-week (IQR 1-3; range 1-8). Although shedding was longer with symptomatic than asymptomatic episodes for both RotaTeq and wild-type detections, these differences were not statistically significant (Table 4.2b).

Figure 4.2. Rotavirus-detections by child age in weeks from birth until the second birthday.



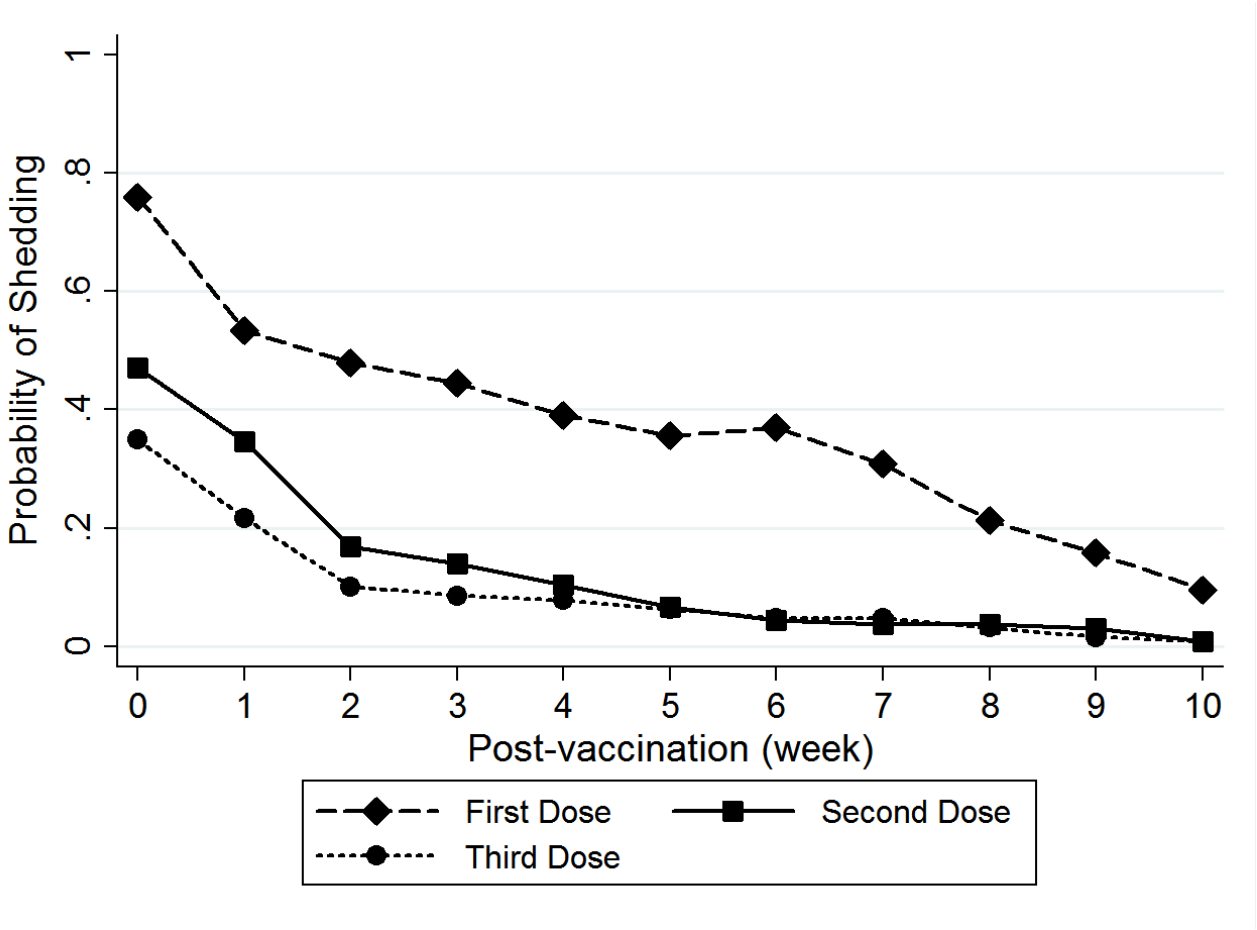
Footnote: RotaTeq represents the multivalent human-bovine reassortant rotavirus vaccine.

Figure 4.3. Wildtype rotavirus-detections by child age in weeks from birth until their second birthday.



Foot note: Please note that due to the small scale some detections are merged and cannot be distinguished from one another. Infant 13 lacked symptom data at the time of their rotavirus episode.

Figure 4.4. Proportion of children shedding vaccine-strain after each multivalent human-bovine reassortant rotavirus vaccine dose.



Footnote: Only vaccine virus shedding within 0-10 weeks following vaccination is displayed. The days where positive samples fell between weeks post-vaccination were rounded down to the preceding week. The cumulative proportions of infants shedding RotaTeq detected at least once after the first, second, and third doses were 87.0% (127/146), 57.4% (78/136), and 47.3% (61/129). This included 28 (19.2%) and 7 (5.1%) infants who were still shedding RotaTeq the week before their second and third vaccine doses respectively. In the first stool swab collected after the second dose, the proportion of infants PCR positive for RotaTeq was 46.3% (50/108) and 85.7% (24/28) for those who had and had not ceased shedding immediately prior to the second vaccine dose respectively. Similarly, in the first stool swab collected after the third dose, the proportion of infants who were RotaTeq PCR positive was 36.1% (44/122) and 57.1% (4/7) for those who had and had not ceased shedding immediately before the third vaccine dose respectively.

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Table 4.2. Rotavirus detections and cycle threshold values associated with genotyping, shedding duration and symptoms

(a). RT-qPCR Ct values and successful rotavirus genotyping.						
Rotavirus strains	**Successfully genotyped detections**		**Non-genotyped detections**		**Mean difference between successful and non-genotyped detections; (95% CI)**	**P-value**
	No. of infants (swabs)	**Ct count; mean (SD)**	**No. of infants (swabs)**	**Ct count; mean (SD)**		
All rotavirus strains	136 (994)	33.4 (4.2)	48 (74)	39.8 (2.0)	**6.4 (5.5 to 7.4)**	**P <0.001**
Vaccine (RotaTeq) strain	136 (951)	33.5 (4.0)				
Wild-type strains	20 (43)	31.1 (7.3)				
(b). Rotavirus shedding duration and symptoms.						
Rotavirus strains	**Asymptomatic episodes**		**Symptomatic episodes**		**Mean difference between symptomatic and asymptomatic episodes; (95% CI)**	**P-value**
	No. of infants (episodes)	**Duration; mean in weeks (SD)**	**No. of infants (episodes)**	**Duration; mean in weeks (SD)**		
All rotavirus strains	132 (252)	3.7 (4.1)	11 (11)	4.1 (5.1)	0.4 (-2.1 to 2.9)	P = 0.781
Vaccine (RotaTeq) strain	131 (236)	3.9 (4.2)	6 (6)	5.3 (6.4)	1.5 (-2.0 to 4.9)	P = 0.346
Wild-type strains	15 (16)	1.7 (1.4)	5 (5)	2.6 (3.0)	0.9 (-1.1 to 2.9)	P = 0.405
(c). RT-qPCR Ct values and symptoms.						
Rotavirus strains	**Asymptomatic episodes**		**Symptomatic episodes**		**Mean difference in Ct values between episodes with and without symptoms ; (95% CI)**	**P-value**
	No. of infants (swabs)	**Ct value[a]; mean (SD)**	**No. of infants (swabs)**	**Ct value[a]; mean (SD)**		
All rotavirus strains	132 (252)	33.2 (5.1)	11 (11)	27.6 (8.6)	5.6 (2.4 to 8.8)	**P = 0.001**
Vaccine (RotaTeq) strain	131 (236)	33.3 (4.8)	6 (6)	32.0 (6.7)	1.3 (-2.7 to 5.2)	P = 0.531
Wild-type strains	15 (16)	32.1 (8.3)	5 (5)	22.3 (8.1)	9.7 (0.8 to 18.6)	**P = 0.034**

Abbreviations: CI, confidence interval; Ct, cycle threshold; RT-qPCR, real-time reverse transcription polymerase chain reaction; RotaTeq, multivalent human-bovine reassortant rotavirus vaccine; SD, standard deviation. [a]The Ct value for the first rotavirus detection in a rotavirus episode.

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Rotavirus-associated symptoms

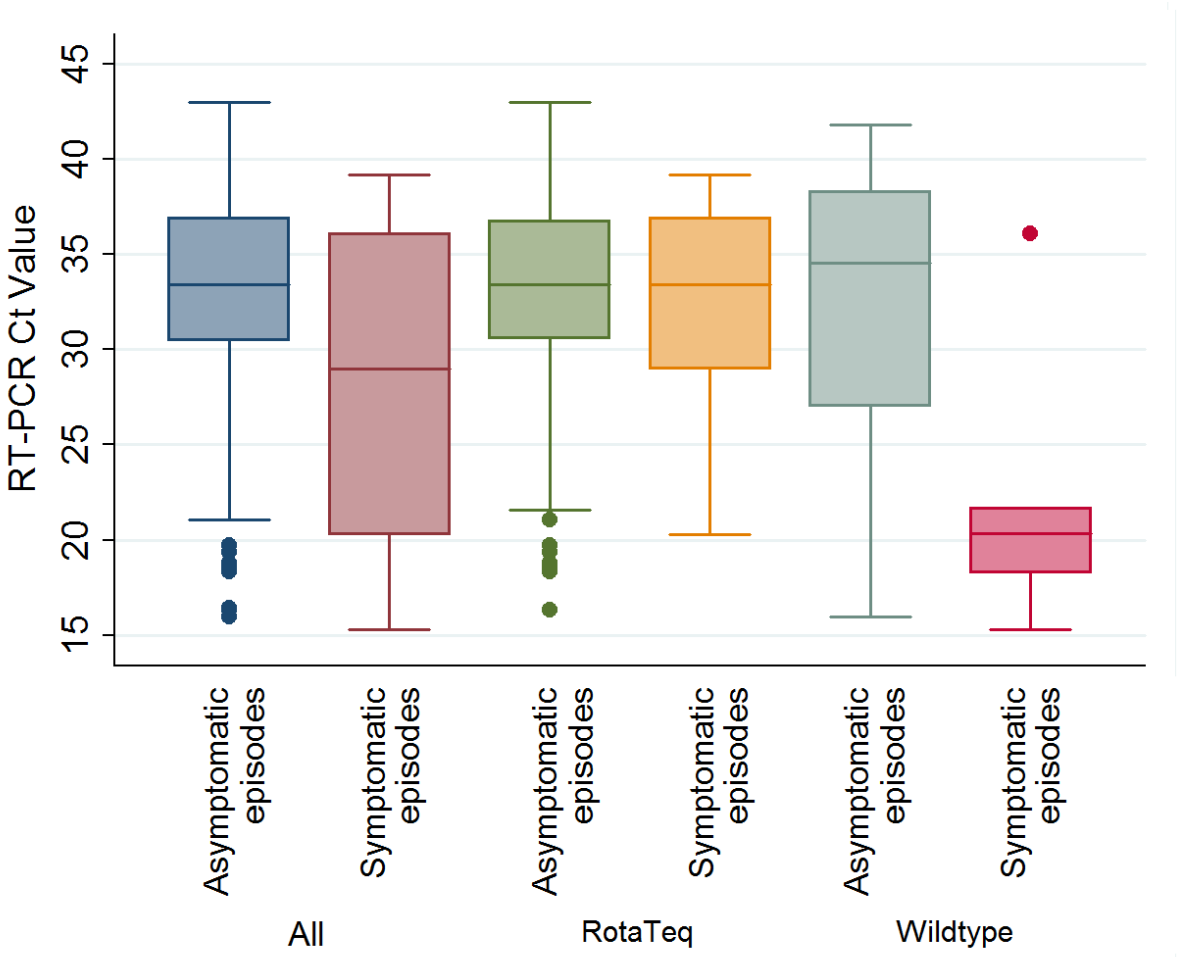
In the ORChID cohort of 263 rotavirus episodes (132 infants) there were 242 RotaTeq (132 infants) and 21 wild-type rotavirus episodes (19 infants) with corresponding symptom data (Table 4.3). Of these, 11 infants had diarrhea within 7-days of the initial rotavirus detection, including 6/242 (2.5%) symptomatic RotaTeq episodes in six infants, resulting in a single family doctor visit, and 5/21 (23.8%) symptomatic wild-type rotavirus episodes in five infants leading to family doctor consultations in three of these infants [5/21 vs 6/242; relative risk 9.6 (95%CI 3.7-25)]. The incidence rate of wild-type rotavirus episodes was 10.3 (95% CI: 6.8-15.6) per 100 child-years of observation, including 2.4 symptomatic episodes (95% CI: 1.0-5.8) per 100 child-years.

Table 4.3. Distribution and percentage of rotavirus symptomatic episodes by rotavirus genotype.

Genotype		No. of infants	No. of rotavirus-episodes	No. of symptomatic-episodes (%)
Wild-type	G1P[8]	2	2	0 (0 %)
	G2P[4]	5	5	2 (40.0 %)
	G4P[8]	1	1	0 (0 %)
	G12P[8]	11	13	3 (23.1%)
	Total	19	21	5 (23.8 %)
Vaccine (RotaTeq) strain		132	242	6 (2.5 %)

The first-detections from the 11 symptomatic episodes had significantly lower Ct values (higher viral loads) than the first-detections from 252 asymptomatic episodes (27.6 vs 33.2; mean difference 5.6; 95% CI 2.4-8.8). This result remained significant for wild-type virus, but not for RotaTeq vaccine virus (Table 4.2c). Nevertheless, some asymptomatic infants also had RotaTeq virus with low Ct values in their stools (Figure 4.5).

Figure 4.5. Box and whiskers plot of cycle threshold (Ct) values for the first detected rotavirus-positive stool samples from infants in the ORChID cohort categorized according to the presence of diarrheal symptoms and genotype results



4.4 Discussion

Employing sensitive PCR assays, we detected rotaviruses in almost 10% of stool specimens collected weekly from ORChID participants during their first 2-years of life. These rotavirus-positive specimens were predominantly RotaTeq vaccine strains detected in asymptomatic infants and intensely clustered around the time of vaccination between 6-weeks and 8-months of age. The proportions of vaccinated infants shedding vaccine virus declined from 87.0%, to 57.4%, and 47.3% following the first, second and third RotaTeq doses respectively, and were still detected up to 14-weeks after the third dose. In contrast, wild-type strains were detected infrequently, but were observed throughout the first 2-years of life, and almost one in four episodes were associated with diarrhea. Mean Ct values were significantly lower in infants with symptomatic wild-type rotavirus episodes than those with either RotaTeq vaccine virus or asymptomatic wild-type detections. However, low RotaTeq

Ct values were also observed in some infants making these semi-quantitative estimates of viral load in our hands an inadequate tool for differentiating between vaccine and wild-type viruses.

Previous studies investigating RotaTeq vaccine virus shedding have yielded mixed results depending upon the laboratory methods employed. Initial investigations relying upon culture-based methods only observed shedding in 8.9%-13% of infants in the week following their first vaccine dose (Vesikari et al., 2006; Dennehy et al., 2007). Subsequently, detections on at least one occasion increased to 21%-56% and 87%-94% of infants when either rotavirus antigen or RT-PCR assays were used respectively to test stools collected for 1-4 weeks after the first administered RotaTeq vaccine dose (Hsieh et al., 2014; Yen et al., 2011; Smith et al., 2011). These time-limited investigations were however not designed to detect prolonged RotaTeq shedding by immunocompetent infants. Our study extends these earlier findings by showing that not only a high proportion of infants had RotaTeq detected in their stools post-vaccination, but the vaccine virus was also found in asymptomatic infants for several weeks afterwards. This supports findings in Finnish infants hospitalized with respiratory infections, but without gastrointestinal symptoms, having RotaTeq detected in their stools up to 12-weeks after their last vaccination (Markkula et al., 2015). It is also consistent with post-hoc studies of the pivotal RotaTeq field trial where the vaccine virus was detected in 75% of re-analyzed rotavirus antigen positive stools collected from infants with gastroenteritis during the period they received the three-doses of the active vaccine (Vesikari et al., 2006; Matson et al., 2014).

Our data indicate there is little rotavirus-associated disease in high-income communities with high vaccine coverage. While the incidence rate of wild-type rotavirus episodes by RT-PCR in the ORChID cohort was 10.3 episodes per 100 child-years, diarrhea associated with these strains in vaccinated infants was only 2.4 episodes per 100 child-years of observation. By comparison, a systematic review of studies before rotavirus vaccines were available estimated the incidence of rotavirus gastroenteritis for children aged <2-years was 24 episodes per 100 child-years (Bilcke et al., 2009). Thus, while rotavirus vaccination confers strong protection, subclinical and occasionally symptomatic infections, may still occur after vaccination. This mimics the natural history of rotavirus where primary infection confers protection against subsequent severe disease and with each new exposure the risk of further rotavirus infection and diarrhea is reduced, but not eliminated (Velázquez et al.,

1996). Nevertheless, there is a theoretical risk from diminished asymptomatic rotavirus circulation as reduced natural boosting could decrease population vaccine-induced immunity leading to outbreaks in older susceptible children. Of further interest in the present study was that symptoms were also found in 2.5% of RotaTeq episodes, showing that vaccine-related diarrhea is uncommon, which is consistent with safety data from large field trials where compared with placebo those receiving RotaTeq had a slightly increased rate of diarrhea (10.4% vs 6.7% respectively) in the week following the first vaccine dose (Dennehy et al., 2007).

Frequent rotavirus detections in asymptomatic infants during the first 8-months of life following RotaTeq vaccination has made interpreting PCR results in infants with diarrhea very difficult when assays do not distinguish between vaccine and wild-type rotavirus strains. This can lead to potential diagnostic delays and confusion in clinical practice (Forrest et al., 2017), while misclassification of cases risks underestimating effectiveness of vaccine programs (Tate et al., 2013). Additional complexity is provided when PCR detects wild-type rotavirus in asymptomatic children (Amar et al., 2007) and for up to 8-weeks after recovering from gastroenteritis (Richardson et al., 1998). Assay diagnostic specificity can be improved by incorporating vaccine-specific probes into multiplex qPCR assays to detect gastrointestinal pathogens (Gautam et al., 2014). Others recommend Ct values of 24-27 as a semi-quantitative measure of specimen viral load and marker of clinical significance (Phillips et al., 2009; Bennett et al., 2015). However, qPCR assay performance characteristics vary and, in this study, although the mean Ct value for wild-type symptomatic episodes of 22.3 was almost 10 cycles lower than asymptomatic wild-type episodes, we were unable to identify a Ct value cut-off for reliably distinguishing symptomatic and asymptomatic wild-type episodes. Moreover, there was no significant difference between the Ct values of RotaTeq symptomatic and asymptomatic episodes.

Rotavirus vaccines provide indirect protective effects on unvaccinated individuals (Lambert et al., 2009). As supported by our community-based study, this is attributed to herd protection from interrupted transmission of wild-type viruses. However, horizontal transmission of vaccine to unvaccinated contacts may also have a role since RotaTeq has been grown from stools collected in the first week post-vaccination (Vesikari et al., 2006), and transmission to siblings has occurred (Payne et al., 2010). Our frequent detection of

RotaTeq following vaccination indicates further studies of vaccine virus transmission using both culture and qPCR methods are needed to better understand this phenomenon.

The strengths of this single center study include its intensive (weekly) sampling of a relatively large number of infants. Approximately three-quarters of infants completed at least 1-year of follow-up, allowing us to comprehensively examine the impact of rotavirus vaccine and rotavirus epidemiological trends in our sample. However, it also has several limitations. First, we relied upon parents to collect samples and to complete the symptom diaries. Although it had excellent completion, given the size and complexity of the study, not all diaries and specimens were returned. Further, it is difficult to ascertain whether all samples were collected and handled appropriately, and if symptom diaries had accurate and complete documentation. Second, as stools swabs were collected weekly we may have under-estimated the duration of RotaTeq shedding post-vaccination. Third, while swabs were taken from stools in diapers this is likely to be similar to sampling from bulk stool specimens (Arvelo et al., 2013). Fourth, although used widely (Tate et al, 2013; Phillips et al., 2009), Ct values from RT-qPCR are a proxy measure of viral load and can be influenced by individual assay performance characteristics and stool fluid composition. Nevertheless, large differences in Ct values are likely to be real. Fifth, we restricted this analysis to rotavirus virus only, and other potential causative agents of diarrhea symptoms have not been investigated at this time, potentially over-estimating rotavirus-specific symptoms. Sixth, with absence of virus culture, we were unable to determine if we were measuring transmissible virus. Seventh, we did not seek to identify vaccine-derived reassortants in infants with either RotaTeq vaccine-associated symptoms or prolonged detection of RotaTeq in their stools (Donato et al., 2012). Finally, this study involved only infants receiving RotaTeq, which does not replicate as well in the gastrointestinal tract as Rotarix, the widely used single, live-attenuated, human-derived G1P[8] strain (Anderson, 2008). The limited data available however, suggest that Rotarix is shed in higher quantities and potentially for longer periods than RotaTeq (Hsieh et al., 2014; Forrest et al., 2017; Anderson, 2008). Hsieh and the colleague found that the mean Rotarix vaccine virus shedding load in vaccine recipients was $1.7x10^9$ genome copy number/g stool, and which was 100 fold-higher than RotaTeq vaccine viruses in RotaTeq vaccine recipients.

In conclusion, our community-based birth cohort study of healthy infants found RotaTeq nucleic acid was readily detected following vaccination, and detection following vaccination

was more prolonged than documented previously in clinical trials and post-licensure studies. This has implications for interpreting diagnostic tests in infants, an age-group commonly tested for rotavirus, and monitoring effectiveness of RotaTeq vaccine programs, while also highlighting the importance of better documentation and understanding of horizontal vaccine virus transmission. Nevertheless, in a setting with high RotaTeq coverage, symptomatic rotavirus disease was uncommon. Ongoing surveillance of circulating rotavirus genotypes remains important for understanding the different epidemiology of RotaTeq and wild-type rotavirus infections and monitoring the effectiveness of rotavirus vaccine programs.

4.5 Acknowledgement

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4.6 Supplementary methods

The real time reverse transcription polymerase chain reaction assay was run for 45 cycles. As with some other researchers in the field (Phillips et al., 2009; Tate et al., 2013), we did not use cycle threshold (Ct) values >40 as a diagnostic threshold in this study. This was because when detecting RotaTeq strains, two independent qRT-PCR assays were used, the NSP3 pan rotavirus screen (Pang et al., 2011)(and the RotaTeq VP6 vaccine assays (Gautam et al., 2014). Of the 85/1068 (8.0%) NSP3 positive samples with Ct values >40, two (2.4%) were confirmed as wildtype G12P[8] and 43 (51%) were confirmed as vaccine type by the RotaTeq VP6 assay: 22/43 (51.2%) had Ct values <40 in this latter VP6 assay. Thus we did not employ the traditional 40 cycle value since it would have led to a slight under-estimate of virus detection and shedding.

CHAPTER 5

Chapter 5 – Discussion and Conclusion

CHAPTER 5

5.1 Summary of findings

Diarrhoeal disease is one of the most common health problems affecting children worldwide, and rotavirus remains a major cause of severe diarrhoeal disease in young children (Tate et al., 2016). In the past 10 years, rotavirus gastroenteritis has decreased substantially in Australia following the introduction of universal rotavirus vaccination (Dey et al., 2012; Field et al., 2010). Despite the successes of rotavirus vaccination and active hospital surveillance, an intensive community-based cohort is required to better understand the post-vaccine epidemiology of rotavirus, especially its impact upon mild disease and asymptomatic circulation of the virus of which relatively little is known.

Rotavirus gastroenteritis diagnosis is heavily dependent upon the accuracy of laboratory methods. In chapter 2, I investigated and identified the basis for an unexplained increase in positive rotavirus testing results and was able to show that the specificity of a commercially available and widely used rotavirus ICT assay was seriously compromised. Indeed, 90.0% (27/30) of the ICT positive-samples were negative in every other assay applied. Furthermore, the performance of a wide range of commercial rotavirus detection methods, including ICT and ELISA assays, and in-house PCR methods were assessed to identify more appropriate methods for routine diagnosis of rotavirus infection in stool specimens. The assay sensitivities were found to range from 80% to 100%, while the specificities were 54.3% for the VIKIA ICT method, and 99.4% to 100% for the other assays. These studies translated into a change in routine rotavirus testing in the state of Queensland and subsequently led to the reformulation of the VIKIA commercial method.

In order to gather initial pilot information about gastrointestinal viral infections in the community in the rotavirus vaccine era, in chapter 3, I applied qPCR assays to assess six enteric viruses (rotavirus, norovirus, adenovirus, HCoV, HBoV-1 and enteroviruses) in the weekly stool samples (N=511) collected over a 2 year period from 5 healthy infants who were vaccinated fully against rotavirus and had fully participated in the ORChID study. The findings showed that wild-type rotavirus was not prevalent amongst these infants and that rotavirus vaccine strains accounted for the majority of the relatively few rotavirus detections (5/7 (71.4%) of rotavirus detections). Other viruses, particularly adenovirus, were more commonly found, and could be shed for up to 3 months without gastrointestinal symptoms. Asymptomatic shedding of one or more of the six viruses was frequently observed. These

data also highlighted the need for caution when using qPCR Ct values as semi-quantitative diagnostic thresholds to predict clinically meaningful viral detections. It also found that only 10.6% of virus-positive samples were associated with gastrointestinal symptoms, and this was a particular issue for adenoviruses and enterovirus.

Following on from chapter 3, my chapter 4 studies involved an extensive investigation of rotavirus genotypes and associated shedding in an unselected community-based birth cohort of Australian infants. To our knowledge this is the largest such study of its kind ever conducted. The notable findings from this study include that, following vaccination, almost all infants had RotaTeq vaccine virus detected on at least one occasion and this was generally unaccompanied by gastrointestinal symptoms with vaccine shedding associated with diarrhoeal symptoms in only 2.5% of such episodes. Prolonged shedding (up to 14 weeks) of rotavirus vaccine viruses was observed in healthy infants following vaccination. The incidence rate of wild-type rotavirus infections during the first 2 years of life in this highly-vaccinated cohort was 10.3 episodes per 100 child-years, of which only about one in four were symptomatic, and suggests that there is substantially reduced rotavirus-associated disease and virus circulation in the community. Our data also showed that symptomatic wild-type rotavirus detections, but not vaccine virus, were associated with higher viral loads. However we were unable to describe an optimal viral load threshold (based on semi-quantitative RT-qPCR Ct values) to differentiate between asymptomatic and symptomatic rotavirus detections, and therefore contradicts previous findings from other rotavirus studies. Overall, the findings from these investigations have important implications for both diagnosis and surveillance of rotavirus infection. Moreover the data highlight that ongoing surveillance of circulating rotavirus genotypes remains important for understanding the different epidemiology of RotaTeq and wild-type rotavirus infections as well as for monitoring the ongoing effectiveness of rotavirus vaccine programs.

CHAPTER 5

5.2 Strengths of these PhD studies

A comprehensive community-based study. The Australian Rotavirus Surveillance Programme was commenced in June 1999 to undertake the hospital-based surveillance and characterisation of rotavirus strains causing annual epidemics of severe diarrhoea in young children. It reports the strain diversity and temporal and geographical changes occurring each year, and has provided important data to inform vaccination strategies. However, the impact of rotavirus vaccines on the natural pattern of circulating rotavirus strains is unknown and difficult to predict, given the different strain contents of each vaccine (RotaTeq, a pentavalent human-bovine reassortant vaccine; and Rotarix, a single-strain human G1P[8] vaccine) (Kirkwood et al., 2009b). Furthermore, there is a lack of active surveillance of less severe cases, which are managed in the community. This PhD project was conducted as a part of a well-organised community-based study with weekly stool sampling, daily symptom diary recordings, and was specifically focused to investigate rotavirus infections and associated shedding in heathy infants (ie. a community rather than hospital-based population) over the first 2 years of life in the rotavirus vaccine era. Prior to this study, there have only been relatively few studies that have investigated the epidemiology of rotavirus infections in the community in the vaccine era, and those that have done so have been somewhat limited in their scope in terms of numbers of patients and associated samples. Therefore, our findings will be beneficial internationally, particularly to countries with similar socioeconomic structures and vaccine programmes, such as the United States, the UK, Europe, New Zealand and potentially high-income countries of East and South East Asia. Compared to high-income countries, rotavirus vaccine uptake is low in low and middle-income countries, including in Asia (Kirkwood & Steele, 2017). Therefore, our data may not directly reflect the scenario of rotavirus infections in these countries, but may still be informative as rotavirus vaccines are introduced progressively in these areas.

Important information to assess the impact of recent changes in vaccination. Since 2007, two types of rotavirus vaccines, RotaTeq and Rotarix, have been administered in different States and Territories in Australia. RotaTeq was administered in Victoria, South Australia, Western Australia (since May 2009) and Queensland, while Rotarix was used in New South Wales, Western Australia (until April 2009), Tasmania, the Northern Territory and the Australian Capital Territory. The unique geographical separation of different rotavirus vaccines implemented in the immunisation programmes made Australia an ideal

place for assessing the influence of rotavirus vaccine and the ongoing evaluation the vaccination programs (Macartney et al., 2009; Davey et al., 2015). However, from 1 July 2017, Rotarix replaced RotaTeq in four jurisdictions. This means all states, and territories will be using Rotarix under the National Immunisation Programme. Our genotyping data will therefore provide valuable information on rotaviruses circulating in the community so as to assess the downstream effects of changes in vaccination. The genotypes observed in our community study (from 2010 to 2014), and reported in chapter 4 were able to be compared to the genotypes in Queensland and nation-wide, which are represented in the rotavirus surveillance programme annual reports (Kirkwood et al., 2011; Kirkwood et al., 2014; Kirkwood & Roczo-Farkas, 2014; Kirkwood & Roczo-Farkas, 2015). Our study documented the changes in the circulating strains in the community which may vary from the strains recorded by hospital-based surveillance. For example, G12P[8] was found circulating in the community in 2011, whereas it was not observed by hospital-based surveillance in Queensland until 2012. It was the dominant community strain in our urban cohort in south East Queensland during 2012 to 2014, but only became the predominant strain a year later (from 2013) in the state-based hospital-based surveillance. Thus, while the genotypes seen in the community are typically consistent with the genotypes observed in Queensland rotavirus surveillance, the proportions of each genotype for each setting may vary.

New data to inform rotavirus diagnostics. Molecular detection methods have helped reduce the diagnostic gap in children with acute gastroenteritis (Amar et al., 2007; Steyer et al., 2016). Clinical virology is moving further towards molecular technology, whether this be qPCR or more recent next generation sequencing technology, for laboratory diagnosis of enteric viruses, including rotavirus. Current existing knowledge about rotavirus infection heavily relied on research conducted well before the recent developments in molecular diagnostics and, as described previously, much of this earlier work focused on severe disease associated with hospitalisation. This PhD project not only compared the performance of qPCR with traditional rotavirus detections methods (ELISA and ICT tests), but also investigated the qPCR detection-symptom associations in community based samples. The results clearly show that qPCR is more sensitive than antigen-based diagnostic methods, however the results also indicate that these gains in sensitivity may not necessarily be beneficial. These issues, including detection of low viral load and shedding of vaccine virus, are discussed further below.

CHAPTER 5

5.3 Issues related to conducting community based studies

This community-based birth cohort study does have some important limitations. Here, I will discuss the factors and problems that may have potentially impacted upon the performance of this study, and where possible highlight means of circumventing these in future investigations.

Subject selection

As is common with these types of studies conducted in high-income countries, families in the ORChID cohort were from more advantaged backgrounds and lived in an urban setting. Many infants in the study were first-born (67%) and came from high-income and highly educated families (Sarna et al., 2016). While this might seem to reduce their risk of acute gastrointestinal infections, a slightly higher proportion (80-85%) attended childcare by 15-24 months of age, compared to 74% in the general Australian population. It should be noted that our study was also more likely to recruit infants from families who engage well with Western healthcare. This creates a potential bias, whereby we were less likely to recruit families from non-English speaking or migrant groups, Indigenous people and also families with economic hardship who may have difficulty accessing healthcare. These groups are recognised as having a higher incidence of infectious diseases and vaccine preventable disease. Further research should be conducted to cover the families with lower socioeconomic status. Nevertheless, our findings for this cohort remain valid and provide important insights into rotavirus vaccine shedding kinetics and wild-type rotavirus exposures and diarrhoea in Australian infants in a subtropical urban setting.

Pre-laboratory issues

A notable element of this study was that it was reliant on parents collecting specimens and recording symptom data. As per the study design discussed earlier, each study family was required to: (1) collect weekly nappy swab samples and return them by surface mail to the research laboratory, (2) complete the daily symptom diary, including recording the daily number of vomits and/or number of loose stools, and (3) complete impact diaries, including any healthcare visits (hospitalisations, diagnostic investigations, use of antibiotics, missed childcare and parental time away from work or usual activities, during acute gastroenteritis episodes) (Lambert et al., 2012). Hence, the reliance on parents for all these critical aspects of the study for specimen was really quite unlike other community-based studies of

diarrhoeal illness where specimens were typically collected by trained healthcare workers upon clinic or home visits. While there are logistical advantages of this approach, which utilises limited research resources and avoids a Hawthorne effect, the obvious issue here is that the quality of the sample/data collection could have been compromised. Potential issues for the sample could include poor or inadequate sample collection (ie. too little or no stool on the swab), inappropriate storage, or even prolonged time between specimen collection and return to the laboratory. Additionally, it is possible that parents, on occasion, failed to record relevant clinical data or mistakes were made in recording the data. A good example of these potential issues relates to participant 86, who had relatively few observations (73/143 days) and participant 127 where no virus was observed (from 68 swabs) for most of the 2 year time period. While it is possible the data are correct, it is also possible that the data and sample collections were incomplete.

Unfortunately, there is probably no easy means of addressing these issues and the impact of human errors is likely to be unavoidable for these types of studies. There are several reasons for this situation. The first is that cost and feasibility are important things to consider when designing large longitudinal community-based cohort studies with frequent sample collection. Using healthcare workers to collect specimens would simply be cost prohibitive, particularly given we analysed in excess of 11,000 stool swabs in this study. Even if the funds could be found, these healthcare worker visits would impose considerable burden upon busy families and would likely see more families either opting not to join the study, or otherwise dropping out soon after. Healthcare worker collection could also introduce a potential new bias in the form of a Hawthorne effect (McCambridge et al., 2014) whereby frequent interactions with study staff lead to alteration of behaviour in participating families during the times they are being observed. Overall, in our setting it would not be logistically or economically possible to conduct a community-based study on this scale without using parent collected specimens and surface mail for returning to the laboratory. In our study, we tried to mitigate any potential issues with sample quality by contacting the participating families on occasion to remind them of the optimal sample collection methods. Perhaps in future studies, digital technology could be used to better manage sample collection and data recording. For example, while we used short message service (SMS) to text each participating family on a weekly basis to remind them to collect and mail samples into the laboratory, a phone app provided to each parent could be used to not only remind parents when study processes are needing to be performed, it could also be used for real-time

monitoring of parent activities. In any event, while we recognise that there are some limitations with the study, we do not believe that any potential issues related to a few individual subjects would likely impact upon the overall population–level study findings.

Laboratory aspects

In this thesis, qPCR was applied for detecting various viral agents, and was the main method used for detecting and characterising rotavirus in the broader community study. The correlation between the viral load and clinical disease severity has been demonstrated in several studies (Fuller et al., 2013; Buckingham et al., 200; Fodha et al., 2007) and was again explored in this study. Here we used the qPCR Ct value as a semi-quantitative measurement of viral load present in the sample. However, it needs to be recognised that there are other factors (ie. beyond viral load) that can affect qPCR results, and in particular can cause the Ct value to vary. These factors include inappropriate sample storage, transition, preparation and extraction of the sample, or even problems encountered in PCR cycling. To address this we applied various quality control procedures. For example, we maintained regular calibration and service of the PCR instruments, we performed QC checks of our primers and probes prior to performing a test, negative and positive controls were included in each test run, and we also used an EHV as an extraction control to assess extraction efficiency and to check for the presence of PCR inhibitors. Nevertheless, prior to extraction we observed a number of nappy swabs with a ‘clean’ appearance ie. not appearing to have any faecal matter on them. A lack of faeces on the nappy swab could of course impact on the detection of the viral targets, and therefore this was a potential concern. In previous studies, an ERV3, which is a marker of human genomic DNA and epithelial cells, has been tested to assess the quality of sample collection. In fact, we used this extensively for the respiratory samples collected from these same patients, and even used the data to facilitate an intervention for optimising sampling techniques in the birth cohort (Alsaleh et al., 2014b). However, unfortunately this approach did not prove useful for the stool samples. While we previously found ERV3 is a good quality indicator of sample collection for respiratory samples, it is not useful for faecal samples. Faeces contain undigested food residue, waste material from food, dead/living bacteria, protein, cellular linings, fats, salts, and substances released from the intestines (such as mucus) and the liver. Moreover, faeces from infants it contain surprisingly little human DNA. In fact, we found over 70% of nappy swabs tests tested negative for ERV3, and of these 11% were positive for rotavirus. Overall this shows that ERV3 is not a good indicator for the quality of nappy

swab collection, and moreover shows that our study really lacks any means of assessing sample quality. To address this potential limitation in future studies, alterative targets would need to be explored. Once potential approach could be to test for the presence of *E.coli* DNA or another similar bacterial stool organism.

Other limitations

In chapter 4, the strains of rotaviruses circulating in the community and their association with acute gastroenteritis was comprehensively assessed. However, while we are confident in the suitability of our RT-qPCR methods to detect rotavirus RNA in stools, there are several limitations associated with RT-qPCR detection;

(1) It is possible that the positive results may simply indicate an incidental infection, a subclinical infection, or prolonged shedding from a previous unrelated diarrhoeal episode rather than the cause of current symptoms. In addition to our chapter 4 findings, it has also been demonstrated by Kapusinszky (Kapusinszky et al., 2012) that there is almost constant shedding of various enteric viruses in healthy infants. Given the analysis performed in chapter 4 was limited to rotavirus only, other potential causative agents of diarrhoeal symptoms (such as other viral or bacterial agents) were not investigated in my study, potentially over-estimating rotavirus-specific symptoms. Further studies of other enteric pathogens on all the nappy specimens from the ORCHID cohort are in the pipeline;

(2) Our study demonstrated that there was an association between rotavirus viral loads (Ct values) with diarrhoeal symptoms, particularly for wild-type virus. However, only small numbers of wild-type-strain rotavirus-positive detections (n=43) were observed. This makes it difficult to validate a Ct value diagnostic threshold for reliably distinguishing between symptomatic and asymptomatic wild-type episodes;

(3) While I was able to provide information on wild-type rotavirus circulating in the community during the study period, the study lacked sufficient power to examine if there is vaccine pressure being exerted on wild-type strains in the community as compared to hospital-based surveillance.

(4) Additionally, in the absence of virus culture, I was unable to determine if we were measuring transmissible virus, or simply just detecting nonviable RNA for many of the

samples. Assessing virus viability would have been particularly interesting in terms of the prolonging shedding that was observed. Notably, this could tell us more about the potential transmissibility of the vaccine strains.

5.4 Future studies and comments

There are several studies that could now be conducted to help extend this work. These include:

Assessing rotavirus reassortment in the community. The fact that rotavirus strains (vaccine and wild-type) are shed for so long (several weeks) provides opportunities for the virus to mutate and with dual infection of cells to undergo reassortment. This could be mutation of an individual virus or even genetic exchange between different rotavirus types. We hypothesise that the risk of a reassortment event occurring may increase as the duration of vaccine virus shedding increases. Previous studies elsewhere indicate that reassortment is common (Bucardo et al., 2012; Rose et al., 2013; Roy et al., 2015; Bezerra et al., 2017). This is potentially important because any impact of the vaccine on the genetic composition and evolution of circulating viruses may pose new challenges to vaccine effectiveness and could also lead to new strains causing human disease. Building on from the rotavirus findings from the chapter 4, we actually conducted a feasibility study using next generation sequencing (NGS) to identify viral reassortment in the community samples (data no shown). NGS, which is also known as high-throughput sequencing, is a term used to describe several different modern sequencing technologies/ platforms including Illumina (Solexa) sequencing, Roche 454 sequencing, Ion torrent sequencing, and SOLiD sequencing. NGS is fast becoming a routine tool in clinical microbiology thanks to relatively simple benchtop technology and efficient library preparation protocols and, compared to Sanger sequencing, has significantly improved the capacity to perform low-cost, efficient whole genome sequencing (Kwong et al., 2015). NGS also allows a variety of pathogens (such as bacteria, virus, fungus, yeast, or parasite to be sequenced in parallel in one run (Motro et al., 2017). NGS amplifies all nucleic acids present in a sample, and without requiring primers for targeted amplification, and can generate whole genome sequence data for epidemiology study (Allcock et al., 2017). In order to address the questions over whether reassortment may be occurring in some of our prolonged vaccine strain shedders (up to 14-weeks

following vaccination), three RotaTeq-positive samples collected over a month after vaccination underwent NGS analysis. Unfortunately, the viral loads in the samples appeared to be below the detection limit of the NGS method, and we didn't detect rotavirus RNA in the samples. All of these samples had Ct values of approximately 30 cycles in the RT-PCR. For this reason, we then tried two additional samples, including a wild-type G12P[8] strain and a RotaTeq strain, with higher viral loads (Ct values around 25 cycles). These samples proved more successful. The sequencing analysis provided data consistent with the expected genotypes (G12P8 and a mixed population for the RotaTeq vaccine strains), however no evidence of recombination was observed. Further studies are needed to address this, but it is likely that the viral load in the faeces sample could prove a problem for current NGS technology. In this PhD project, rotavirus genotypes were determined from both a PCR-based genotyping method targeting the rotavirus VP4 and VP7 regions in combination with gel electrophoresis and Sanger sequencing. While this method proved useful to characterise the majority of positive samples, it lacks the resolution of NGS and therefore cannot be used to identify vaccine-derived or vaccine-wild-type reassortants, which may be present in infants with either RotaTeq vaccine-associated symptoms or prolonged detection of RotaTeq in their stools. It is likely that these issues will be resolved as NGS technology improves, and the technology becomes more suitable for direct application on clinical samples rather than just isolates. NGS is a promising tool that to be used in clinical diagnostics in the near future, but, for now, the technology is still too complex and expensive to replace current simple PCR methods for routine screening.

Ongoing diagnostic issues associated with PCR. Diarrhoeal disease is a non-specific illness that can be caused by a range of different enteropathogens (bacterial, protozoal, and viral). In order to identify these enteropathogens, several methods (including bacterial culture, immunosassay, microscopy, and PCR) are typically employed by pathology laboratories. These methods all vary in their performance characteristics, technical requirements, and turn-around time. Molecular-based diagnostics are being used increasingly for routine identification of gastrointestinal infections, especially as they conveniently fulfil several needs, including being relatively cost-effective, rapid, have high throughput capabilities, and also being able to quantify pathogen load. However, there are potential problems that can arise with a sudden switch to more sensitive test technology, such as is now occurring for rotavirus. As detailed in chapter 2, in 2012 concerns were raised in Queensland about the specificity of a widely used rotavirus ICT assay, following feedback from clinicians. In

chapter 2, I investigated this problem, and was able to demonstrate that two in-house PCR methods (as well as a range of antigen-based assays) were more appropriate methods in terms of performance characteristics for the diagnosis of rotavirus infection. As a result of these studies, PCR then replaced the previous ICT assay for routine rotavirus testing in the state of Queensland. Unfortunately this did not resolve all the concerns of local clinicians. Not long after PCR introduction, we received considerable clinical feedback that we were again frequently detecting rotavirus without clinical association of disease. While we were confident that we were indeed detecting rotavirus RNA, we became concerned that the PCR methods may be detecting subclinical, low load infections through their increased sensitivity. Based on our chapter 4 studies showing frequent shedding of vaccine virus, these concerns raised by clinicians were not surprising. Since detecting rotavirus RNA in stools may not be the cause of current symptoms, there was some suggestion from clinicians that the less sensitive antigen detection methods may correlate better with symptomatic episodes. However, given it appears that detection of vaccine virus is the main problem, we believe that it may be better to simply incorporate vaccine-specific probes into multiplex RT-qPCR assays to detect rotavirus. This way a clinician can indeed know if the infection is wild-type of not. Another option is to further explore the possibility of using Ct values to indicate rotavirus illness. Our findings from this PhD show that it is not possible to find a suitable Ct-based diagnostic threshold that neatly correlates with viral load and symptoms. However, rather than correlating with symptoms, it may be possible to simply identify a Ct value that correlates with ICT positivity. If we used this approach then there would be several limitations that clinicians would need to be made aware of, including that it may still be possible that a rotavirus infection could still be the cause of disease despite being below (ie. higher Ct value, but lower viral load) the threshold.

The above questions relating to rotavirus load and Ct-threshold values almost certainly apply to other enteric pathogens. Further studies, for example with larger sample sizes including more gastroenteritis cases and controls are required to ascertain the contribution of these to diarrhoeal disease.

Transmission of vaccine to unvaccinated contacts? Our study described that a high proportion (47%-87%) of infants had RotaTeq detected in their stools post-vaccination, and the vaccine viruses were detected in asymptomatic infants for several weeks (up to 14 weeks) afterwards. There is clearly a possible risk of horizontal transmission of vaccine to

unvaccinated contacts. It has been reported that the RotaTeq-vaccine virus can be associated with gastrointestinal symptoms in unvaccinated recipients following close contact (Hemming & Vesikari, 2014; Payne et al., 2010; Boom et al., 2012). Miura and team have monitored vaccine viruses spreading in a foster home setting, however found that there was no transmission of vaccine virus in such a close contact environment (Miura et al., 2016). Possible alternative investigations could be established in some large families or certain childcare settings where recently vaccinated infants and older children are being cared for in close contact environment. Ideally, such studies would include use of tissue culture and NGS in order to comprehensively ascertain transmission of viable virus. Further community studies will help to understand herd immunity and waning protective immunity.

5.5 Final comments

The changing landscape of rotavirus vaccine programmes and circulating strains requires continued and enhanced surveillance so as to better assess vaccine effectiveness. The current Australian rotavirus surveillance programme is hospital-based and only captures the more severe rotavirus infections where physician behaviour determines whether or not stool samples should be sent to the laboratory for rotavirus testing. Although our community-based longitudinal cohort extended our knowledge on what happens in the community, as indicated above the size of our study (158 participants) makes it impossible to answer questions such as if there is vaccine-related selective pressure. It has been suggested that protection of rotavirus vaccination may not be sustained in the second year of life, despite high vaccination coverage (Mohan et al., 2017). While this observation is limited to disadvantaged communities living in areas with a high rotavirus-disease burden where interference with oral polio vaccines, maternal antibodies, malnutrition, the intestinal microbiome and environmental enteropathy may all play a role (Cunliffe et al., 2016), waning protective immunity may also emerge in high-income communities with high vaccination rates for very different reasons. My study in chapter 4 raises this as a potential scenario as we saw limited wild-type rotavirus circulation in the infant cohort. As vaccination mimics the natural history of rotavirus infections and sustained protection may depend upon repeated rotavirus exposure and boosting of immunity, it is possible that over time there will be an increasing group of individuals susceptible to rotavirus infections. Indeed, rotavirus notifications in Queensland are increasing again across all age groups and clinician

feedback is of cases of severe rotavirus gastroenteritis in older, immunised children (Queensland Health Weekly Communicable Diseases Surveillance Report and Weekly Influenza Report, 2017). A key question to be explored is therefore whether the rotavirus vaccine protection is sustained as earlier studies from Europe (Vesikari et al., 2010) suggested or if after many years of a successful vaccine programme it begins to wane? Ongoing hospital and community-based surveillance of less severe cases will help to address this question properly and even suggest how it might be resolved.

5.6 Conclusions

In this PhD thesis, a problem related to rotavirus diagnostics was addressed and led to a change in pathology testing practice. Unlike pre-vaccine times, rotavirus was no longer the one of the most prevalent gastrointestinal pathogens in our cohort, and rotavirus vaccine strain shedding was very common and more prolonged than previously documented in clinical trials and post-licensure studies. Prolonged shedding of vaccine virus and increases in asymptomatic detections may be a potential problem for PCR diagnostics. The work also showed that symptomatic rotavirus detections were associated with higher viral loads compared to asymptomatic detections, but that (in the absence of methods that specifically distinguish vaccine from wild-type infection) may not necessarily resolve the problem of PCR diagnosis. Overall these data highlight that genotyping remains important tool to understand rotavirus prevalence and epidemiology in the vaccine era.

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