THE UNIVERSITY OF QUEENSLAND

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RESPIRATORY VIRUS INFECTIONS DURING THE FIRST YEAR OF LIFE

A LONGITUDINAL COMMUNITY-BASED DYNAMIC BIRTH COHORT STUDY

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Abstract

Background:

Viral acute respiratory infections (ARIs) are the commonest illnesses experienced by all age groups, especially in infants where infection rates are highest. Nevertheless, during the molecular era, outside of hospital-based studies, little is known about the current aetiology and community burden of viral ARIs in infants and young children.

The observational research in childhood infectious disease (ORChID) project is a prospective community-based birth-cohort study of healthy Australian infants and children. It began in 2010 to investigate respiratory virus infections until two-years of age. My PhD established laboratory quality control techniques for studies of this nature and describes the respiratory viruses and molecular epidemiology of human rhinoviruses (RV) during the first year of life in a nested subgroup of this cohort.

The hypotheses were:

(i) During the first year of life and in otherwise healthy infants, RV is the most commonly detected respiratory virus in respiratory secretions.

(ii) Various factors impact upon successful viral detection, including the ability of parents to collect appropriate samples and other laboratory-based technical issues.

(iii) Repeated detection of RV-RNA in respiratory secretions over periods of more than 4-weeks results from genotype replacement and new infection events rather than from prolonged shedding of the same genotype.

(iv) Many RV genotypes circulate in a single location in 1 year period.

Methods

The ongoing ORChID study completed sample collection at the end of 2014. Parents were approached antenatally and asked to collect weekly anterior nasal swabs from the time of their child's birth until their second birthday. Swabs were mailed to the laboratory where they were stored at -80^{o}C until analysis. Parents also completed a daily symptom diary, which was submitted monthly. My PhD focused on samples collected from an infant subgroup within this cohort and the first two-years of the ORChID study.

Samples were extracted using an automated robotic system after spiking each sample with equid herpes virus (EHV-1). The extraction quality and presence of human DNA in extracts were assessed by real-time PCR for EHV-1 and endogenous retrovirus 3 (ERV-3) respectively. Respiratory virus

PCR testing included: RV, influenza viruses (IFVs: A/B), parainfluenza viruses (PIV: 1-3), respiratory syncytial virus (RSV; A/B), human metapneumovirus (hMPV); human coronaviruses (hCoV; NL63, 229E, OC43 and HKU); human polyomaviruses (PyV: WU and KI), adenovirus (AdV), and human bocavirus (hBoV).

In a subset of 3366 nasal swab samples, the impact of ERV-3 load upon respiratory virus detection was determined. Mould was observed incidentally in some samples reaching the laboratory. The impact of different mould levels upon ERV-3 and respiratory virus detection was therefore investigated.

The influence of sequence variation upon target sequences was assessed for HAdV detection. Two new HAdV real-time PCR assays utilising combinations of degenerate oligonucleotides were tested in parallel with a previously designed and well established real-time PCR assay. ORChID (n=8800) and routine clinical (n=779) samples were then tested and the results compared.

The nature and shedding patterns of respiratory viruses, including the molecular epidemiology of RV was investigated in the nested infant cohort. Viral protein regions 4 and 2 were targeted to investigate RV-genotypes.

Simple descriptive statistics and regression models analysed associations and outcomes of interest.

Results

My nested subgroup of 72 infants provided 3446 swabs. Of these, RV (19.1%) had the highest detection rates followed by PyV-KIV (1.7%), hBoV (1.6%), AdV (1.1%), PyV-WUV (0.9%), RSV-A (0.6%), hCoV-OC43 (0.3%), PIV-3 (0.3%), hCoV-NL63 (0.3%), RSV-B (0.2%), hMPV (0.2%), PIV-1 (0.1%), IFV-A (0.09%), IFV-B (0.06%), hCoV-229E (0.06%), PIV-2 (0.03%) and hCoV-HKU1 (0.03 %).

Failure to detect ERV-3 was associated with 60% reduction in virus detection rates in nasal swabs. Mould was observed in 23% of samples and associated with delays in transportation, season and reduced ERV-3 and respiratory virus detection.

Degenerate oligonucleotides may overcome season-to-season variation in viral gene targets. Compared with the established assay, the new HAdV assays provided similar qualitative, but superior quantitative results.

Serial RV-detection for more than 3-4 weeks was from genotype replacement rather than prolonged shedding. Although detected in asymptomatic infants, an association was found between RV and respiratory symptoms, especially for the RV-C species.

Conclusions

Longitudinal studies help further understand respiratory virus detection, viral shedding and disease burden in the community. The quality of nasal swab collection and transportation can be monitored in real-time using the human DNA marker ERV-3. Gene target variation is a potential problem for longitudinal studies and was addressed successfully in HAdV real-time PCR assays by using combinations of degenerate oligonucleotides. This developmental work allowed me to show that in otherwise healthy Australian infants RVs were the dominant respiratory pathogens, followed by the DNA respiratory viruses. The apparent prolonged shedding of RVs over more than 3-4 weeks was from genotype replacement rather than persistent infection. RV-C appeared more pathogenic than the other RV-species and if confirmed this will help identify a viral target for future novel therapeutic and public health interventions.

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.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted *to qualify for the award of any* other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

- Alsaleh, A. N., Grimwood, K., Sloots, T. P., & Whiley, D. M. (2014). A retrospective performance evaluation of an adenovirus real-time PCR assay. Journal of medical virology, 86(5), 795-801. doi:10.1002/jmv.23844
- Alsaleh, A. N., Whiley, D. M., Bialasiewicz, S., Lambert, S. B., Ware, R. S., Nissen, M. D., Sloots, T. P., et al. (2014). Nasal swab samples and real-time polymerase chain reaction assays in community-based, longitudinal studies of respiratory viruses: the importance of sample integrity and quality control. BMC infectious diseases, 14(1), 15. doi:10.1186/1471-2334-14-15

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- Alsaleh AN. Patterns of viral respiratory infections during the first two years of life in a community cohort. Queensland Children Medical Research Institute. (August-2013). Format: Poster presentation.
- Alsaleh AN. The impact and epidemiology of respiratory viral infections during the first two years of life- An observational prospective community-based cohort study. Queensland Children Medical Research Institute. (December-2011). Format: Oral presentation
- Alsaleh AN. A preliminary investigation of PCR negative clinical respiratory samples using the Abbott Ibis system. Australian Society of Microbiology Annual Scientific Meeting and Exhibition. (July-2012). Brisbane. Format: Poster presentation
- Alsaleh AN. Improving quality assurance for PCR diagnostics: using adenovirus as an example. Queensland Children Medical Research Institute, Students expo (August-2012) –Format: Oral presentation.
- Alsaleh AN. Respiratory viruses during the first year of life. Queensland Children Medical Research Institute. (September-2012).Format: Oral presentation.
- Alsaleh AN. The ORChID study; an observational research in children infectious disease; quality issues. Capricornia Medical Science Association 5th Scientific conference. (June-2013) Rockhampton. Format: Oral presentation.
- Alsaleh AN. The Influence of Suboptimal Swab Sample Collection and Transport for Community-Based Studies of Respiratory Viruses. Australian Society of Microbiology Annual Scientific Meeting and Exhibition. (July-2013). Adelaide. Format: Poster presentation.
- Alsaleh AN. Molecular epidemiology of human rhinovirus during the first year of life. Queensland Children Medical Research Institute. (November-2013). Format: Oral presentation.

Publications included in this thesis

The publications are incorporated in this thesis as per UQ policy (PPL 4.60.07 Alternative Thesis Format Options) and the contributions of all authors to the publication, as required in the UQ Authorship Policy (PPL 4.20.04 Authorship), are described below

- Publication 1: incorporated as Chapter 4.

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Contributor	Statement of contribution
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Contributions by others to the thesis

My PhD was derived from the ORChID project an ongoing prospective, community-based dynamic birth cohort study, which was designed by: Keith Grimwood, Stephen Lambert, Robert Ware, Michael Nissen, Theo Sloots and David Wang. The initial laboratory set up for the ORChID project was done by myself (25%), Seweryn Bialasiewicz (25%), Jane Gaydon (20%) and Hannah Cox (30%). For the cohort samples presented in this thesis, approximately 30% of general respiratory virus screening and 70% of the RV screening was performed by me. All RV genotyping and all other experiments included in this thesis were performed by me. The data analyses reported in chapters 4 and 6 were performed under the supervision of Robert Ware and Stephen Lambert as specified in the publications section. The Results interpretation for chapter 6 was conducted with the assistance of Ian Mackay.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Community-based, longitudinal, birth-cohort, sequence variation, respiratory viruses, nasal swab quality, rhinovirus.

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FoR code: 1103 Clinical Sciences 20%

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Abbreviations

µl	Microliter
µM	Micromolar
5`UTR	Five prime untranslated region
AOM	Acute otitis media
ARIs	Acute respiratory infections
BAL	Bronchoalveolar lavage fluid
BHQ	Black hole quencher
CF	Cystic fibrosis
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
Ct	Cycle threshold value
dA	Adenine nucleotide
dC	Cytosine nucleotide
dG	Guanine nucleotide
DNA	Deoxyribonucleic acid
DNA-VDE	DNA-virus detection episode
dT	Thymine nucleotide
dY	Pyrimidine
EHV-1	Equid herpesvirus-1
EPR	Extract pool racks
ERV-3	Endogenous retrovirus-3
EV	Enterovirus
FAM	6-carboxyfluorescein dye
FRET	Forster resonance energy transfer
HAdV	Human adenovirus
HAdV-DE	Human adenovirus detection episode
hBoV	Human bocavirus
hBoV-DE	Human bocavirus detection episode
hCoV	Human coronavirus
hCoV-229E	Human coronavirus 229E
hCoV-NL63	Human coronavirus NL63
hCoV-NL63-DE	Human coronavirus NL63 detection episode

hCoV-OC43	Human coronavirus OC43
hMPV	Human metapneumovirus
hMPV-DE	Human metapneumovirus detection episode
HVR-7	High variable region 7
ID number	Identification number
IFV	Influenza virus
IFVA-DE, IFVB-DE	Influenza virus A or B detection episode
IQR	Inter quartile range
ITS	Internal transcribed spacer
KIV	KI polyomavirus
KIV-DE	KI polyomavirus detection episode
LLS	Liquid level sensing
LNA	Locked nucleic acid
LRTIs	Lower respiratory tract infections
mM	Millimolar
Mod-PCR	Modified PCR assay
NAATs	Nucleic acid amplification tests
NPA	Nasopharyngeal aspirates
nt	Nucleotide
NV	Novel viruses
OME	Otitis media with effusion
OR	Odds ratios
ORChID	Observational Research in Childhood Infectious Diseases
ORF	Open reading frame
PCR	Polymerase chain reaction
PIV	Parainfluenza virus
PIV1, 2 or 3-DE	Parainfluenza virus 1, 2 or 3 detection episode
PyV	Polyomavirus
QPID	Queensland paediatric infectious disease laboratory
RNA	Ribonucleic acid
RNA-VDE	RNA-virus detection episode
RSV	Respiratory syncytial virus
RSVA-DE, RSV-DE	Respiratory syncytial virus A or B detection episode
RV	Rhinovirus

RVA, B or C-TDE	Rhinovirus A,B or C typed detection episode
RV-DE	Rhinovirus detection episode
SID	Subject Identifier
SD	Standard deviation
TAMRA®	Tetramethylrhodamine
TDE	Typed detection episode
URTIs	Upper respiratory tract infections
VDE	Virus detection episode
VPg	Viral protein gene
VP1, 2 or 4	Viral protein 1, 2 or 4
VTM	Viral transport medium
WUV	WU polyomavirus
WUV-DE	WU polyomavirus detection episode

Chapter 1

Review of the literature

1.1 Background

Viral acute respiratory infections (ARIs) encompassing upper respiratory tract infections (URTIs) and lower respiratory tract infections (LRTIs) are the most common illnesses experienced by people of all ages. The highest rates of these illnesses occur during the first 24 months of life with annual episodes of 6-8 ARIs causing many complications and a high incidence of infant and child mortality worldwide (Monto, 2002). Indeed, for respiratory syncytial virus (RSV) alone, it is estimated that each year in children under five years of age there are 34 million episodes of LRTI, 3.4 million hospitalisations and as many as 199,000 deaths globally (Nair et al., 2010). Locally in Australia during 2003, almost half of those younger than five years of age were taken to their family doctor for ARI symptoms or were advised by a primary care health consultant (Lambert et al., 2008b; Lambert et al., 2007). Nearly 40% of new primary care consultations in children in the same age group are for respiratory illnesses (Bridges-Webb et al., 1993). Young children transmit respiratory viruses to their family and other household members (Peltola et al., 2008). In a population study of healthy Australian adults, the risk of household respiratory virus transmission was higher if a child lived in the house (McCaw et al., 2012). Complication rates are also higher in younger children. The risk of acute otitis media (AOM) complicating URTIs can be as high as 33%, while as many as 13% of URTIs can also be complicated by sinusitis (Revai et al., 2007). Viral LRTIs are responsible for hospitalising 3-5% of infants with bronchiolitis, pneumonia, croup or from secondary bacterial pneumonia (van Woensel et al., 2003).

Traditionally, the common viruses associated with childhood ARIs are RSV, parainfluenza viruses (PIVs), influenza viruses (IFVs) and adenoviruses (HAdVs) with human rhinoviruses (RVs) causing most URTIs (Lambert et al., 2008b; Monto, 2002). However, with the recent development of molecular-based detection techniques, such as polymerase chain reaction (PCR) assays, several new human viruses have been discovered in patients with respiratory symptoms since 2001. These include human metapneumovirus (hMPV), human bocavirus (hBoV), two novel human coronaviruses, (hCoV), hCov-NL63 and hCoV-HKU1, and two polyomaviruses (PyVs), WUV and KIV (Kahn, 2006; Lambert et al., 2008a; van der Hoek et al., 2006; Woo et al., 2006). Studies of RV revealed a new human rhinovirus C (RV-C) species after the complete coding sequence of a member of a divergent cluster of RV strains was reported in 2007 (McErlean et al., 2007)**.** RVs have also been identified as important causes of asthma exacerbations (Busse et al., 2010; Hayden, 2006; Saraya et al., 2014). Deployment of highly sensitive molecular assays has raised concerns over the co-detection of multiple respiratory viral pathogens in samples from both symptomatic patients and healthy controls, as well as the persistence of some viruses in respiratory secretions following a full clinical recovery (Fleming and Elliot, 2007; Hayden, 2006). Further questions

emerge when comparing hospital and community-based populations. Recently, PCR-based methods were used in community-based cohort studies to identify viruses in 69-83% of infants and young children with ARI (Lambert et al., 2007; Lambert et al., 2008). However, only about half of these children received medical attention during their illness and less than 2% were hospitalised (Kusel et al., 2006; Lambert et al., 2007).

Community-based studies have determined the incidence and prevalence of viral ARIs in early life. However, as outlined in subsequent sections many have important methodological limitations or have studied only a few agents. The Observational Research in Childhood Infectious Diseases (ORChID) study is an ongoing, prospective community-based, dynamic, longitudinal cohort study that began in 2010 to investigate the patterns of respiratory virus detections in healthy infants during their first two years of life. Parents collected weekly nasal swabs from their children and mailed them directly to the laboratory where they were comprehensively screened for 17 respiratory viruses, including six newly identified viruses. In addition, parents record a daily symptom diary for their child, which they return monthly to the ORChID project coordinator. The infants are followed from birth until their second birthday. This study will help to provide new insights into the nature of respiratory viruses infecting young Australian children, including their shedding characteristics and relationship with respiratory illnesses. My PhD thesis describes the respiratory viruses detected within a subgroup of infants nested within the ORChID cohort. It also focuses upon laboratory factors that may impact upon longitudinal community-based studies of respiratory viruses, including the importance of establishing laboratory quality control techniques, before finally describing the molecular epidemiology of RV during the first year of life.

1.2 Community-based studies

1.2.1 The importance of community-based cohort studies compared with hospital-based studies

Much more needs to be done to understand the contemporary causes of viral ARIs within the community. Most studies published recently have been confined to either hospital cohorts or closed populations (Arden et al., 2010; Lambert et al., 2007; Lambert et al., 2008; Sloots et al., 2006). In paediatric hospital-based studies, more than 80% of cases are younger than two years of age representing the sickest 2-3% of young children seen and are thus not representative of the general child population (Lambert et al., 2007) (Table 1.1).

Table 1.1 Distribution of viruses detected by RT-PCR in hospital and community-based studies in several regions

	% of respiratory virus detection in hospital-based studies in Australia	% of respiratory virus detection in previous community-based studies							
Virus	QPID (n =2866)† 0-97 yrs‡	UK (n=123) < 1 yr	Switzerland (n=112) < 1yr	Perth (n=976) < 1yr	Perth controls (n=456) < 1yr	Melbourne (n=543) 0-4 yrs	Netherlands symptomatic (n=165) 0-7 yr	Netherlands asymptomatic (n=65) 0-7 yr	Peru (n=3957)
RSV	11	27	15	11	5	7	2	0	3.3
IFV A,B or C									4.7
IFV-A and B	4		4	4.5	0	3.7	1	0	
PIV		13		5.2	0.9	4.1			5.5
PIV-1	1.1		3						
PIV-2	0.4		4						
PIV-3	2.8		11						
HAdV	6		3	1.5	0.4	3.1	1	0	5.5
hMPV	7		13	1.8	0.2	3.7	0	0	2.1
RVs	31	46	23	49	11	41	23	22	31.8
hCoV				5.5	4.4		9	8	
hCoV-229E	0.8	6.5	3				4	2	
hCoV-OC43	0.5	2.4	6				4	6	
hCoV-NL63	2.0		8			1.5	2	0	
hCoV-HKU1	0.8		1						
hBoV	5		5						
KIV	2.6								
WUV	4.5								
Enterovirus	< 1.0		1				4	3	
M. pneumoniae		2.4		1.4	2.6		0	0	
C. pneumoniae		2.4		1.3	1.1		1	5	
Co-detections	10	20	20	11	1.5	10	17	3	13.7
No pathogens detected	20	17	21	31	76				33.08
References	(McErlean et al., 2007)·(Arden et al., 2006; Sloots et al., 2006)· (Lambert et al., 2007)	(Legg et al., 2005)	(Regamey et al., 2008)	(Kusel et al., 2006)	(Kusel et al., 2006)	(Lambert et al., 2007)	(van der Zalm et al., 2009)	(van der Zalm et al., 2009)	(Budge et al., 2013)

† No. of hospital-based samples tested in the Queensland Paediatric Infectious Disease Laboratory-Brisbane. ‡ 78% aged less than five years.
HAdV: human adenovirus; **hBoV**: human bocavirus; **hCoV:** human coronavirus; **HMPV**: human metapneumovirus; **IFV**: influenza virus; **PIV**: parainfluenzavirus; **PyV:** polyomavirus; **RSV**: respiratory syncytial virus; **RV**: rhinovirus.

The accurate identification of ARI disease burden and it's causes in the community is important for developing public health interventions, such as vaccination programmes, and avoiding possible bias caused by overestimating the severity of a particular virus, especially during pandemics (Hayward et al., 2014). Recent community-based studies (Table 1.1) have further characterised the aetiology of viral ARIs using modern molecular diagnostic techniques.

In studies of infants at high risk of atopy (Kusel et al., 2006; Lemanske et al., 2005) or those attending day-care (Fairchok et al., 2010; Martin et al., 2013; Regamey et al., 2008), RV was identified as the most common respiratory virus associated with ARIs, a finding which needs to be confirmed in other studies recruiting healthy infants (van der Zalm et al., 2009). A recent study by van der Zalm and colleagues investigated the nature of frequent RV detections in 18 healthy children, younger than eight years of age (van der Zalm et al., 2011). Over a six month winter period, fortnightly nasal swab specimens were collected by parents and mailed to the research laboratory. RV was detected in 101/272 (37%) respiratory samples identifying 27 different RV subtypes. This longitudinal study confirmed the diversity of RV subtypes in the study population, and identified the importance in future studies of determining the pathogenic role of RV subtypes causing respiratory infections (van der Zalm et al., 2011).

Thus extrapolating data only from hospital-based studies leads to an underestimate of the causes of ARI and the overall disease burden. Even for IFVs, that represent one of the most studied groups of viral respiratory pathogens, the economic burden of infection have been estimated from assumptions or retrospectively collected data, usually from surveys, rather than from the results of community-based studies (Lambert et al., 2007; Lambert et al., 2008).

From two early cohort studies that relied, partially, on molecular methods, the prevalence of RV in asymptomatic infants was 11-26% (Copenhaver et al., 2004; Kusel et al., 2006). Discounting the potential for technical false positive results, the detection of viral sequences by PCR in immunocompetent, asymptomatic individuals could be explained by (i) mild or unrecognised symptoms of an actual symptomatic infection, (ii) nascent infection before symptoms develop, (iii) post infection shedding of residual viral genetic material, or (iv) genuine subclinical infection (Jartti et al., 2008; Peltola et al., 2008). The importance of PCR positive findings in asymptomatic children is yet to be clarified, as there are no large, comprehensive molecular-based studies intensely sampling cohorts of unselected, healthy children. Such studies are important when seeking to determine the relevance of viral concentrations in respiratory secretions before, during, and after the onset of respiratory symptoms.

Longer-term, community-based studies (e.g. exceeding one year) are important when determining the seasonal distribution and variation of respiratory viruses. Olofsson *et al* (2011) identified the

seasonal distribution of ten respiratory virus species from five different virus families by comparing several studies that used molecular techniques. In the Northern Hemisphere, both picornaviruses and HAdVs were detected all year round, as were IFVs in the Tropics. In contrast, RSV, IFVs, hMPV and hCoVs had noticeable seasonal peaks during the winter and early spring months in the temperate regions of both the Northern and Southern Hemispheres. Little information is available for seasonal patterns from the newly described hCoVs (NL63 and HKU1) and PyVs (WUV and KIV). Seasonality is strongly dependent upon geographic location, and with the limited data available on the patterns of respiratory infections occurring in communities from the Southern Hemisphere and tropical regions in particular, additional studies using molecular detection methods in these areas are needed (Olofsson et al., 2011).

1.2.2 Cohort studies performed to date and critique

The few published community-based studies in infants have important methodological limitations, such as using less sensitive approaches for viral detection (e.g. cell culture-based methods or serological testing) or reporting on a limited number of closely related or common agents (Budge et al., 2013; Legg et al., 2005; Manoha et al., 2007; van Woensel et al., 2003). For example, the epidemiology of RSV, hMPV and RV in children aged younger than three years was studied for two winter seasons in France (Manoha et al., 2007). Although the study found a significant difference in the prevalence of hMPV between the two seasons (10.1% and 3.3%), it excluded viral co-detections (Manoha et al., 2007). Another, more recent study, investigated ARIs in children younger than three years of age living in high altitude regions of the Andes mountains, but testing was limited to ten of the established respiratory viruses (Budge et al., 2013).

In addition to geographic diversity and seasonal variation, methodological differences can result in different outcomes for community cohort studies. For example, in five recent infant birth cohort studies (Table 1.2), no demographic data were available for one (Regamey et al., 2008), while two studies recruited only infants with atopic parents (Kusel et al., 2006; Legg et al., 2005), the fourth study recruited healthy urban infants (van der Zalm et al., 2009) while the fifth study recruited families with children under three years of age from rural communities in the Andes (Budge et al., 2013).

Table 1.2 Comparison between five community study designs of infant birth cohorts

Location (Ref). **Duration**	**UK** (Legg et al., 2005) **<1y**	**Perth** (Kusel et al., 2006) **<1y**	**Switzerland** (Regamey et al., 2008) **<1y**	**Netherlands** (van der Zalm et al., 2009) **<1y**	**Peru** (Budge et al., 2013) **Median follow-up 14.5 mths**
Similarities	• The four studies recruited small families with high socioeconomic status				• Families in rural agricultural areas.
Demographic data	At least one atopic, asthmatic parent	At least one atopic, asthmatic parent	Not reported	Healthy infants	Not provided
Timing of specimen collection	Onset of symptoms, done by study team	Within 48h of symptom onset, done by study team	At onset of symptoms (cough or wheeze), done by study team	On the 2nd day of illness, done by parents	During seven days of ARI symptoms.
Samples and collection	Nasal lavage	Nasopharyngeal aspirate	Nasal and throat swabs	Nasal and throat swabs	Nasal swabs
Viral shedding	Not reported	Not reported	Single sample collected 3 weeks later	Not reported	Not reported
Control samples	None	Two control samples (one in winter and another in summer, but only if no symptoms were recorded in previous four weeks)	None	None	None
Novel respiratory viruses.	Not tested	hMPV	hCoV-NL63 and HKU-1 hBoV and hMPV	hCoV-NL63 and hMPV	hMPV.
Bacterial agents	*C. pneumoniae* & *M. pneumoniae*	*C. pneumoniae* & *M. pneumoniae*	Not reported	*C. pneumoniae* & *M. pneumoniae*	Not reported

The first four studies had enrolled predominantly small families with a high socioeconomic status where subjects were more likely to be breast-fed and less likely to be exposed to tobacco smoke. The fifth study recruited families with distinct characteristics; families were labourers, mainly in agriculture living in small, apparently, crowded households (Budge et al., 2013). Different types of samples were collected in each study. Nasal lavage (Legg et al., 2005) and nasopharyngeal aspirates (NPA) (Kusel et al., 2006) were collected by research staff during an ARI in two of the studies, while in the remaining two combined nasal and throat swabs (Regamey et al., 2008) (Marieke M. van der Zalm et al., 2009) were collected by parents. For the fifth study, trained research staff collected nasal swabs when ARI were identified in child subjects (Budge et al., 2013). Control samples were collected in just one study. They were collected from subjects (one in winter and another in summer) when they were without respiratory symptoms for at least a preceding four week period (Kusel et al., 2006). Viral shedding was measured in another study, but this was from a single specimen collected three weeks after an ARI (Regamey et al., 2008). Limitations of all five studies included: none tested for all the newly discovered respiratory viruses, subjects were followed for no more than one year, except for the fifth study where subjects were followed for 28 months, and in one study observations and microbiological sampling were confined to just the first winter (Legg et al., 2005; van der Zalm et al., 2009) or the only first ARI with cough and wheeze (Regamey et al., 2008).

The overall incidence and prevalence of respiratory viruses in asymptomatic infants in these community-based studies cannot be determined as either samples were not collected at all or not collected routinely throughout the year. As only three of these studies recruited over several years (Budge et al., 2013; Kusel et al., 2006; van der Zalm et al., 2009) data on season-to-season variation are limited. In addition, the impact of newly discovered viruses on ARIs in the cohorts studied was not explored and the possibilities of new agents were also not investigated. Although different specimen collection methods were used across each of the five studies, all had similar detection rates for RV; and of these methods nasal swabs were the ones most preferred by parents (Heikkinen et al., 2002; Ipp et al., 2002; Regamey et al., 2008).

1.3 Respiratory viruses in early childhood

There is now considerable evidence to indicate a relationship between viral ARIs in the first two years of life and intermittent wheeze triggered by respiratory viruses in nearly one-third of preschool children (Arden et al., 2010; Carroll and Hartert, 2008; Jackson et al., 2008; Lambert et al., 2007; Lemanske et al., 2005). This relationship is underpinned by the occurrence of viral respiratory infections during the critical phase of alveolar and airway development. In addition, the

combination of these infections with allergic sensitisation and other environmental exposures (e.g. noxious gases, including tobacco smoke) may lead to airway injury and remodelling in genetically susceptible individuals and ultimately to asthma and even (non-smoking) chronic obstructive pulmonary disease (COPD) (Tregoning and Schwarze, 2010). Sly et al suggested that the contribution of respiratory viral infections in early childhood and atopic sensitisation may lead to asthma (Arbes et al., 2007; Oddy et al., 2002; Sly et al., 2008). *M. pneumoniae* and chlamydial respiratory infections have also been implicated in the aetiology of chronic pulmonary disorders, including asthma (Principi and Esposito, 2001; Webley et al., 2009). Taken together, ARIs in children result in enormous costs to the healthcare system, families and society (Lambert et al., 2008).

These infections are also responsible for several complications involving both the upper and lower respiratory tracts. A recent longitudinal cohort study showed that 61% of URTIs were complicated by either AOM (33%) or otitis media with effusion (OME) (24%) (Chonmaitree et al., 2008). The same study showed that 59% of tested specimens were positive for one or more respiratory viruses and that the most common viruses detected in OME were RSV and RV (Chonmaitree et al., 2008). Viral agents are also responsible for disrupting mucociliary clearance and damaging the ciliated epithelial cells that line the eustachian tubes, the latter being shorter, straighter and narrower in infants (Revai et al., 2007). Middle ear pressure is also reduced by eustachian tube dysfunction and this draws respiratory secretions, mucus and bacteria into the middle ear. These changes in the middle ear environment increase the chance of AOM after a viral ARI (Revai et al., 2007). Although complications with sinusitis are less common than OM, about 5-13% of viral ARIs are complicated by sinusitis (Alho, 2005; Brook and Journal, 2005; Steele, 2005). Substantial radiographic abnormalities in the para-nasal sinuses can be found early in the course of viral ARIs as many imaging studies in both adults and children have shown (Alho, 2005). RV, IFV, HAdV and PIV are the most common viruses associated with sinusitis (Brook and Journal, 2005). Although many of these viral infections resolve spontaneously, respiratory viruses may predispose to sinusitis by microbial synergy and inducing local inflammation that blocks the sinus ostia. This mechanism increases the chance of bacterial attachment to epithelial cells and disturbs local innate immune defence mechanisms (Alho, 2005; Brook and Journal, 2005).

LRTIs, which lead to 3-5% of infants being hospitalised, include bronchiolitis, pneumonia, croup and secondary bacterial pneumonia (van Woensel et al., 2003). Annually, 120,000 infants are hospitalised in the United States with bronchiolitis, the most common acute LRTI and the main cause of infant hospitalisations worldwide (Carroll and Hartert, 2008; Nair et al., 2010). Although many viruses are responsible for bronchiolitis, different viruses may act at different ages. For example, RSV infections that cause 60-90% of bronchiolitis episodes, are more common in younger

infants than the closely related hMPV (Calvo et al., 2010; Kahn, 2006). RSV, RV, hMPV, PIVs, hBoV and hCoVs are all known to be associated with wheezing in the very young, but RV seem to be the most important viral risk factor for subsequent preschool wheezing and diagnosis of asthma (Calışkan et al., 2013).

1.3.1 Aetiological agents

The established respiratory viruses include HAdVs, RSV, IFVs, PIVs and RV. These agents have been well studied previously and are known to affect all age groups with epidemics and outbreaks recorded annually worldwide for RSV and IFVs. In addition, the advent of molecular detection techniques has resulted in the discovery of several new respiratory viruses and provided new insights for established pathogens, especially RV. These are summarised below.

1.3.1.1 Human metapneumovirus (hMPV)

Using a randomly primed PCR technique, hMPV was first identified in 2001 from children with ARIs in the Netherlands. Further, the initial data indicated that hMPV was a common human respiratory pathogen. Screening of banked sera and parallel use of serological tests, based on both immunofluorescence and viral neutralization assays, suggested that hMPV was circulating in the human population and was not a mutated animal virus. Further, almost all individuals tested showed evidence of hMPV infection by five years of age (Kahn, 2006).

hMPV is an RNA virus of the family *Paramyxoviridae*, subfamily *Pneumovirinae* and is related to RSV. Based on phylogenetic analysis and genomic sequencing of five hMPV genes (N,M,F,G or L), two distinct lineages were subsequently identified for hMPV (A and B), with two serotypes for each (A1, A2, B1 and B2) (Kahn, 2006; Manoha et al., 2007; Williams et al., 2004). With no cross-protection between types, hMPV infections may recur on more than one occasion (Kahn, 2006).

A strong association between hMPV infection, wheezing exacerbations and hospitalisation has been found among children aged younger than three years. However, this association decreases in children older than three years of age (Busse et al., 2010). hMPV is closely related to RSV and is detected worldwide in 5-20% of young children with ARI or asthma exacerbations (Hayden, 2006). In a small study of 30 children with severe RSV bronchiolitis, 70% of cases were co-infected with hMPV (Greensill et al., 2003). In Queensland, Australia, of 10,125 respiratory samples collected between 2001-2004 from patients presenting to health facilities with symptoms of LRTIs (78% were children younger than five years of age) 7.1% had hMPV detected in the respiratory secretions and co-detection of hMPV with other respiratory viruses was only 6.8% (Sloots, 2006a). Diseases caused by hMPV are the same as those associated with RSV, with seasonal outbreaks (mainly in spring) and variation of severity from year to year. However, the peak of hMPV activity often

interferes with or follows the peak of RSV activity (Kahn, 2006; Williams et al., 2004). The most common LRTIs associated with hMPV are bronchiolitis and pneumonia. However, the severity of infections caused by hMPV is less than that observed with RSV in young children.

In the upper respiratory tract, hMPV is responsible for 5-15% of ARIs in children. In three studies of AOM, hMPV appeared to play a role in 6-50% of cases (Arden et al., 2010; Greensill et al., 2003). In contrast, hMPV is uncommonly detected in healthy children and adults (Williams et al., 2004). Taken together, these data confirm that hMPV is a major respiratory pathogen in children hospitalised because of ARI and is a predisposing factor for AOM. However, little is known about its overall impact in the community.

1.3.1.2 Human bocavirus (hBoV)

Since the original description of hBoV in 2005, the presence of this agent in children with ARIs and asthma exacerbations has been confirmed worldwide with most studies detecting hBoV DNA in 1.5-19% of those with symptoms of an ARI (Choi et al., 2008; Uršič et al., 2012). The virus was identified by "molecular virus screening", a procedure based on DNase treatment of nasopharyngeal specimens, random amplification and cloning, followed by large scale sequencing and bioinformatic analyses (Allander, 2008). hBoV has also been detected in faeces from children with gastroenteritis. More recently two further species have been identified, hBoV2 and hBoV3, in association with acute diarrhoea in children (Arthur et al., 2009).

Until the identification of hBoV, human parvovirus B19, the causative agent of fifth disease, had been the only human virus in the family *Parvoviridae*. The subfamily *Parvovirinea* includes two genera: *Bocavirus* and *Erythrovirus*. hBoV was included in the genus *Bocavirus* according to its similarity in genomic and amino acid sequences shared with other viruses in this genus. The members of the family *Parvoviridae* are small, nonenveloped viruses with isometric nucleocapsids, 18 – 26 nm in diameters, and include a single molecule of either linear, positive or negative sense, single-stranded DNA. The complete length of the genome for this family is between 4,000 to 6,000 nucleotides (nt). Although the complete genome length for hBoV has not yet been determined, 5,299 nt have already been identified in one of the reference strains (Choi et al., 2008; Schildgen et al., 2008). It is speculated that the genomic DNA of hBoV is flanked by hairpin structures as has been shown for other parvoviruses (Schildgen et al., 2008).

To date, detection of hBoV in clinical samples has been by PCR-based methods and no virus culture method, animal model of infection or antibody preparation for antigen detection is available currently. In addition, the optimal sampling site is yet to be identified, although the viral DNA may be readily detected in both respiratory and gastrointestinal specimens. For detection purposes, PCR primers usually target more conserved genetic regions and the limited variability of hBoV has

provided multiple targets, including NS1, which is the most preferred, followed by the NP1 gene (Schildgen et al., 2008). Using quantitative PCR, high loads of hBoV are often present when it is the sole agent detected in an acutely wheezing child, while low or moderate viral loads are often found in association with other respiratory viruses being present (Allander et al., 2007). In a study by Redshaw et al. (2007), the virus was detected in samples from eight of 230 (3.5%) New Zealand children hospitalised with bronchiolitis. No other agent was detected in five of these samples, while RSV was found in the remaining three (Redshaw et al., 2007). In Brisbane, 18% of 96 respiratory samples collected from children with ARI had hBoV detected (Tozer et al., 2009). Nevertheless, the role of this virus as a respiratory pathogen is still to be confirmed. In larger studies it was co-detected with other respiratory viruses in 40-60% of 225 cases and in 43% of 100 asymptomatic children (Longtin et al., 2008). Examining 1154 NPA specimens collected from children with ARIs and healthy controls, 10% of case samples provided positive results for hBoV, while 17% of healthy controls were also positive. Also, when hBoV was identified, at least one other pathogen was detected in 75% of cases. Other studies however support hBoV as a causative agent of ARIs because in high viral loads it is usually the only agent found and is accompanied by viraemia and serological evidence of primary infection (Christensen et al., 2010; Kantola et al., 2008). To help further confirm hBoV as a respiratory pathogen, the duration of viral shedding from the respiratory tract and how often it is present in asymptomatic controls needs to be determined systematically.

1.3.1.3 Human coronaviruses (hCoVs)

hCoVs are members of the subfamily *Coronavirus* of the *Coronoviridae* family, and are enveloped viruses with a large plus-strand RNA genome. Originally, two serologically distinct groups were identified, hCoV-229E and hCoV-OC43 in the 1960s and were considered responsible for only mild URTIs. However, a third group was identified earlier this century with the discovery of SARS-CoV as the aetiological agent for the severe acute respiratory syndrome (SARS) (Williams, 2007; Woo et al., 2009). Subsequently, two novel coronaviruses hCoV-Nl63 and hCoV-HKU1 were discovered (Fielding, 2011). Recently, a new hCoV was identified as the aetiological agent for Middle East respiratory syndrome (MERS), and is now termed MERS-CoV (Zaki et al., 2012).

hCoV-NL63 was first isolated in 2004 from a Dutch child with bronchiolitis and since its discovery, several studies have demonstrated it to be associated with both upper and lower respiratory symptoms. However, in a birth cohort study of 82 healthy infants followed throughout the first year of life, the virus was detected in six (7%) infants experiencing their first symptoms of a LRTI and in three of the six cases the virus was cleared within three weeks of symptom onset (Kaiser et al., 2005). The study also reported that it was the first ARI episode for two of the six hCoV-NL63 positive cases and in each instance it was the only agent detected, thereby supporting the role of

hCoV-NL63 as a respiratory pathogen. The seroprevalence of hCoV-NL63 in six to 12 month old infants is in the range of 29-40%, and by three and a half years the age 75% of children are seropositive (Dijkman and van der Hoek, 2009; Shao et al., 2007). Overall, in 2-9% of children the virus is associated with many different ARI illnesses, including the common cold, bronchiolitis, pneumonia and croup (Hayden, 2006).

The early studies on hCoV-NL63 reported seasonal variations (Fielding, 2011; van der Hoek et al., 2006). In contrast, in tropical climates the virus was detected throughout the year (Fielding, 2011). In one population-based study for children aged under three years with LRTI, it was found that winter was more likely to be the peak season for hCoV-NL63 (van der Hoek et al., 2010). The results also indicated an inter-epidemic period of two years (van der Hoek et al., 2010). In Queensland, 2% of 315 children hospitalised with ARI were found to have hCoV-NL63 and in 38% of these children, another virus was detected (Arden et al., 2006).

A second novel coronavirus, hCoV-HKU1, was isolated in 2005 from an adult with pneumonia (Woo et al., 2005). It too is distributed worldwide in both adults and children causing a range of respiratory symptoms and illness including, the common cold, wheezing, bronchiolitis and pneumonia (Kahn, 2006; Pyrc et al., 2007). The virus may also play a role in gastrointestinal disease as it was detected in stool samples from two patients with acute enteric symptoms (Pyrc et al., 2007). Two distinct groups (genotypes A and B) of hCoV-HKU1, and another (genotype C) the result of recombination events between genotype A and B viruses, circulate globally (Sloots et al., 2006; Woo et al., 2006, 2005). Isolates from Australia belong to genotype A (Sloots et al., 2006). In Brisbane hCoV-HKU1 was detected in 3% of 324 specimens collected from children presenting to hospital with severe ARI (Sloots et al., 2006). Co-detection of other viruses was reported in 11-43% of specimens containing hCoV RNA (Arden et al., 2006; Kuypers et al., 2007; Sloots et al., 2006). Overall, the newly discovered hCoVs appear to be more prevalent than other hCoVs, they are associated with more severe disease and appear to be acquired at an early age.

1.3.1.4 Human polyomaviruses (PyVs)

PyVs are small, double stranded circular DNA viruses that can infect several mammals and birds. Two human PyVs (JCV and BKV) are widely distributed in the human population and are thought to be acquired either via the respiratory tract or the oral-faecal route early in life (Oddy et al., 2002). They can cause persistent infection and have oncogenic potential in both animals and humans. Using “shotgun sequencing”, two novel PyVs were identified from respiratory secretions of hospitalised patients with ARI (Allander et al., 2007; Gaynor et al., 2007). Swedish scientists found KIV in 6/637 (0.9%) NPA specimens from patients with ARI; half of whom were younger than two years of age and in five of the six cases another respiratory virus was also present (Allander et al.,

2007). Shortly afterwards, WUV was first described following its detection in respiratory specimens from children hospitalised with severe ARI in St Louis (6/890; 0.7%) and Brisbane (37/1245; 3.0%) (Gaynor et al., 2007). Most cases were aged under three years with bronchiolitis, pneumonia and croup reflecting the patient population being tested. However, as with KIV, 72% of samples had other respiratory viruses detected. Follow-up studies from Brisbane and St Louis detected WUV in 128/2866 (4.5%) and 70/2637 (2.7%) in various respiratory specimens (Bialasiewicz et al., 2008, 2007; Gaynor et al., 2007). Global prevalence rates in young children with ARI are 0.5-2.7% for KIV and 0.4-7.1% for WUV, with 55-80% co-detection rates for other viruses and 0-6.4% in asymptomatic controls (Bialasiewicz et al., 2007; Gaynor et al., 2007; Lee et al., 2007). The high co-detection rates are similar to those found in hBoV and the role of all three viruses in ARIs is still uncertain (Bialasiewicz et al., 2008). WUV was found in a one day old infant delivered by caesarean section who developed respiratory distress with pulmonary infiltrates raising the possibility of vertical transmission (Le et al., 2007). A limited six month study (18 children, aged 0-7yrs), using fortnightly parent collected nasal/throat swabs detected WUV and KIV in 44% and 17% of children with and without respiratory symptoms respectively, and both viruses were shed for two-to-eight weeks (van der Zalm et al., 2008). The picture emerging is of human PyV infection early in life, followed by prolonged viral shedding, the clinical significance of which is uncertain, but requires a prospective, longitudinal study for confirmation.

1.3.1.5 Newly identified rhinoviruses, including novel RV-C/QPM

RVs are well recognised as the most common cause of ARIs, and amongst respiratory viruses, they are the most commonly detected viruses in respiratory secretions from asymptomatic individuals (Monto, 2002; Winther et al., 2006). They circulate year round with noticeable peaks in both spring and autumn. Although they affect all age groups, the highest rates of symptoms due to RV infections occur in the very young (Peltola et al., 2008). Previously, it was thought that they just caused the common cold and upper respiratory symptoms, but there is now an increasing recognition of a role of these viruses in LRTIs in infants, the elderly and the immunocompromised (Mackay, 2007). After RSV, they are the second most commonly detected respiratory viral agent in infants hospitalised with an ARI and the most frequent agents associated with ARI and wheezing illness. For the first three years of life, RV infections are more severe and are more likely to result in wheeze in those at high risk of developing asthma than are other respiratory viruses (Jackson et al., 2008). Moreover, RV-related illness may predict subsequent asthma development and it may play a major role in the "double hit" hypothesis of the aetiology of asthma (Jackson et al., 2008; Sly et al., 2008). Recently, a novel RV, RV-QPM, was discovered amongst a cluster of unusual picornavirus nt) sequences (McErlean et al., 2007). Overall, 17/1244 (1.4%) respiratory specimens

collected from patients in Brisbane presenting to a public hospital or to family doctors with ARI had RV-QPM RNA detected. This new genotype was closely associated with hospitalised bronchiolitis cases and appeared to be part of a novel RV clade, genogroup C. This was subsequent recognised as a third species RV-C being present worldwide and associated in some, but not all studies, with more severe disease (Denlinger et al., 2011; Khetsuriani et al., 2008; Mackay et al., 2008). This further emphasises the importance of RVs as a cause of ARI and reinforces suggestions that biological variations in RVs may also result in different clinical severity (Brownlee and Turner, 2008).

RV RNA can nonetheless be detected in 15-35% of asymptomatic individuals, but the clinical significance of these findings is unknown (Mackay et al., 2008; Peltola et al., 2008; Winther et al., 2006). Whether viral RNA detected weeks after a symptomatic illness is from persistent shedding or a subclinical infection from a newly acquired RV strain has yet to be fully resolved (Jartti et al., 2008). However, this is now achievable by sequencing of isolates from well characterised, serially collected specimens. Such data will better define the role of RVs when detected in asymptomatic individuals or with other viruses during an ARI.

1.4 Recent developments in respiratory virus detection

The four main approaches for detecting respiratory viruses are: virus culture, serology, immunofluorescence/antigen detection and nucleic acid amplification-based tests (NAATs). Virus culture, which is based on infecting cell lines with a clinical sample, and serology that relies upon blood samples for detecting viral specific antibodies and testing biological fluids or tissues for viral antigen, are both labour intensive and time consuming. For tissue culture, expertise is required for interpreting results, that may take up to ten days, and for serological methods seroconversion can take two weeks or more to develop (Beck and Henrickson, 2010; Tregoning and Schwarze, 2010). For many novel viruses traditional detection techniques are demanding. For example, limited cell types are suitable for culturing hMPV and adding trypsin is required for viral replication, a reagent which is not routinely used in viral diagnostic laboratories (Williams, 2007). While for the recently identified RV-C, cell culture was not feasible until 2011 by using a cell culture system based on propagation of RV-C in sinus mucosal organ culture (Bochkov et al., 2011); a method that is not practical for routine laboratories. Antigen detection relies upon detecting aetiological agents from various respiratory specimens by using monoclonal antibodies directed against specific viral target antigens. The viral antigens are detected using a conjugated enzyme or fluorescence (Tregoning and Schwarze, 2010). This method is relatively sensitive and rapid. However, the main difficulty limiting the comprehensive use of these techniques is that suitable monoclonal antibodies are only available for a limited number of targets, especially for novel respiratory agents (Fox, 2007).

Although antigen detection tests are still used widely, they are being replaced by the more rapid, sensitive and highly specific NAATs. Compared with tissue culture and serology, NAATs are able to provide results within two to 24 hours from the time of sample collection (Beck and Henrickson, 2010). Well optimised assays are more sensitive than culture by 5-10%, even with low viral concentrations (Mahony, 2010). Using genetic markers, NAATs are able to identify changes in virus characteristics, such as detecting antiviral resistance in IFVs and with the ability to multiplex assays by using different targets, these tests are superior to all other detection methods. The continuous development and enrichment of genetic information allows the rapid update of NAAT approaches (Beck and Henrickson, 2010).

Generally, NAATs rely upon three major steps: extracting the genetic material (DNA/RNA) from a clinical sample by releasing it from infected cells and virus components, amplifying the extracted genetic molecule and obtaining results by detecting the amplified genetic material (Mackay, 2007). Different techniques use different endpoints to detect the amplified genetic material (Mahony, 2010). In conventional polymerase chain reaction (PCR) assays, two primers are used to thermally amplify the genetic target and gel electrophoresis is employed to detect the amplified target. For real-time PCR, visualising the different phases of amplification is possible by either using a DNA intercalating fluorescent dye, such as SYBR® green or a fluorescent-labelled oligonucleotide probe (Beck and Henrickson, 2010). There are many types of probes available. However, TaqMan™ probes are preferred for respiratory virus detection, being more specific and less sensitive to possible mismatches in genome targets compared to other fluorescent dyes (Gunson et al., 2006).

Multiplexing NAATs allows the rapid detection of several viruses concurrently (Mahony, 2010). The principle of multiplexing relies upon using different sets of primers to amplify several gene targets, either from different pathogens or, as in the case of the novel virus KIV, amplifying different targets from the same pathogen (Bialasiewicz et al., 2007; Busse et al., 2010).

The original community-based cohort studies relied upon serology or cell culture-based methods. Kusel et al, applied the first comprehensive study using molecular based methods in 2006 (Kusel et al., 2006). Since that time the use of these techniques in epidemiological studies has been widely reported. However, PCR does still have some limitations and potential problems should be considered when using these techniques in long-term cohort studies that are conducted over several years. The major potential problem when using PCR in such studies is sequence variation in gene targets, which is considered one of the main challenges for respiratory virus screening (Mackay, 2007). For example, in a study investigating hMPV during different seasons (2000-2004), the rate of detection failure increased from 19% to 76% during the study period resulting in a significant decrease in prevalence estimates from 7% to 1.6% (Mackay, 2007; Mahony, 2010). For quantitative

PCR, sequence variation in primers targets can have a negative impact on the accuracy of the amplification and, as a result, underestimate viral loads. This is especially important when mismatches were located in the 3` end of a primer (Whiley and Sloots, 2005). For real-time PCR using fluorescence dyes, mismatches in the probe location may decrease the sensitivity of the assay (Whiley and Sloots, 2006). However, variation will not necessarily affect the performance. Over a period of 21 months, hBoV showed some genetic variation within two target genes, NP1 and NS1. Nevertheless, the performance of the amplification and overall prevalence was unaffected by this variation (Arnold et al., 2006).

1.4.1 Determining pathogenicity of novel agents in the molecular era

Koch's postulates provided traditional standards to identify the role of a new agent as a potential pathogen. However, it became necessary to modify these postulates as recent and rapid development in molecular detection techniques challenged orthodox views of viruses and their roles various infectious diseases. Fredreicks and Relman presented a modification in Koch's postulates to establish a relationship between a new agent and a disease. This may be occur if:

> *"" i) A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease. Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased (i.e., with anatomic, histologic, chemical, or clinical evidence of pathology) and not in those organs that lack pathology. (ii) Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease. (iii) With resolution of disease (for example, with clinically effective treatment), the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur. (iv) When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship. (v) The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms. When phenotypes (e.g., pathology, microbial morphology, and clinical features) are predicted by sequence-based phylogenetic relationships, the meaningfulness of the sequence is enhanced. (vi) Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located. (vii) These sequence-based forms of evidence for microbial causation should be reproducible".* (Fredreicks and Relman, 1996).

In terms of real-time PCR and for novel respiratory viruses other guidelines should also be considered. These were highlighted by Mackay et al and focused on defining the aspects of an infection in order to establish a relationship between the novel virus and each of the following: (i) the illness/symptoms, (ii) PCR results, and (iii) the presence of other respiratory viruses (Mackay, 2007).

Molecular methods played a significant role, not only with identifying and characterising new respiratory agents, but also in determining the impact of viral loads on disease severity. A strong association between high viral load and severe respiratory symptoms was indicated in a study testing 1154 samples for hBoV from children with ARIs and 162 control samples from asymptomatic children (Christensen et al., 2010). In this particular study hBoV was detected in 10% of patients and 17% of the control samples. In 75% of hBoV positive samples, another respiratory virus was detected. The study then categorised the hBoV positive samples into one of three categories; high (10^6-10^{10} copies/ml), moderate (10^4-10^6 copies/ml) and low viral loads (10^3-10^4 copies/ml). After adjusting for age, gender and other viruses, a high viral load was associated with LRTI (odds ratio 3.6, 95% confidence interval 1,2-10.7). Viraemia was found in 45% of these cases, and in none of the controls, and was confined to patients younger than two years of age (Christensen et al., 2010). Other studies have also found an association between high RV viral load and asthma exacerbation. A reduced type1 interferon response is thought to lead to high viral loads and delayed viral clearance in infants as the immune system in early life is skewed to a hyporesponsive phenotype. However, higher viral loads might also result in greater proinflammatory stimuli and this may explain the more severe symptoms (Tregoning and Schwarze, 2010).

1.4.2 Respiratory specimens and transport for pragmatic community-based studies

To avoid the methodological limitations of the previous studies, the ORChID study utilises real-time PCR to detect 17 respiratory viruses, including six recently described viruses. Parental weekly collection maximises respiratory episode capture and overcomes Hawthorne effects, when behaviour of research participants changes due to their awareness of being monitored or examined. This may influence compliance with specimen collection (Lambert et al., 2008a; van der Zalm et al., 2006). Weekly sample collections allow the dynamic relationships between; (i) pathogen acquisition, (ii) co-detection with other infectious agents, (iii) shedding of pathogen(s) in respiratory secretions, and (iv) symptoms of ARI to be examined systematically. However, there are some potential challenges with ensuring consistent quality of nasal swab collection and transportation.

Although combined nose and throat swabs were considered as alternatives, the traditional specimens for viral detection by PCR are NPAs and in certain circumstances bronchoalveolar lavage fluid (BAL) (Schuller et al., 2010). For community-based studies that rely on parental collection, simpler and more acceptable approaches are required to optimise cohort retention and regular specimen collection. Thus evaluating an alternative approach became necessary. Lambert et al compared PCR assay results for respiratory viruses from parent-collected combined nose and throat swab specimens with NPA samples collected by health care workers from 303 children presenting to hospital with respiratory symptoms (Lambert et al., 2008b). Samples were screened for eight respiratory viruses and both sampling techniques performed similarly with virus detection sensitivity levels of 91.9% and 93% respectively. Other studies indicated that nose swabs are well tolerated by subjects, easy to perform, and cause few problems for parents (Lambert et al., 2008b; Lambert et al., 2008; van der Zalm et al., 2006) Earlier studies have shown that using nasal swabs is the collection method preferred by most parents and will likely maximise cohort retention and specimen collection (Lambert et al., 2007). Importantly, studies are now emerging that show the sensitivity of PCR-based techniques for detecting respiratory viruses is not substantially compromised by using nasal swab specimens when compared with those obtained by NPA (Meerhoff et al., 2010; She et al., 2010; Waris et al., 2007) .

A study by O'Grady et al. explored the effect on respiratory virus detection by PCR of transporting nasal swabs from remote communities in Central Australia to a research laboratory in Brisbane, Queensland (O'Grady et al., 2011). Two anterior nasal swabs were collected from each study participant. The left nostril specimen was mailed to the laboratory by the routine postal services at ambient temperature, while the right nostril specimen was transported frozen. The extracts were then screened by real-time PCR for 16 respiratory viruses and similar results were obtained from the two groups, which confirmed that mailing unfrozen respiratory swabs over large distances within Australia did not adversely affect the PCR results.

Sampling regularly from individuals throughout the year helps to determine the influence of seasonal factors and also allows subjects to act as their own controls, helping to account for demographic features and factors such as family structure, environmental conditions and socioeconomic determinants. In the Melbourne community-based study, surveys indicated that approximately 90% of families were willing to continue the study for two more years even with using more invasive throat swabs (Lambert et al., 2008b).

Weekly parent collected nasal swabs are expected to increase the likelihood of obtaining samples during an ARI, especially for episodes that might be missed by the inconvenience of having to organise visits by research personnel (van der Zalm et al., 2006). It also allows the exploration of the temporal relationships between respiratory viruses and symptomatic illness, making this

particularly helpful when interpreting virus co-detections and their shedding in asymptomatic children.

1.5 Summary and development of hypotheses

Overall, relatively little is known about the nature and types of viral ARIs in otherwise healthy children whose early life respiratory illnesses are managed within the community. The need of more comprehensive community-based studies is essential in providing more data about the role of recently identified novel respiratory viruses as causes of ARIs. The high prevalence of RV in both symptomatic and asymptomatic children and the range of co-detections of various viruses and RV strains both raise many questions over pathogenicity and strain replacement. Some data suggest a significant association between the high loads of respiratory viruses and the severity of associated ARIs (See hBoV section). Therefore, the use and development of accurate real-time PCR assays is important in community-based studies. Little information is available on the shedding kinetics of respiratory viruses, although some have suggested for certain viruses it is for a period of up to four weeks after their first detection. Despite the recent and continuous developments in NAAT, many technical and logistical challenges remain when choosing to study respiratory viruses longitudinally in community settings.

1.5.1 Aims and objectives

The main aims of this thesis are to

1- Describe respiratory virus infections and ARIs during the first year of life in a subset of 72 healthy infants from the ORChID study,

2- Examine the effect of some technical issues upon respiratory virus detection during this longitudinal study, such as the quality of nasal swab samples collection and transportation and sequence variation in DNA viruses,

3- Describe the nature of any observed sequential RV infections.

1.5.2 Hypotheses

(i) During the first year of life and in otherwise healthy infants, RV is the most commonly detected respiratory virus in respiratory secretions.

(ii) Various factors impact upon successful viral detection, including the ability of parents to collect appropriate samples and other laboratory-based technical issues.

(iii) Repeated detection of RV-RNA in respiratory secretions over periods of more than four weeks results from strain replacement and new infection events rather than from prolonged shedding of the same genotype.

(iv) Many RV genotypes circulate in a single location in one year period.

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Chapter 2

The Observational Research in Childhood Infectious Diseases (ORChID) Study:

General methodology and laboratory protocols

2.1 Methods

The following provides an overview of the principal methods that underpin the ORChID study and form the basis of my PhD studies. These comprise sample collection, nucleic acid extraction, real-time PCR testing and data analysis. Note that additional procedures used during the course of my PhD studies, but not central to the main ORChID study are described in subsequent chapters where relevant.

2.1.1 Study Design and cohort

ORChID is an unselected, dynamic, community-based birth cohort study designed originally to determine the burden and aetiology of ARIs in healthy Australian infants during their first two years of life (Lambert et al., 2012). Parents were asked to record a daily symptom diary and to collect weekly nasal swab specimens from their infants. This began within the first few days of life and continued until they reached their second birthday. The symptom diaries were submitted monthly, while the weekly nasal swab specimens were mailed to the laboratory as soon as possible after their collection. The study was designed to avoid any unnecessary contact with the study team, in terms of sample and data collection, so as to minimise any potential bias due to a Hawthorne effect discussed in chapter-1; section 1.4.2). The study recruitment, cohort maintenance, ongoing oversight of specimen collection and transport, and recording of socio-demographic and clinical data were conducted by the QPID clinical trials staff as part of the ORChID project.

2.1.1.1 Antenatal recruitment

Recruitment of parents and infants was conducted by the study nurses at participating antenatal clinics in either the Royal Brisbane and Women's or the North Western Private Hospitals. The two hospitals are located in the north of Brisbane, a subtropical Australian city of more than 2.2 million people. Annually, each hospital has about 5,200 and 1,700 deliveries respectively. From September 2010 to October 2012, 165 healthy babies were recruited antenatally. The progressive recruitment plan was designed to enrol infants from throughout the year, so as to account for seasonal and year-to-year variation in respiratory virus activity.

Prior to enrollment, and during antenatal visits, a description of the study objectives and tasks was provided to parents. The Queensland Children's Hospital and Health Service, Royal Brisbane and Women's Hospital, and the University of Queensland Medical Research Ethics Committees approved the study.

2.1.1.2 Inclusion and exclusion criteria

Eligible infants were healthy full-term infants of English-speaking parents who anticipated living in the Brisbane metropolitan region for the next two-years. The full enrolment inclusion and exclusion criteria are summarised in Table 2.1.

Table 2.1 Enrolment inclusion and exclusion criteria

Inclusion criteria	**Definition and or explanation.**
Pregnant women expecting a healthy term baby	Gestational age >36 weeks, without evidence of an underlying congenital disorder.
Written informed consent	This is provided initially by the enrolled woman during pregnancy and is confirmed following the birth of her baby.
Sample collection agreement	Parents are expected to collect single weekly anterior nasal swabs (from both nostrils) from their study child and to return them to the QPID laboratory by regular mail using one of the self-addressed padded envelopes provided by the study team.
Sufficient English skills	This is needed to ensure the parents or guardians understand the tasks required of them during the study.
Exclusion criteria	
Premature babies	Gestational age ≤ 36 weeks
Neonates and infants with major congenital abnormalities or underlying chronic disorders	Including chronic heart, respiratory (excluding asthma), neurological or immunological disorders (congenital immunodeficiency, human immunodeficiency virus infection, or if receiving immune system suppressing medications, other than inhaled corticosteroids).
Planning to move from Brisbane during the course of the study.	

2.1.2 Sample and data collection

2.1.2.1 Initial visits

Once an enrolled baby was born, and while both mother and their newborn baby were still in hospital, an initial visit was undertaken by the study nurses. During this visit, parents provided written, informed consent to confirm their participation in the study.

The parents then received written instructions on the tasks they were being asked to perform (see below) as well as various study materials. At the same time they underwent instruction on how to collect nasal swab specimens from both anterior nares (Lambert et al., 2012). Baseline demographic and medical history details were also recorded at this visit. These included the infant's date of birth, gender, ascribed ethnicity, parental occupation and educational level, maternal obstetric history, family history of asthma and atopy, tobacco smoke exposure, breast feeding, medications,

immunisations, day care attendance, number of siblings, illness in other household members and household size.

The study nurses also collected an initial anterior nasal swab by demonstrating the optimal method of nasal swab collection. In addition, nasal swabs were collected from both parents during this visit. All swabs were delivered at ambient temperature by the study nurses to the QPID research laboratory immediately after collection where they were stored at -80ºC until the time of analysis.

2.1.2.2 Post discharge

Follow-up commenced once the mother and baby were discharged from hospital. Parents were asked to complete several tasks that included a daily symptom diary and weekly nasal swab collection and mail out. ARIs were defined '*a priori'* by the study investigators and parents were taught how to identify and differentiate between the various symptoms they recorded in the daily symptom diary. URTIs were identified as any episode of nasal discharge or congestion, or cough, without breathing difficulty, wheezing, or rattly breathing, while LRTIs were defined as any episode of breathing difficulty, wheeze or rattly chest (Kusel et al., 2006). Parents were instructed to identify breathing difficulty (chest wall retractions), wheeze (high pitched whistling sound from the chest in expiration) and rattly chest (wet, noisy breath sounds from the child's chest). For the purposes of demarcating ARIs, a new episode occurred only if there had been more than three symptom-free days since the end of the previous ARI episode (Lambert et al., 2007).

2.1.2.2.1 Cohort maintenance

Participants received text messages and phone calls from the ORChID project manager to remind them of their study tasks, to collect further data or to complete any missing data. Text messages were sent every week to remind parents of sample collection and mailing, while monthly text messages were sent as reminders for mailing in symptom diary cards. Every three months, phone calls were made to collect updates about immunisation status, breastfeeding, childcare attendance and changes in household composition. Additional phone calls could be made if the ORChID study manager encountered any gaps in the monthly diary card data. The timing for these additional phone calls was not fixed and to ensure the accuracy of the provided data, the calls were made shortly after the arrival of cards where gaps in data were observed. Biannually, a study newsletter was sent to the participating families either by email or by post in accordance to family preference. Every issue included information about the progress of the study, attaining important milestones and, where relevant, any changes in contact information.

2.1.2.2.2 Symptom diary card

As outlined previously, parents were provided a simple daily symptom diary card, which recorded the presence or absence of various respiratory symptoms (Appendix 2-1). The design of the diary card was based on one used successfully in a previous community-based study for a twelve month period in Melbourne preschool children (Lambert et al., 2007). Each group of symptoms was presented in a distinct colour to help differentiate between upper and lower respiratory symptoms. In addition to teaching parents how to distinguish between each symptom, they were also instructed how to complete the card using tick boxes and numbers. When the infant showed no symptoms, parents were asked to tick an additional box to also record the infant's wellbeing. Whenever fever was reported as a symptom for a specific day, the infant's axillary temperature was also recorded at that time. This was accomplished using a digital thermometer provided to each participating family, all of whom had been instructed on its use. The diary card was returned each month to the ORChID project manager and its contents double entered onto the customised study subject database.

2.1.2.2.3 Nasal swab collection

Parents were asked to collect a nasal swab from their infants on a weekly basis. The study nurses demonstrated the optimal method of collection to the parents during the initial visit and written instructions were also provided for the long-term sample collections. The plastic-shaft, rayon budded swab came with its own transport tube, which contained a foam pad reservoir soaked with viral transport medium (VTM) located in the base of the tube (Virocult MW950, Medical Wire & Equipment, Wiltshire, England). Rayon budded swabs complied with Australia Post regulations (see below) and were chosen following their successful deployment in other community-based studies (Lambert et al., 2007; O'Grady et al., 2011; Yin et al., 2011). After performing hand hygiene and ensuring the child was positioned comfortably and securely, parents placed and then rotated the sterile cotton swab against the internal anterior walls of one nostril before using the same swab to repeat the same procedure in the other nostril. When the child had a nasal discharge at the time of collection, this too was swabbed by the parent. The swab was then placed in the tube and the cap pushed down until the lid was sealed securely. The base of the tube was squeezed to release the VTM, which then bathed the swab. Some basic information including: the infant's name, date of birth, date and time of collection, who had taken the sample and any other comments were recorded on a collection form. This form and the accompanying nasal swab were placed first into a biohazard bag and then into a padded envelope before being sent as soon as possible by regular postal mail (in accordance with Australia Post regulations (Australia Post, 2009) at ambient temperature to the QPID research laboratory.

2.1.3 Sample processing and screening

2.1.3.1 Overview

Sample processing, screening and archiving were all located at the QPID laboratory. The QPID laboratory provided access to: (i) -80^{0}C freezers for long-term storage of swabs, processed samples and extracts; (ii) validated and purpose programmed automated extraction and liquid handling systems for high-throughput sample processing; and (iii) various conventional PCR and real-time PCR instruments for sample testing. Once a sample had arrived at the laboratory, a database, specially designed for swab lodgement, was used to enter the data provided in the collection form and the swabs were stored at -80°C until analysis (see section 2.1.8 of this chapter for databases and data management). Figure 2.1 illustrates the laboratory workflow for this part of my PhD project.

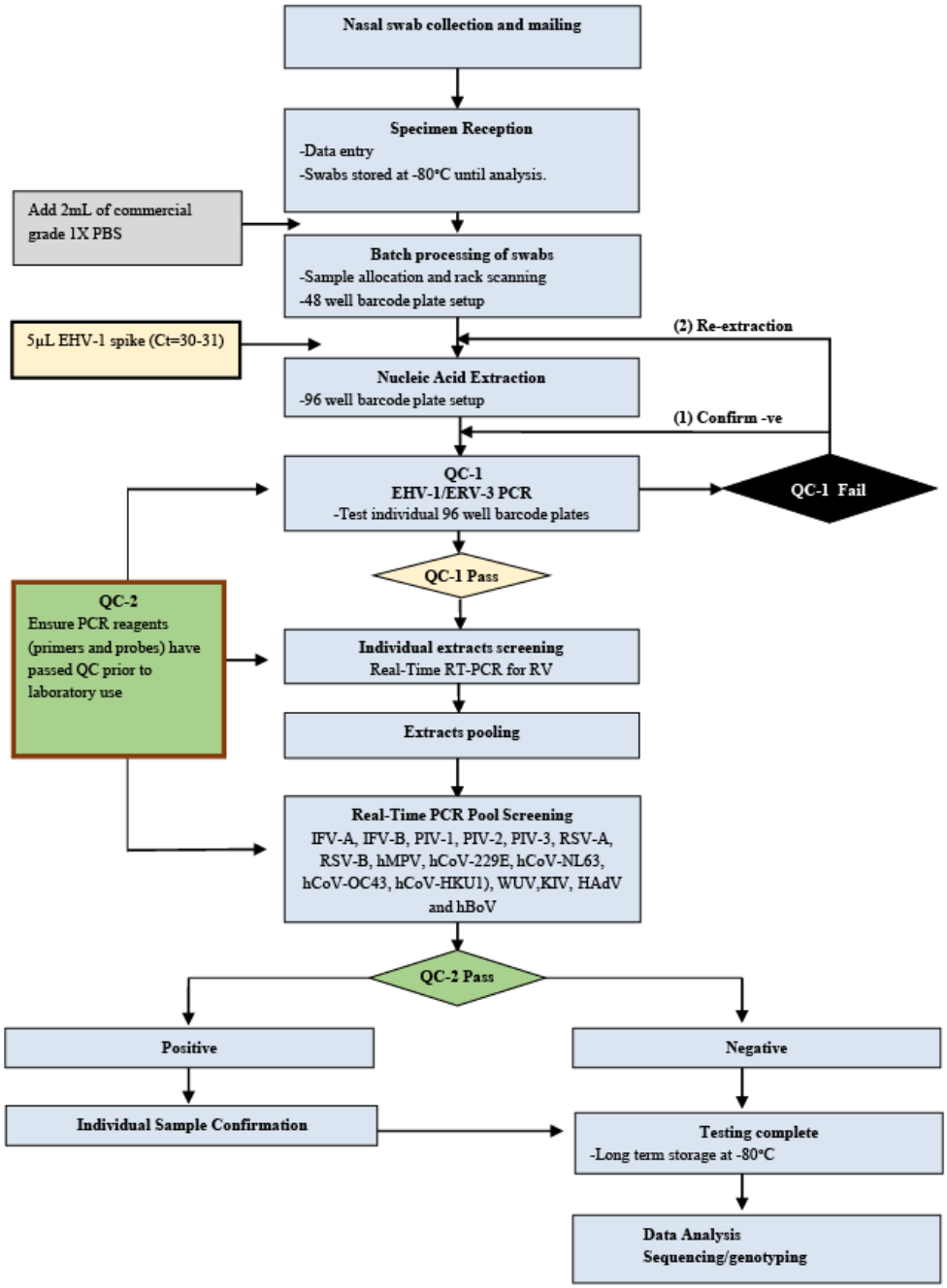

Figure 2.1 Workflow of ORChID study's respiratory specimens from collection by parents until data analysis.

2.1.3.2 Specimen processing

The swabs were processed in batches of 96, which included 92 nasal swabs and 4 negative controls. For each swab, 2mL of phosphate buffered saline (PBS) was added, the pad in the base of the swab's transfer tube was squeezed and the tube was vortexed for ten seconds. The suspension was then transferred to a 2mL tube (Sterile SmartScan tubes, Thermo Scientific, Australia) and the swab and its tube were discarded. The 2D barcoded tubes came in a 48-place rack format, which allowed for efficient sealing and long-term sample archiving at -80°C. All samples were handled in physical containment level 2 laboratory (PC2 laboratory). Samples were opened and aliquots were prepared in Class II Biological Safety Cabinet in accordance with IBC safety approvals.

2.1.3.3 Nucleic acid extraction

Nucleic acid extraction employed the QIAxtractor robotic extraction system (Qiagen, Australia), along with the genomic DNA extraction kit (DX, Qiagen, Australia). Each extraction run was performed with a single batch of specimens. To prepare an extraction run, 200µL of each sample was manually loaded into the 96-well lysis plates provided by the manufacturer and spiked with 5µL of an Equid herpesvirus-1 (EHV-1) culture that was normalised to produce a Ct value of approximately 30 cycles in the standard conditions of the ORChID study's real-time PCR. This served to detect the presence of inhibitors within the sample and to assess the quality of extraction. Purification was performed following the manufacturer's instructions. Briefly, fully automated lysing, binding, washing and elution steps, resulted in the allocation of specimen extracts into a 96-well 2D barcoded extraction storage rack (Matrix, Thermo Scientific, Australia). This semi-automated system allowed the purification of nucleic acid components without extensive manual interference and minimised human error when processing large numbers of samples. It also reduced the turnaround time, requiring only two hours per 96 specimen extractions instead of approximately eight hours required for manual extraction). After extracting the samples, the final elution volume of 150µL was checked visually to ensure that the elution step was consistent for each of the 96-wells.

2.1.3.4 Individual extract screening and pooling approach

To screen sample extracts, two approaches were applied, which depended upon the expected proportion of the pathogen of interest in the community. The first approach tested individual extracts in each real-time PCR assay. The second approach screened pooled sample extracts, which involved combining aliquots from ten extracts into a single tube and then testing that by PCR. The pooling approach is useful in maximising throughput, while also saving resources and minimising

costs. Such pooling strategies have been used successfully to screen for sexually transmitted pathogens (Currie et al., 2004; Johnson et al., 2002; Kacena et al., 1998), and more recently for respiratory viruses (Johnson et al., 2002; Van et al., 2012).

In the ORChID study, samples in each batch were screened individually to assess the quality of nucleic acid extraction. According to specific criteria (see section 2.1.4.2), extracts that passed the quality control test were then approved for pathogen testing. The first respiratory virus group to undergo screening were the human rhinoviruses (RV; figure-2.1). Here the extracts were screened individually because based upon the literature, and our experience at QPID, I anticipated RVs to be present in 18-30% of our community-based samples.

In contrast, the extracts were pooled for the remaining respiratory viruses. This was performed using the CAS1200 liquid handling system (Corbett Robotics, Australia), which is a compact robotic workstation for automated pipetting and pre-PCR liquid handling. During a pooling run, the special liquid level sensing (LLS) tips allowed the electronic detection of liquid levels in real-time. An initial calibration was required to adjust the dimensions of the 96-well 2D barcoded extraction rack (Matrix, Thermo Scientific, Australia) to the platform. The CAS1200 software was then programmed to pool 10µL aliquots from ten extracts both by row and by column. All pools were then added to an extract pools rack (EPR). This allowed each extract to be tested in the EPR twice; once via the pool for the corresponding row and another by pool for that extract's column (Appendix 2-2).

Respiratory virus real-time PCR testing (except for RV testing) was performed initially on an EPR. Once a pooled tube was found positive, the row or column in the corresponding EPR was subject to confirmatory testing. If two pools from a row and a column were found positive, only the tube located in the junction point would undergo confirmatory testing (Appendix 2-2).

2.1.4 Real-time PCR

Real-time PCR is a highly sensitive and specific technique for amplifying, detecting and quantifying targeted nucleic acid components. It is now used commonly for detecting respiratory viruses (Mackay, 2004). The principle of real-time PCR is similar to conventional PCR in terms of amplification, with the only difference being with how amplicons are detected. By combining the usual PCR reagents with fluorescent dyes or labelled oligonucleotides, this technique allows kinetic detection of the amplified target nucleic acids, decreasing both turnaround time and risk of cross-contamination, (Espy et al., 2006) which are critical factors for the high-throughput processing

required for this study. Several fluorescent dyes have been used for respiratory virus detection, however the most widely used involve dual-labelled probes (e.g. TaqMan™ probes). TaqMan™ probes have a linear structure specific to the targeted gene and contain a fluorescent dye molecule located in the 5` base and a quenching dye molecule on the 3` base. Through the Förster resonance energy transfer (FRET) mechanism, the close proximity between the two molecules allows a non-radiative energy transfer from the excited fluorescent dye, which acts as a donor, to the quenching dye, which acts as an acceptor. The separation of the two molecules occurs during the elongation step of the PCR reaction via the nuclease activity of the polymerase enzyme. This separation omits the FRET activity and allows the detection of the signal from the fluorescent dye and according to the rate of probe cleavage, the signal increases in each PCR cycle (Gunson et al., 2006; Mackay, 2007, 2004). In ORChID, TaqMan™ dual-labelled probes were used for all of the 17 real-time PCR assays. The basis of selecting the real-time PCR reaction mixes are described in appendix 2-3. Under standardised conditions, the real-time PCR runs were performed on several instruments located at the QPID laboratory and included; ABI7500 (Applied Biosystem, Australia), ViiA-7 (Applied Biosystem, Australia), LightCycler480 (Roche Applied Science, Australia) and Rotor-Gene 6000 and Rotor-Gene-Q (Qiagen, Australia). These instruments were used to increase the productivity during this dynamic study, which required screening large numbers of samples for many real-time PCR assays.

2.1.4.1 Singleplex and multiplex real-time PCR assays

Multiplex real-time PCR assays were also adopted to increase sample throughput and reduce the expense of sample screening. In this approach, several oligonucleotides that target different respiratory viruses were combined into a single reaction tube. The multiplex assays were designed and optimised previously. Five multiplex assays were employed to screen for 12 respiratory viruses (described in detail below). Additional, minor optimisation for each TaqMan™ probe used in these assays was undertaken to further enhance PCR performance when processing large numbers of samples or when used as multiplex assays. This involved choosing appropriate fluorophore dyes with distinct emission maxima to avoid crosstalk between channels in the multiplex real-time PCR assays. For the accompanying quencher, the Black Hole Quencher family (BHQ1-BHQ3) was used as an alternative to the more conventional tetramethylrhodamine dye (TAMRA®) (Heim et al., 2003; Matsuzaki et al., 2009; van Elden et al., 2003). This was because TAMRA® is unable to quench emissions that exceed 560nm. Also, using TAMRA® as a quencher prevents the designation of donor fluorophores at higher wavelengths, further limiting its role in multiplex real-time PCR assays.

2.1.4.2 Quality control of real-time PCR assays

Two tests were used to monitor the quality of sample extraction and collection: EHV-1 and endogenous retrovirus-3 (ERV-3) PCR assays respectively. EHV-1 was added to the samples prior to extraction, ERV-3 as an endogenous proviral DNA sequence located near to the centromere of chromosome 7 in every human cell (Lower et al., 1996; Shih et al., 1991) and its detection was used as an internal control to determine the presence of human DNA in each extract. It was utilised as a marker of sample quality indicating the successful extraction of nucleic acids from human epithelial cells and/or white blood cells present in the nasal swab specimen (Chin Yuan et al., 2001).

A duplex PCR was used to test for both targets, and included two TaqMan™ probe assays that target the nucleocapsid gene of the EHV-1 genome (Bialasiewicz et al., 2009) and the envelope gene in the ERV-3 genome (Chin Yuan et al., 2001) (see table 2.2 for oligonucleotides).

Table 2.2 Oligonucleotide sequences, reporting and quenching dyes and the target genes used in each real-time PCR assay for respiratory virus detection

Name	Sequence	Target in the genome	Type of assay	References
EHV-1-F[a] **EHV-1-R**[b] **EHV-1-TM**[c]	GATGACACTAGCGACTTCGA CAGGGCAGAAACCATAGACA **QUASAR670**-TTTCGCGTGCCTCCTCCAG-**BHQ3**	Nucleocapsid	Duplex	(Schuller et al., 2010)
ERV-3-F **ERV-3-R** **ERV-3-TM**	CATGGGAAGCAAGGGAACTAATG CCCAGCGAGCAATACAGAATTT **FAM**-TCTTCCCTCGAACCTGCACCATCAAGTCA-**BHQ1**	envelope		(Chin Yuan et al., 2001)
RV-F **RV-R** **RV-TM**	CY+AGCC+TGCGTGGY GAAACACGGACACCCAAAGTA **FAM**-TCCTCCGGCCCCTGAATGYGGC-**BHQ1**	5`UTR	Singleplex	(Arden and Mackay, 2010) (Khetsuriani et al., 2008)
IFV-A-F **IFV-A-R** **IFV-A-TM**	CTTCTAACCGAGGTCGAAACGTA GGTGACAGGATTGGTCTTGTCTTTA **QUASAR570**-TCAGGCCCCCTCAAAGCCGAG-**BHQ2**	Matrix	Duplex	(Whiley and Sloots, 2005)
IFV-B-F **IFV-B-R** **IFV-B-TM**	GCATCTTTTGTTTTTTATCCATTCC CACAATTGCCTACCTGCTTTCA **FAM**-TGCTAGTTCTGCTTTGCCTTCTCCATCTTCT-**BHQ1**	Matrix		(Whiley and Sloots, 2005)
PIV-1-F **PIV-1-R** **PIV-1-TM**	TTTAAACCCGGTAATTTCTCATACCT CCCCTTGTTCCTGCAGCTATT **FAM**-TGACATCAACGACAACAGGAAATCATGTTCTG-**BHQ1**	Hemagglutinin-neuraminidase	Multiplex	(Whiley and Sloots, 2006)
PIV-2-F **PIV-2-R** **PIV-2-TM**	AGAGTTCCAACATTCAATGAATCAGT CTCAAGAGAAATGTCATTCCCATCT **YAK**-CCTCTGTATTGCTCATGCATAGCACGGA-**BHQ1**	Nucleocapsid		(Whiley and Sloots, 2006)
PIV-3-F **PIV-3-R** **PIV-3-TM**	CGGTGACACAGTGGATCAGATT AGGTCATTTCTGCTAGTATTCATTGTTATT **QUASAR670**-TCAATCATGCGGTCTCAACAGAGCTTG-**BHQ3**	Nucleocapsid		(Whiley and Sloots, 2006)
RSV-A-F **RSV-A-R** **RSV-A-TM**	AGATCAACTTCTGTCATCCAGCAA TTCTGCACATCATAATTAGGAGTATCAAT **FAM**-CACCATCCAACGGAGCACAGGAGAT-**BHQ1**	Polymerase (L)	Duplex	(van Elden et al., 2003)
RSV-B-F **RSV-B-R** **RSV-B-TM**	AAGATGCAAATCATAAATTCACAGGA TGATATCCAGCATCTTTAAGTATCTTTATAGTG **YAK**-TATGTCC+AGG+TTAGGAAG+G+G+AA-**BHQ1**	Polymerase (L)		(van Elden et al., 2003)
hMPV-F **hMPV-R** **hMPV-TM**	CATAYAARCATGCTATATTAAAAGAGTCTCA CCTATYTCWGCAGCATATTTGTARTCAG **FAM-** CAACHGCAGTRACACCYTCATCATTRCA **-BHQ1**	Nucleocapsid	Singleplex	(Maertzdorf et al., 2004)

Table 2.2-Continued. Oligonucleotide sequences, reporting and quenching dyes and the target genes used in each real-time PCR assay for respiratory virus detection

Name	Sequence	Target in the genome	Type of assay	References
hCoV- 229E-F	CAGTCAAATGGGCTGATGCA	Nucleoprotein	Multiplex	(Dare et al., 2007; Schuller et al., 2010; van Elden et al., 2004).
hCoV- 229E-R	AAAGGGCTATAAAGAGAATAAGGTATTCT			
hCoV- 229E-TM	**FAM**-CCCTGACGACCACGTTGTGGTTCA-**BHQ1**			
hCoV- NL63-F	ACGTACTTCTATTATGAAGCATGATATTAA	1a		
hCoV- NL63-R	AGCAGATCTAATGTTATACTTAAAACTACG			
hCoV- NL63-TM	**YAK**-ATTGCCAAGGCTCCTAAACGTACAGGTGTT-**BHQ1**			
hCoV- OC43-F	CGATGAGGCTATTCCGACTAGGT	Nucleocapsid		
hCoV- OC43-R	CCTTCCTGAGCCTTCAATATAGTAACC			
hCoV- OC43-TM	**QUASAR670-** TCCGCCTGGCACGGTACTCCCT-**BHQ3**			
hCoV- HKU1-F	CCTTGCGAATGAATGTGCT	Replicase 1b	Singleplex	(Lambert et al., 2008; Schuller et al., 2010)
hCoV- HKU1-R	TTGCATCACCACTGCTAGTACCAC			
hCoV- HKU1-TM	**FAM**-TGTGTGGCGGTTGCTATTATGTTAAGCCTG-**BHQ1**			
WU-F	GCCGACAGCCGTTGGATATA	VP-2	Multiplex	(Dang et al., 2011)
WU-R	TTTCAGGCACAGCAAGCAAT			
WU-TM	**FAM**-AGGGTCACCATTTTTATTTCAGATGGGCA-**BHQ1**			
KIV-D-F	CACAGGTGGTTTTCTATAAATTTTGTACTT	Small T antigen		(Dang et al., 2011)
KIV-D-R	GAATGCATACATCCCACTGCTTC			
KIV-D-TM	**YAK**-TGCATTGGCATTCGTGATTGTAGCCA-**BHQ1**			
KIV-E-F	GAACTTCTACTGTCCTTGACACAGGTA	Regulatory region		(Dang et al., 2011)
KIV-E-R	GGATTAGAACTTACAGTCTTAGCATTTCAG			
KIV-E-TM	**QUASAR670**-TGGGAAACATCCGGTTTCCTCTCACTTCC-**BHQ3**			
hBoV-F	GGCAGAATTCAGCCATACTCAAA	VP-1	Singleplex	(Tozer et al., 2009)
hBoV-R	TCTGGGTTAGTGCAAACCATGA			
hBoV-TM	**QUASAR670**-AGAGTAGGACCACAGTCATCAGACACTGCTCC-**BHQ3**			
HAdV-TM1	TCGG**R**GTACCT**S**AG**T**CCGGGTCTGGTGCA	Hexon	Singleplex	(Alsaleh et al., 2013)
HAdV-TM2	TCGGAGTACCTGAG**C**CC**S**GG**K**CTGGTGCA			
HAdV-F1	GCC**S**CA**R**TGGGCATACATGCACATC			
HAdV-F2	GCC**G**CAGTGG**K**C**K**TACATGCACATC			
HAdV-F3	GCC**C**CAGTGG**K**C**K**TACATGCACATC			
HAdV-R1	GCCACTGTGGGGTTTCTAAA**Y**TT			
HAdV-R2	GCCAC**S**GTGGGGTT**Y**CTAAACTT			
HAdV-R3	GC**T**ACGGT**R**GG**A**TTTCTAAACTT			

[a] F:Forward primer **;** [b] R: Reverse primer **;** [c] TM: Taq-Man probe

Once an extraction run was completed, the batch of 96 sample extracts was screened individually (i.e. not pooled) with this duplex assay. The following criteria were applied to assess the quality of sample extraction. For EHV-1, the value of an extract's Ct in a specific batch should not exceed ±2 standard deviations from the average Ct value calculated for this batch. Results with Ct values beyond this range, or where EHV-1 was not detected, were considered extraction failures and this step was repeated. For ERV-3, positive or negative results indicated the presence or absence of human DNA respectively in that sample and was recorded in the database.

2.1.4.3 Human rhinovirus real-time reverse transcription (RT)-PCR assay

Samples that passed the quality control test were screened individually for RV using a comprehensive real-time RT-PCR assay developed in 2008 (Lu et al., 2008) The assay targeted the 5`untranslated region (5`UTR) in the RV genome, which contains the most highly conserved stretches of nt conservation among the genus *Enterovirus*. The primers were designed to amplify ~212 nt of the 5`UTR. The original assay had a relatively short forward primer of 14 nt that was stabilised using two locked nucleic acid (LNA™) nt; an Adenine (dA) and a Thymidine (dT) (Table 2.2 for oligonucleotides). Unlike the common nucleic acid analogues, the ribose ring in an LNA™ is locked with a methylene bridge linking the 2`-O atom and the 4`-C atom. This structure allows rapid annealing to a complementary strand, increasing the stability of the duplex, and by raising the melting temperature, it optimises the use of short oligonucleotides and balances the melting temperature between the two primers. The modification applied to the forward primer involved replacing the Cytosine nucleotide (dC) in the 3` end of the primer with a pYrimidine (Y) derivative, which is a degenerate base that mimics a dC/dT mix (Lu et al., 2008). The aim of this designation was to avoid possible mismatches with more recently characterised RV types. For the TaqMan probe, the 5` end was labelled with 6-carboxyfluorescein (FAM) as a reporter and a BHQ-1 molecule at the 3` end as a quencher. To perform real-time reverse transcription (RT)-PCR, a SensiFast No Rox one-step RT kit (Bioline, Australia) was used. The reaction mix consisted of a total reaction volume of 20.0µL, including 10.0µL of SensiFast No Rox one-step mix (Bioline-Australia) and 2.0µL of sample extract, 0.1µM of the TaqMan probe, 0.4µM of each of the forward and reverse primers and 2mM of $MgCl_2$. Amplification was performed under the following cycling conditions: a RT step at 45ºC for 20 minutes followed by an activation step of 94ºC for 2 minutes, then 55 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step.

2.1.4.4 Influenza A and B virus (IFV-A and IFV-B) real-time RT-PCR duplex assay

The testing for IFVs (A and B) involved screening EPRs and then confirming positive pools. For this purpose, a previously described and optimised real-time RT-PCR duplex assay was used (Lambert et al., 2008; Whiley and Sloots, 2005). For IFV-A, the assay comprised one forward and one reverse primer and a TaqMan™ probe that was labelled with Quasar 570 at the 5` end and BHQ2 as a quencher at the 3` end. This assay targeted a highly conserved sequence in the *M1* gene that encodes the matrix protein in the IFV-A (Neumann and Kawaoka, 2011). Similarly, the IFV-B assay comprised one forward and one reverse primer and a TaqMan™ probe, which was labelled with FAM as a reporter in the 5` end and BHQ-1 as a quencher at the 3` end. The IFV-B assay targeted the M2 gene, which encodes the integral membrane protein BM2 (Neumann and Kawaoka, 2011). The 20 µL reaction mix comprised: 10.0µL of SensiFast No Rox one-step mix (Bioline-Australia) and 2.0µL of sample extract, 0.4µM of each primer, 0.32µM of IFV-A TaqMan™ probe and 0. 16µM of IFV-B TaqMan™ probe. The amplification was performed under the following thermo cycling conditions: a RT step at 45ºC for 20 minutes followed by an activation step of 94ºC for 2 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using similar reaction and cycling conditions.

2.1.4.5 Parainfluenza virus (PIV) 1, 2 and 3 multiplex real-time RT-PCR

EPRs were screened for three PIVs. These were; PIV-1, PIV-2 and PIV-3. The multiplex, that was previously designed and optimised, (Lambert et al., 2008; Whiley and Sloots, 2006) comprised a single set of primers in addition to a single TaqMan™ probe for each of the three assays. For PIV-1, the assay amplified approximately 81 nt from the HN gene that encodes the Hemagglutinin-neuraminidase protein. The TaqMan™ probe for PIV-1 assay was labelled with FAM as a 5` reporter and BHQ1 as a 3`quencher. The PIV-2 assay targeted nearly 111 nucleotides of the NP gene that encodes the nucleocapsid protein. The TaqMan™ probe for PIV-2 assay was labelled with YAK fluorescent dye as a 5` reporter and BHQ1 as a 3`quencher. The PIV-3 PCR assay amplified approximately 104 nt of the NP gene, which similar to PIV-2 encodes the nucleocapsid protein. The TaqMan™ probe was labelled with Quasar670 fluorescent dye as a 5` reporter and BHQ3 as a 3`quencher. The 20µL multiplex reaction mix comprised the following: 10.0µL of SensiFast No Rox one-step mix (Bioline-Australia) and 2.0µL of sample extract, 0.4µM of each primer and 0.16µM of each TaqMan™ probe. The amplification was performed under the following thermo-cycling conditions: a RT step at 45ºC for 20 minutes followed by an activation step of 94ºC for

2 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using similar reaction and cycling conditions.

2.1.4.6 Human respiratory syncytial viruses (RSV; A and B) duplex real-time RT-PCR

Pools were screened for both RSV-A and RSV-B using a duplex assay that was slightly modified from its original version. The two assays were first developed and optimised in 2003, (van Elden et al., 2003) however the RSV-B TaqMan™ probe was replaced with a LNA probe to assist in developing the duplex. Similar to the previous multiplexes, a single set of primers and a single TaqMan™ probe were used in each assay. The TaqMan™ probes were labelled with FAM for RSV-A and YAK for RSV-B at the 5` ends, whilst BHQ-1 molecules were used as a quencher dye for both. The 20µL multiplex reaction mix comprised the following: 10.0µL of SensiFast No Rox one-step mix (Bioline-Australia) and 2.0µL of sample extract, 0.4µM of each primer and 0.16µM of each TaqMan™ probe. The amplification was performed under the following thermo-cycling conditions: a RT step at 45ºC for 20 minutes followed by an activation step of 94ºC for 2 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using similar reaction and cycling conditions.

2.1.4.7 Human metapneumovirus (hMPV) singleplex real-time RT-PCR

One of the most targeted genome regions for designing real-time RT-PCR assays for detecting hMPV is a well conserved region of 162 nt located within the *N* gene that encodes the nucleocapsid protein (Maertzdorf et al., 2004; Matsuzaki et al., 2009). However, even for this highly conserved sequence, there is a considerable difficulty in designing diagnostic assays for the comprehensive detection and quantification of different hMPV genotypes. Therefore, we optimised a previously developed assay that consisted of several degenerate bases to cover the high numbers of mutationswithin the N gene of different hMPV types (Matsuzaki et al., 2009). Pools were tested for hMPV using this singleplex assay in similar reaction compositions and cycling conditions. The 20µL singleplex reaction mix comprised the following: 10.0µL of SensiFast No Rox one-step mix (Bioline-Australia) and 2.0µL of sample extract, 0.4µM of each primer and 0.16µM of the TaqMan™ probe. The amplification was performed under the following thermo-cycling conditions:

a RT step at 45ºC for 20 minutes followed by an activation step of 94ºC for 2 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using the same reaction and cycling conditions.

2.1.4.8 Human coronaviruses (hCoV) 229E, NL63 and OC43 multiplex real-time RT-PCR

To test the pools for the three hCoVs 229E, NL63 and OC43, a previously developed and evaluated multiplex assay was used (Schuller et al., 2010). The original assays for both hCoV-229E and hCoV-OC43 were designed to amplify approximately 75 nt of the *N* gene that encodes the nucleocapsid protein in the two viruses, (van Elden et al., 2004) while for hCoV-NL63, the assay amplified 102 nt of the same gene (Dare et al., 2007). The multiplex, as in the previous multiplexes, used a single set of primers and a single TaqMan™ probe for each assay. The probes were labelled with FAM for hCoV-229E, YAK for hCoV-NL63 and Quasar670 for hCoV-OC43 as fluorescent dyes at the 5`ends, and BHQ1 for hCoV-229E and hCoV-NL63, and BHQ-3 for hCoV-OC43, as quenching dyes at the 3` ends. The 20µL multiplex reaction mix comprised the following: 10.0µL of SensiFast No Rox one-step mix (Bioline-Australia) and 2.0µL of sample extract, 0.4µM of each primer and 0.16µM of each TaqMan™ probe. The amplification was performed under the following thermo-cycling conditions: a RT step at 45ºC for 20 minutes followed by an activation step of 94ºC for 2 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using the same reaction and cycling conditions

2.1.4.9 hCoV HKU1 singleplex real-time RT-PCR

Pools were tested for the hCoV HKU1 using a singleplex assay that was designed and optimised previously (Dare et al., 2007; Lambert et al., 2008; Schuller et al., 2010). The assay targets approximately 76 nt of the Pp1a gene that encodes the replicase polyprotein 1a. A single set of primers was used in this assay along with a single TaqMan™ probe that was labelled with FAM as a fluorescent dye and BHQ1 as a quenching dye at the 5` and 3` ends respectively. The 20µL singleplex reaction mix comprised the following: 10.0µL of SensiFast No Rox one-step mix (Bioline-Australia) and 2.0µL of sample extract, 0.4µM of each primer and 0.16µM of the TaqMan™ probe. The amplification was performed under the following thermo-cycling conditions:

a RT step at 45ºC for 20 minutes followed by an activation step of 94ºC for 2 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using the same reaction and cycling conditions.

2.1.4.10 Human polyomaviruses (PyV) WU and KI real-time PCR

Pools were tested for the two novel human PyVs WU and KI (WUV and KIV) using a multiplex of three previously described and evaluated real-time PCR assays (Bialasiewicz et al., 2007; Dang et al., 2011; Payungporn et al., 2008). WUV real-time PCR assay targeted approximately 71 nt of the VP2 gene that encodes a nucleocapsid protein and was comprised of a single set of primers and a single TaqMan™ probe that was labelled with FAM and BHQ1. While for KIV the lack of sequence data for this virus meant that two real-time PCR assays were used. Each of these assays was specific for a different sequence in the genome. The first real-time PCR assay amplified 82 nt of the small T antigen gene. This assay comprised a single set of primers and a TaqMan™ probe that was labelled with YAK and BHQ1 (KIV-D real-time PCR assay in table 2.2). The second KIV assay was designed to amplify 72 nt located in the regulatory region and, similarly, it comprised a single set of primers and a TaqMan™ probe labelled with Quasar670 and BHQ3 (KIV-E real-time PCR assay in table 2.2). The 20µL multiplex reaction mix comprised the following: 10.0µL of SesiMix™ II Probe Mix (Bioline-Australia) and 2.0µL of sample extract, 0.4µM of each primer and 0.16µM of each TaqMan™ probe. The amplification was performed under the following thermo-cycling conditions: an activation step of 94ºC for 10 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using the same reaction and cycling conditions.

2.1.4.11 Human bocavirus (hBoV) real-time PCR

A singleplex assay was used to screen the pools for hBoV. Similar to the previous assays, the hBoV real-time PCR assay comprised a single set of primers, that amplified 96 nt of the VP1 gene, and a TaqMan™ probe that was labelled with FAM and BHQ1 (Tozer et al., 2009). The 20µL singleplex reaction mix comprised the following: 10.0µL of SesiMix™ II Probe Mix (Bioline-Australia) and 2.0µL of sample extract, 0.4µM of each primer and 0.16µM of each TaqMan™ probe. The amplification was performed under the following thermo-cycling conditions: an activation step of

94ºC for 10 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using the same reaction and cycling conditions.

2.1.4.12 Human adenovirus (HAdV) real-time PCR

Pools were tested for HAdV using a singleplex real-time PCR assay, which was designed and evaluated specially for this study (Alsaleh et al., 2013). This assay comprised a set of three forward primers, three reverse primers and a set of two TaqMan™ probes. The oligonucleotide sequences (forward and reverse primers and TaqMan™ probe) were identical to a previously described and widely used real-time PCR assay (Heim et al., 2003). However, several degenerate bases were incorporated at specific positions to accommodate sequence mismatches. The two TaqMan™ probes were labelled with FAM and BHQ1. The 20µL reaction mix consisted of: 10.0µL of SesiMix™ II Probe Mix (Bioline-Australia) and 2.0µL of sample extract, 0.13 µM of each of the three forward primers (mod1-F1, F2 and F3; Table 2.2), 0.13 µM of each of the three reverse primers (mod1-R1, R2, and R3; Table 2.2), and 0.16µM of each probe (Mod1-P1 and P2; Table 2.2). The amplification was performed under the following thermo-cycling conditions: an activation step of 94ºC for 10 minutes, then 45 cycles of (1) a denaturation step at 94 ºC for 15 seconds and (2) annealing and elongation steps at 60ºC for 60 seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step. Once a pool tube provided a positive result (as described previously), the confirmatory testing was performed on the individual samples using the same reaction and cycling conditions.

2.1.5 Real-time PCR quality control approaches

To ensure the quality of real-time PCR data, strict quality control measures were applied throughout the study. One of the main aspects of quality control was to standardise all real-time PCR reaction components to maximise reproducibility and to minimise variability, each of which negatively impact upon the generated data.

2.1.5.1 Standardised positive control

All positive controls for the respiratory viruses of interest were prepared by the study team in the QPID laboratory. Briefly, nucleic acids from a previously archival respiratory virus culture or positive sample of interest were extracted using the Roche High Pure Viral Nucleic Acid extraction kit (Roche, Australia) following the manufacturer's instructions. The extract was then tested by

real-time PCR assays. Depending on the Ct values obtained, the extract was diluted using Baxter water to obtain a Ct value of 20-22 cycles noting that a ten-fold dilution increases the Ct value by one Log (approximately 3.3 cycles). The diluted extract was tested under standard conditions to further ensure the optimal concentration was obtained. Positive controls were stored in labelled 50µL aliquots and placed in a -80ºC freezer for long-term storage. No pooled positive controls were used in this study.

2.1.5.2 Quality control of real-time PCR reagents

Real-time PCR reagents, including newly purchased reaction kits and oligonucleotides, were tested under standard conditions before being used to test study samples. This was to address lot-to-lot variation amongst the reagents. Newly arrived oligonucleotides were first dissolved in RNase/DNase free water to stock concentration solutions of 200 µM for primers and 100µM for probes. New oligonucleotides were then run in parallel with old oligonucleotides in singleplex reactions to examine their sensitivity and performance. For a standard singleplex, three new oligonucleotides (one each of a forward and a reverse primer and a Taqman™ probe) were each optimised separately. All runs were performed under standard conditions and using the corresponding positive controls.

2.1.6 Rhinovirus genotyping

Samples that provided positive results for RV were subjected to genotyping using the sequences obtained from a viral protein (VP) or 5`UTR targets. The variable region VP4/VP2 genes was amplified using a nested PCR assay that comprised two sets of primers as previously described (Wisdom et al., 2009). Samples that could not be amplified twice by the VP4/VP2 assay, were further investigated by amplifying a 390nt from the 5`UTR (Gama et al., 1989). PCR products from either VP4/VP2 or 5`UTR were purified using the QIAquick PCR purification kit (Qiagen, Australia) and then submitted for DNA sequencing to the Australian Genome Research Facility (The University of Queensland, Brisbane, Australia). Sequence data were analysed using Geneious version 5.5.7 (Biomatters Ltd) and blasted against Genbank. To assign a sequence to a specific subtype, VP4/VP2 sequences should share ≥ 90% nt identity while 5`UTR sequences should share ≥ 96% nt identity (Gama et al., 1989; Wisdom et al., 2009). Phylogenetic trees were constructed using MEGA version 5.2 and parameters are further discussed in chapter-6 (Tamura et al., 2011).

2.1.7 Real-time PCR results interpretation

The interpretation of real-time PCR results relied upon numerical Ct values. The Ct value is defined as the cycle number at which a fluorescent signal exceeds an arbitrarily defined signal threshold. The Ct value fort each cycle is proportional to the accumulated level of PCR products. It can also be used as an indirect and approximate measure of the initial concentration of the gene target in a reaction (i.e. target load) (Mackay, 2004; Schuller et al., 2010). The threshold line in every real-time PCR run was manually set for each reporter dye by placing the threshold line across all exponential amplification curves and above the background noise lines (figure 2.2).

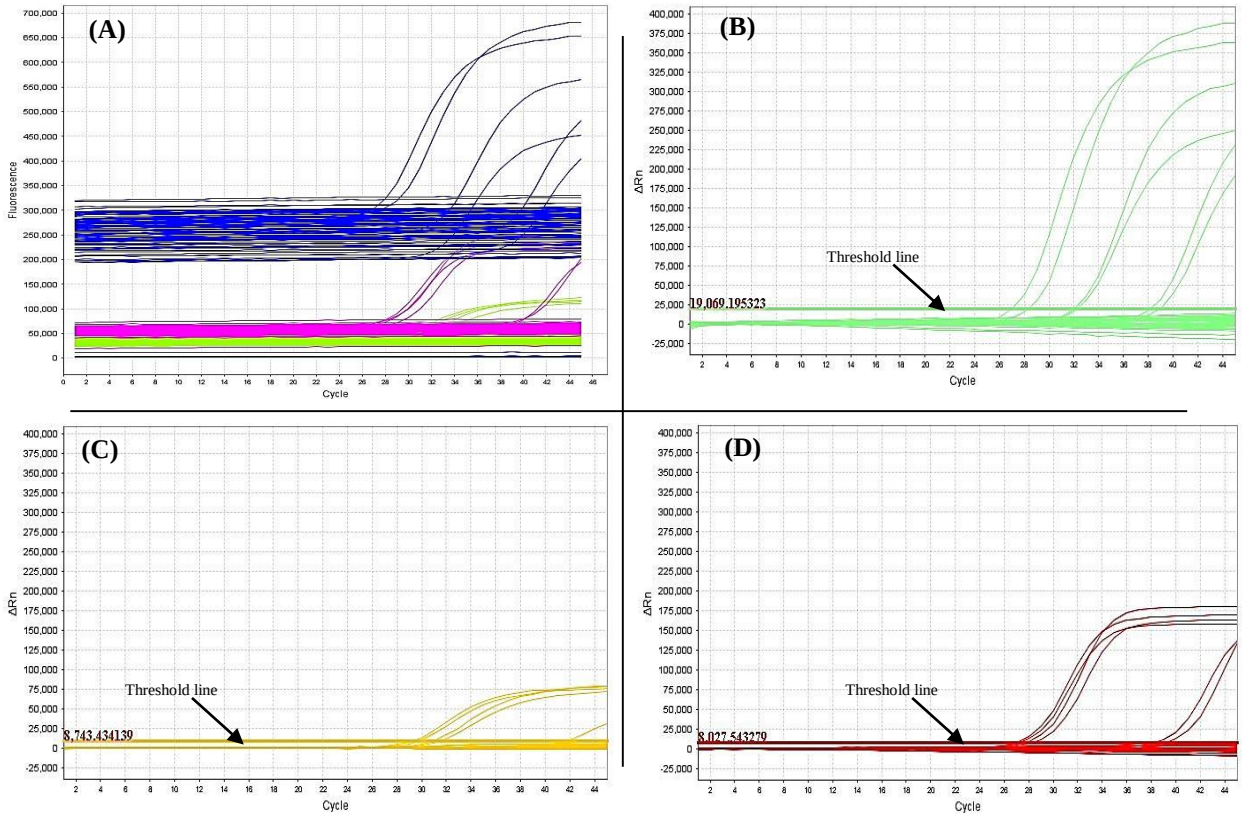

Figure 2.2 The above are examples of the WUV and KIV real-time PCR assay; (A) Illustrates the amplification curves results prior to analysis. The multicomponent plot shows the three reporter dyes; FAM, YAK and Quasuar 670. In (B), (C) and (D) threshold lines were set manually to cross all exponential amplification curves.; (B) amplification curves from PyV-WU real-time PCR assay, (C) amplification curves from KIV-D real-tie PCR and (D) from KIV-E real-time PCR.

The manual threshold setting avoided over-estimating the number of positive results that could arise from signal drifts, which was defined as a non-exponential rise in the fluorescent signal unrelated to amplification of the target sequence (Wong and Medrano, 2005; Yuan et al., 2006). Signal drifts can also occur from non-specific breakdown of the TaqMan™ probe, poorly made probe or from accumulation of reaction traces towards the end of a real-time PCR run (Gunson et al., 2006). The latter can cause confusion when interpreting true positives with relatively low target load as often obtained from screening pooled samples. Therefore, to interpret positive results from screening pooled samples, any curves that showed an exponential increase in signal were considered a true positive, even if they were obtained after 42 cycles. When such a situation arose, individual samples were then tested to confirm the positive result.

Real-time PCR testing was performed using several instruments that have different sample loading and sealing formats. Some real-time PCR tests were performed using Rotor-Gene thermo-cycler series (RG-6000, RG-Q; Qiagen, Australia). Reaction mixes for these particular instruments were loaded into 100-well disks using the CAS1200 liquid handling system, which were then closed with heat sealing films. For other real-time PCR tests, alternative instruments with a 96-well plate format and complementary clear sealing films were used, such as ABI7500, ViiA 7 (Applied Biosystems, Australia) and the LC480 (Roche, Australia).

2.1.8 Sample and extract databases and data management

Starting with samples collected by parents and ending with their various real-time PCR results, samples in each stage of the study were monitored and their details recorded in customised databases. Upon receiving nasal swabs and collection forms, a simple excel database was used for sample and data lodgement. For each swab received, the infant identity ID number, nasal swab number and collection form details were recorded, as well as the -80ºC specimen batch rack where the swab was stored. Additional details recorded during the lodgement by the laboratory team included; the date received and stored frozen in the laboratory, days between swab collection and freezing, and whether any visible mould was present according to a semi-quantitative score using a sliding scale (0-3; where 0 = no mould observed, 1 = low, 2 = medium, and 3 = high levels of visible mould present). When needed, any further comments were included to assist describing the condition of the swab upon arrival.

Another simple Microsoft Excel database was designed to assist monitoring swab return rates per month for each infant. For swab processing and extraction, a different electronic record was used to

assign and combine the 2mL 2D barcoded tubes in the 48-place racks (Sterile Smart Scan Racks, Thermo Scientific, Australia) and the 96-well 2D barcoded extraction storage rack (Sterile Matrix, Thermo Scientific, Australia) by recording the infant's identifying information (study ID number and date of birth), swab information (the swab number and date of collection) and specimen batch rack details. This information was imported manually from the sample log database.

For real-time PCR screening, quality control results were recorded in a separate database whereby Ct values obtained from EHV-1 and ERV-3 individual extract testing were recorded next to the tube's 2D barcode numbers only. This allowed easier examination of *Ct* values and a direct application of the quality control criteria using simple descriptive statistics such as mean ±2 standard deviation values. For respiratory viruses, real-time PCR results were obtained and recorded for each EPR (i.e. four individual extract racks) separately. A pooled sample result file included (1) the EPR map, which is the layout of the four extract racks used in the EPR along with the 2D barcode IDs for all of the racks used; and (2) respiratory virus real-time PCR results for all individual, pool and confirmatory testing. The *Ct* values obtained from respiratory virus real-time PCR were recorded after importing sample identifiers from the processing and extracting database. All databases were designed using Microsoft Office Excel (version 2010).

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Chapter-2 Appendices

Appendix 2-1

Symptom Diary Card

The symptom diary card was designed to collect ARI symptoms from participating infants every day and to return the cards to the ORChID project manager on a monthly basis. A separate diary card was used every month until the child reached their second birthday. Each participating family was therefore provided a total of 24 diary cards. For each diary card, the month and year were indicated at the top right-hand corner of the card, while the infant's basic information (Name and number in the study) was typed by either the care provider or study personnel. The card layout directed parents to record the type of symptoms they observed from their child. To differentiate between LRTIs and URTIs, a colour coding system was used whereby the yellow colour illustrated LRTIs and the blue colour illustrated URTIs. Parents were also asked to record the axillary temperature whenever fever was reported, a sign of a systemic reaction. This was accomplished using a digital thermometer provided to each participating family, each of whom had been instructed on its use.

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

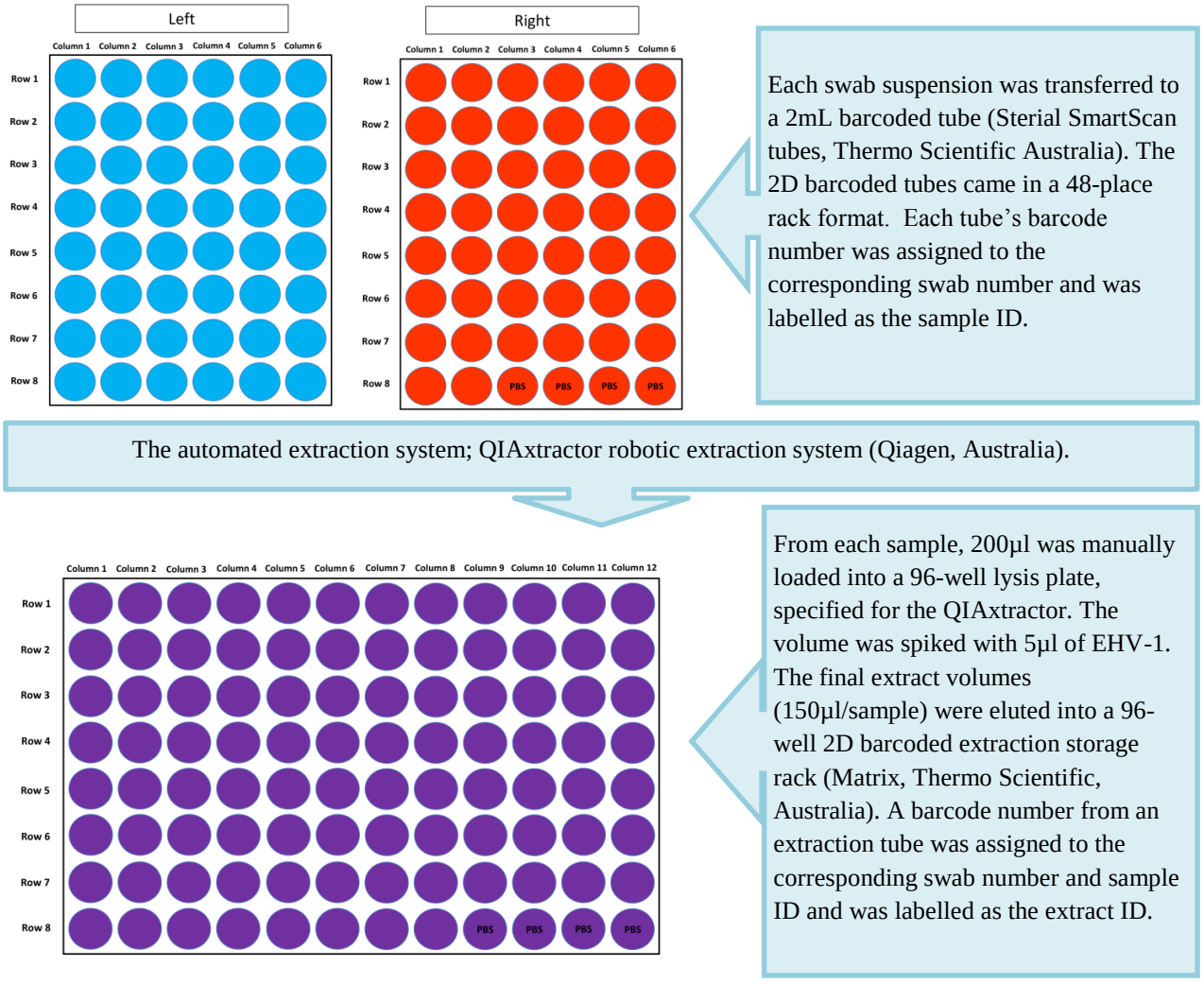
ORChID daily symptom diary card: version 1, 01 March 2010

Qpid Queensland Paediatric Infectious Diseases Laboratory

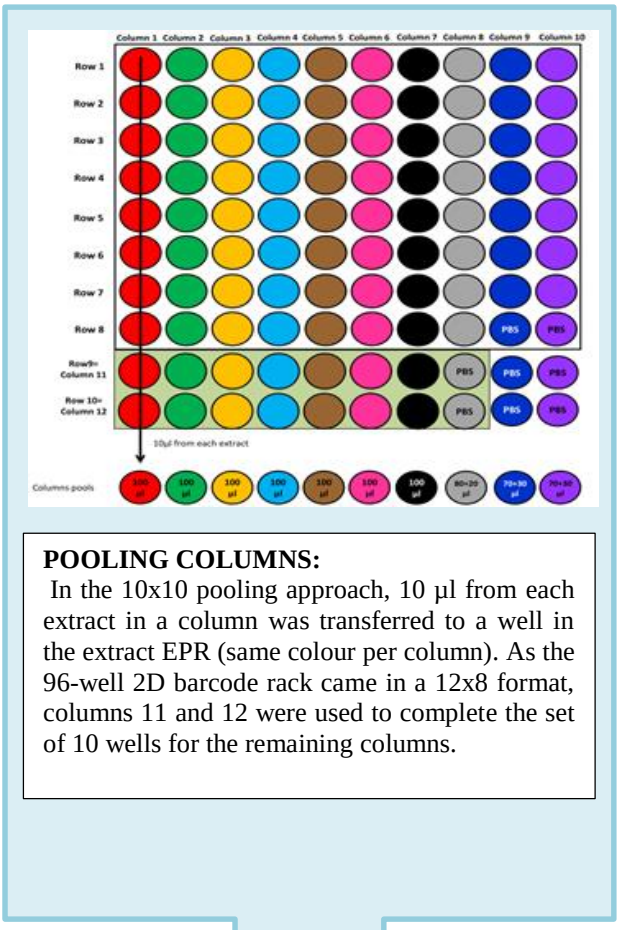
Appendix 2-2

Sample Processing and Pooling Approach

Parents were asked to collect anterior nasal swabs from their children on the same day of every week (e.g. sample on Sunday) and to mail them (eg. mail on Monday) as soon as possible to the QPID laboratory. The study materials provided to each participating family included swab labels with identical numbers to further assist in data management. Once collected, parents labelled the swab, completed the swab collection form and sent it by the regular mail at ambient temperature to the QPID laboratory where the data were reviewed and lodged and the swabs stored at -80ºC until analysis. Nasal swab samples were processed in batches of 96 that comprised 92 nasal swabs and four negative controls. A batch was given an identical number and the frozen swabs moved to 4ºC for defrosting, after which each swab was vortexed once 2mL of PBS was added to the sample tube.



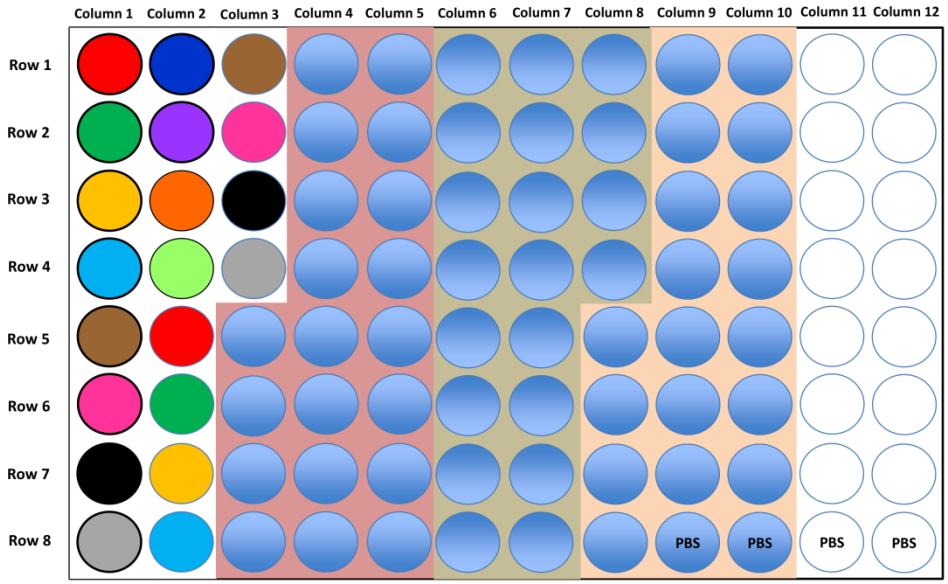
After passing quality control testing and screening for RV, an extract rack was pooled in a new 96 well 2D barcoded extract storage rack (Matrix, Thermo Scientific, Australia) using the single channel robotic liquid handling system: CAS1200 (Qiagen, Australia). The software was programmed specially for the ORChID study to accomplish a 10x10 pooling approach whereby 10µL aliquots from ten extracts in a defined column were combined in a defined single well into the extract pools rack. Similarly, 10µL aliquots from ten extracts in each row were combined into one well.



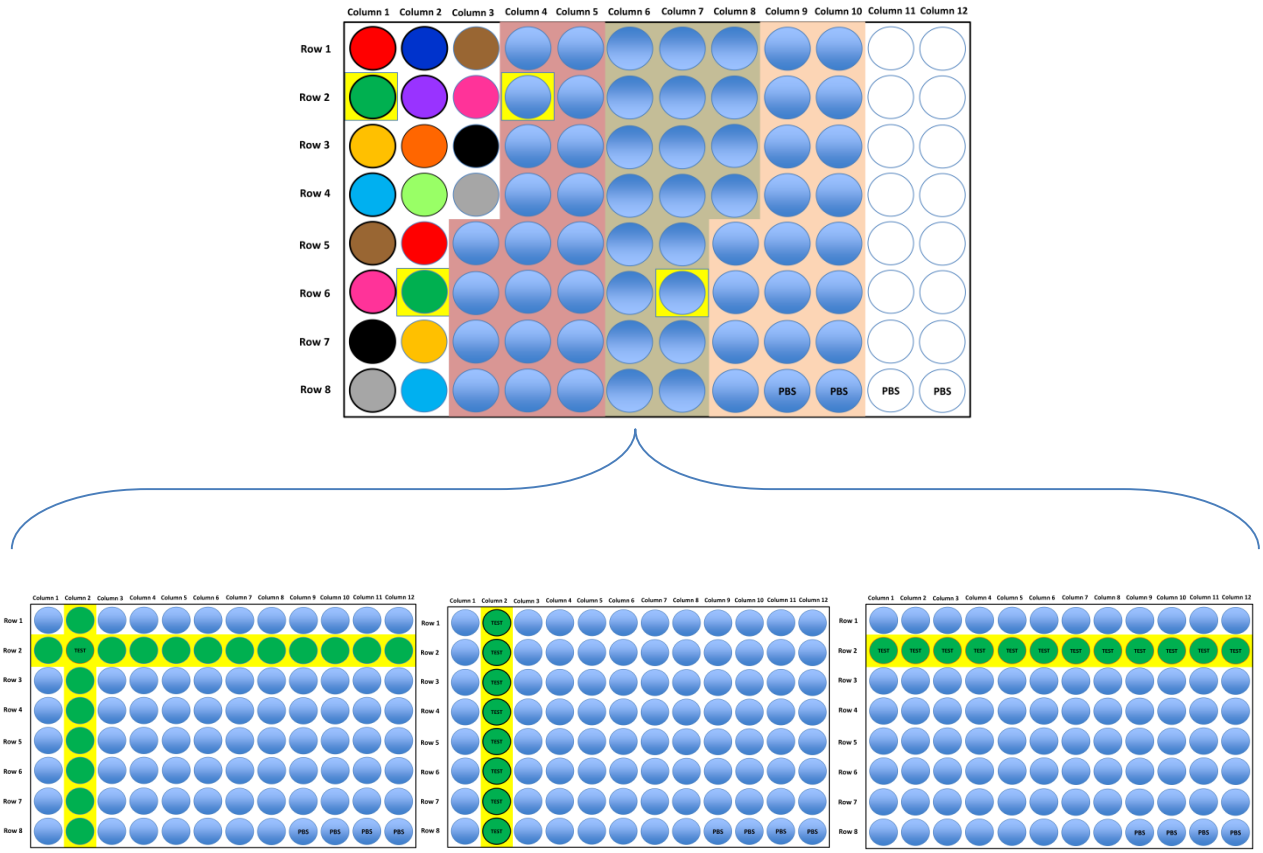
POOLING COLUMNS:
In the 10x10 pooling approach, 10 µl from each extract in a column was transferred to a well in the extract EPR (same colour per column). As the 96-well 2D barcode rack came in a 12x8 format, columns 11 and 12 were used to complete the set of 10 wells for the remaining columns.

POOLING ROWS:
In the 10x10 pooling approach: 10 µl from each extract in a row was transferred to a defined well in the EPR. As the 96-well 2D barcode rack came in a 12x8 format, columns 11 and 12 were used to complete the set of 10 rows.

The pooling technique resulted in compressing 96 wells (92 extracts and four negative controls) into 20 wells of an EPR pooled extract rack. Consequently, four extract racks could be combined into one EPR.



Pooled extract racks were screened for respiratory viruses other than RV. Appropriate respiratory viruses positive controls were loaded into the free wells of the examined pools rack according to the real-time PCR or real-time RT-PCR assay performed. When positive results were obtained from a column pools well and a row pools well from a specific extract rack, the extract located in the joint well was tested to confirm positivity. When a positive result was obtained from a column pools well only or a row pools well only, the whole column or row in the corresponding extract rack was tested.



Appendix 2-3

Kit Evaluation.

The quality control work included an evaluation of six commercial PCR and RT-PCR kits (three kits each) to help choose those providing the optimal sensitivity, specificity, reproducibility time and price. Table-2.3 summarises the main features of these kits and some of our testing details.

Table 2.3 Comparing three PCR kits and three RT-PCR kits regarding their price, reproducibility time, manufacturer names and some of our testing details (number of assays tested, performance of multiplex assays)

	Kit	Reproducibility time (standard conditions/run)*	Price/ 20 µL reaction ($AUD)	Manufacturer	Testing details		
					No. Of assays	Multi-plexes?	Clinical samples?
PCR	QuantiFast Probe PCR	56 min.	1.52	Qiagen	1	✓	X
	QuantiTect Probe PCR*	1:50h.	1.79	Qiagen	2	✓	✓
	SensiMix II Probe	1:38 h	1.51	Biloine	2	✓	✓
RT-PCR	QuantiFast Probe No-ROX 1-Step	1:38 h	2.12	Qiagen	1	✓	X
	One-step RT-PCR*	2:20 h	2.85	Qiagen	4	✓	✓
	SensiFast Probe RT-PCR	2:02 h	1.51	Bioline	4	✓	✓

*Reproducibility time does not include nucleic acid extraction or master mixes preparation
*PCR refers to amplifying DNA targets and RT-PCR to amplifying RNA target runs that include a cDNA step.
*Both Quantitect probe PCR and One-step RT-PCR (Qiagen) are used in our lab and provide good performance. As initial assessments of the Quantifast kits were unsuccessful, no further evaluation of these kits were performed.

The initial evaluations were performed using the PyVs WU/KI and PIVs 1-3 triplexes and serial dilutions of previously characterised positive controls. When good performance was observed, further evaluations were held utilising: the quality control duplex (EHV-1/ERV-3) assay for competitive inhibition effects, hCoV triplex (hCoV-229E, hCoV-NL63 and hCoV-OC43), hMPV singleplex and RV singleplex assays, using serial dilutions of positive controls, and positive and negative clinical samples. The competitive inhibition effect was tested using the (EHV-1/ERV-3) assay. The reaction mixes were spiked with 20µL of EHV-1 (Ct=28*)* and ERV-3 was used as the gradual template.

Overall, the best performance and pricing was observed with the two bioline kits (SensMix II probe and SensiFast Probe RT-PCR). Therefore, these two kits were used in this longitudinal study. To ensure consistency of performance, an arrangement was reached with the manufacturer to supply the same batches used in the earlier evaluation for the rest of the study.

Chapter 3

Respiratory virus infections during the first year of life:

General results

3.1 Background

Our current knowledge of respiratory viruses in young children is derived mainly from hospital-based studies, many relying upon testing specimens of convenience (Arden and Mackay, 2010; Sloots et al., 2006). Such studies sample from only a small fraction of respiratory virus infections and so community-based studies are needed to gain a greater understanding of the true burden of disease. However, older community-based studies had only classical culture techniques available to them and are likely to have under-estimated the true burden of respiratory virus disease (Monto , 2002) when compared with contemporary, highly-sensitive molecular-based detection techniques used increasingly during the last decade (Calvo et al., 2007; Chonmaitree et al., 2008; Hall et al., 2009; Heymann et al., 2004; Jackson et al., 2008). One of the best methods of determining the true community burden of viral respiratory infections in early life, which is a critical developmental phase in lung growth, is to prospectively follow infant birth cohorts. Unfortunately, the few recent studies involving infant cohorts and employing molecular-based detection assays have suffered from several methodological limitations. For example, many have sampled from highly selected populations (especially those deemed to be at high risk of atopy), lacked adequate controls, had limited sampling frequency and observation periods or tested for a limited range of viruses (Jackson et al., 2012; Kusel et al., 2006; Legg et al., 2005; Regamey et al., 2008; van der Gugten et al., 2013). Consequently, there remain important knowledge gaps regarding the frequency and types of respiratory viruses affecting healthy infants in their first year of life. The ORChID study described in earlier chapters seeks to address these limitations.

The main aims of this chapter are to describe the nature and shedding patterns of respiratory viruses detected in the first year of life from a cohort of 72 healthy infants nested within the ORChID study. I also describe the association between virus detection, ARI episodes and viral loads. I wish to address the following hypotheses:

- RV is the most frequently detected respiratory virus associated with symptoms in otherwise healthy infants.
- Various host and environmental factors influence rates of respiratory virus infections and associated symptoms; including age, gender, breast feeding, household size, exposures to other children and to environmental tobacco smoke, and season.
- ARIs that involve multiple respiratory viruses last longer than ARIs involving single viruses
- Ct values obtained from RV real-time RT-PCR (a semi-quantitative marker of viral load) are not closely associated with the presence or severity of symptoms.

3.2 Methods

Samples for these analyses were collected weekly from birth until the end of the first year of life. The overall recruitment and management strategies for the cohort, as well as respiratory virus testing, were described in detail in chapter-2. Below are the additional methods relating specifically to this chapter.

3.2.1 Study cohort and episode definitions

ORChID study infants born between September 2010 and November 2011 inclusive comprised this nested cohort. For the work described in this chapter, an "ARI" was defined as any URTI or LRTI episode. Any episode with any combination of dry cough, runny nose, nasal congestion or ear infection alone was considered an URTI, whereas episodes associated with any of the following: (i) breathing difficulty, (ii) wheeze, or (iii) wet cough or rattly chest were considered to be a LRTI (Kusel et al., 2006). An ARI was considered febrile only if body temperature data were provided and exceeded 37.5°C. A "new ARI episode" occurred if there were more than three symptom-free days between ARI episodes. Respiratory virus detection was performed by real-time PCR, as described previously (Chapter-2). A "new viral detection episode" (VDE) was defined when a virus was detected 14 days or more since the last detection; this included two negative intervening nasal swabs. If one of these intervening samples was missing, 30 days were required to define a new infection episode.

3.2.2 Outcomes of interest and confounders

The outcomes of interest for this analysis included subcategories of all VDEs; (i) viral ARIs, which were defined as any ARI episode associated with a VDE and (ii) asymptomatic VDEs, which were any VDEs that was not associated with ARI symptoms and/or signs. For both outcomes, eight confounding variables related to infant characteristics were included: the age quarter of which the outcomes of interest occur, gender, exposure to smoking, maternal vaccination status, number of siblings in the household, feeding status, childcare and season. The variable exposure to smoking included three categories depending upon: parental smoking (mother or father) and the absence of this exposure. For feeding status, three categories were included; (i) if the infant was exclusively breastfed, (ii) if other milk was introduced and (iii) after solids have been introduced. For childcare status, the outcome of interest was any infant who attended (i) formal childcare, (ii) informal childcare, (iii) both formal and informal childcare and (iv) no childcare. Informal childcare was defined as care arranged by a parent and provided by an unpaid carer (family member of friends). Three additional variables were included to examine outcomes for asymptomatic VDEs, two of

which were related to infant characteristics: (i) order of infant in the family, and (ii) household size, while the third was related to the outcome characteristic, namely the number of viruses detected.

3.2.3 Statistical analysis

The association between variables of interest and outcomes was investigated using mixed-effects logistic regression models, with infants included as a random intercept to account for the possibility of correlated outcomes within each infant. Both univariate and multivariate analyses were conducted, with the multivariate analysis being adjusted for all potential confounding variables. Analyses were conducted using Stata statistical software v.11.0 (StataCorp, College Station, TX, USA).

3.3 Results

3.3.1 Study cohort and demographic data

From September 2010 to November 2011, 94 infants were recruited into the ORChID study. Of these, 9 infants were lost to follow-up and 13 were withdrawn for personal reasons within the family (these include caring responsibilities, postnatal depression, illness of a family member or separation for 10 infants or moving from the study area for another 3 infants). The remaining 72 infants (37 female) remained within ORChID during their first year of life coinciding with the time available for my PhD studies and therefore were included in this analysis. Their median gestational age was 40.2 weeks (range 37-42 weeks) and the median birth weight 3505g (range 2794-4830; figure 3.1).

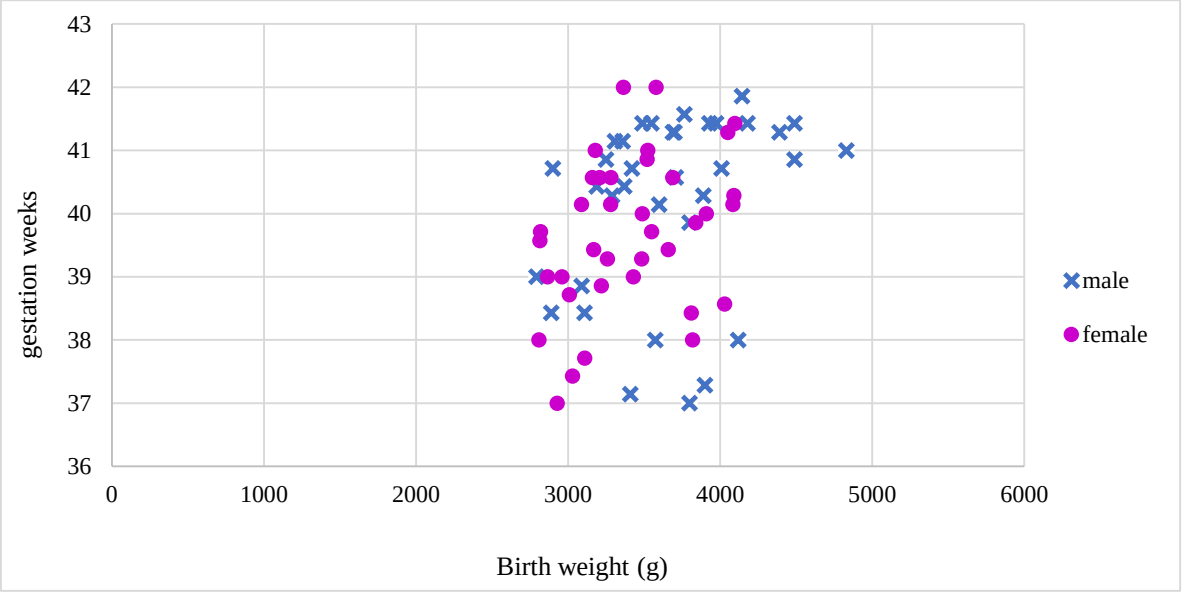


Figure 3.1 Gestation (weeks) and birth weight (grams), stratified by gender. There were no significant differences in gestational age or birth weight between male and female infants.

Most (n=49, 68.1%) were first born children. The median household size for this cohort was three (range 3-9). All of the infants lived with both parents in the household and six lived with at least one additional adult. Twenty four infants shared the household with at least one other child (range 1-6 children). Nine infants (12.5%) lived with at least one parent who was an active smoker (1 mother and 8 fathers). Most mothers (n=53, 73%) reported receiving the seasonal IFV vaccine at least once. Of them, 33 mothers specified receiving the vaccine during the 12 months prior to their infant's enrolment in the study.

3.3.2 Symptom data and ARI episodes

Overall, the retained cohort provided 25,808 of a possible 26,322 (98%) person-days of symptom data during the first year of life. The days per infant ranged from 209-366 (day of birth was included) with an average of 357.4 days (Table 3.1).

Table 3.1 Observation days provided by 72 infants of the one year old cohort and their ARI episodes

SID	Days available	Missing days	Symptoms days	Symptoms free days	No. ARIs reported	Median duration
001	366	0	63	303	8	8
002	366	0	48	318	6	9.5
003	366	0	98	268	9	8
004	366	0	124	242	7	7
005	366	0	49	317	8	4.5
006	366	0	56	310	8	5.5
010	366	0	141	225	6	11.5
012	294	72	54	240	4	12
013	366	0	5	361	4	1
015	366	0	0	366	0	0
016	360	6	205	155	8	18
017	366	0	116	250	8	17.5
018	366	0	60	306	8	6
019	223	143	68	155	2	34
020	366	0	34	332	5	7
021	366	0	113	253	9	12
023	366	0	61	305	11	4
024	366	0	5	361	1	5
025	366	0	82	284	6	7
027	366	0	176	190	7	13
028	366	0	33	333	1	33
029	366	0	134	232	7	23
030	366	0	63	303	10	5
031	366	0	18	348	5	2
032	366	0	33	333	6	4
034	366	0	52	314	6	9
035	366	0	55	311	9	6
038	366	0	18	348	3	5
039	365	1	0	365	0	0
041	366	0	125	241	7	11
042	366	0	36	330	3	4
043	366	0	58	308	8	5
044	210	156	0	210	0	0
045	364	2	32	332	2	16
046	330	36	10	320	2	5

Table 3.1 continued. Observation days provided by 72 infants of the one year old cohort and their ARI episodes

SID	Days available	Missing days	Symptoms days	Symptoms free days	No. ARIs reported	Median duration
048	366	0	0	366	0	0
049	366	0	54	312	15	2
050	366	0	34	332	3	11
051	366	0	31	335	5	6
052	366	0	46	320	5	12
054	366	0	109	257	9	13
055	327	39	21	306	5	4
056	366	0	123	243	10	5
058	366	0	50	316	7	8
060	366	0	50	316	3	14
061	279	87	6	273	2	3
062	366	0	76	290	7	9
063	365	1	13	352	4	3
064	366	0	110	256	8	11.5
065	366	0	44	322	11	4
066	366	0	94	272	9	10
067	366	0	27	339	4	6
068	366	0	270	96	6	23
071	366	0	26	340	3	9
072	366	0	54	312	10	3.5
075	366	0	39	327	5	2
076	366	0	4	362	1	4
077	366	0	40	326	9	3
078	366	0	22	344	5	3
079	366	0	57	309	5	11
080	366	0	53	313	11	3
081	366	0	3	363	2	1.5
084	366	0	113	253	7	8
085	366	0	90	276	8	11
087	366	0	17	349	6	2.5
088	366	0	36	330	5	6
089	365	1	77	288	9	11
090	366	0	21	345	4	3.5
091	366	0	31	335	3	9
092	366	0	35	331	5	7
094	366	0	131	235	10	11.5
095	366	0	102	264	9	11
Total	25808	544	4334	21474	424	7

The missing symptom data were from eleven infants (15.2%); five of whom failed to provide symptom data for less than seven days at the end of the study. The remaining six infants did not provide symptom data for a total of 533 days (mean 88.8 (SD 55.0); median 79.5) days per infant; range 36-156).

In total, 424 ARIs were identified in 68 infants (94.4%) who experienced at least one ARI during their first year of life. The data provided by the remaining four infants did not meet the definition of an ARI (infants 015, 039, 044 and 048; Tables 3.1 and 3.2). The median number of ARIs per subject was six (range 0-15 ARIs) with a median symptom duration for each episode of seven (range 1-192) days. This gave a total of 4334 person-symptom days that represent 16.8% of the total observation period (Table 3.1). The incidence rate was 1.64 ARIs per 100 child-days (95% confidence interval (CI); 1.49-1.76) or six ARIs per child-year (95% CI 0.46-11.54). ARI episodes were observed during the first week of life in two infants. One experienced fever on their second day of life and the other had several days of nasal discharge.

Overall, two peaks for ARI episodes were observed during the two year study period. The first was identified in August-2011 while the second occurred between May-2012 and July-2012. Similarly, ARI episodes declined twice during the same period. The first was in January-2012 and the second in August-2012 (Figure 3.2).

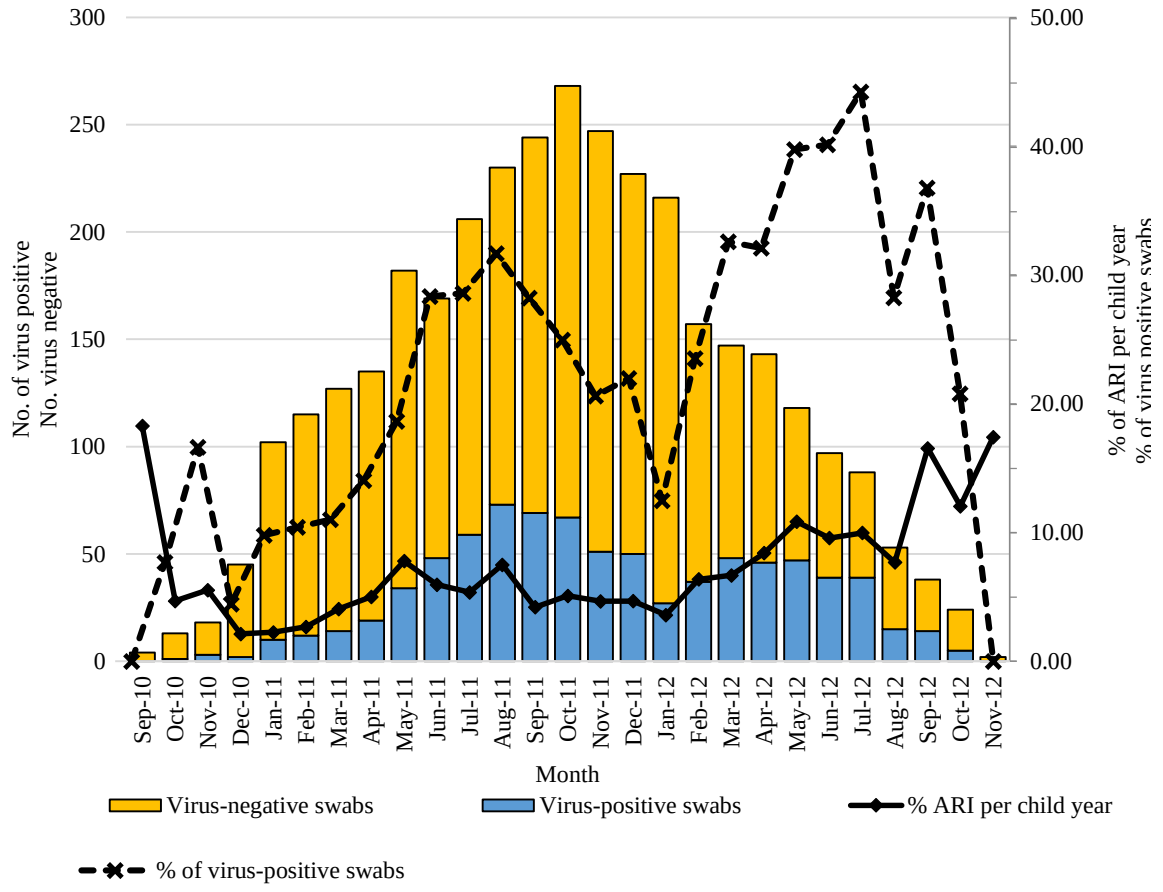


Figure 3.2 The number of swabs positive and negative for any respiratory virus (columns) and associated percentages (lines) for the duration of the study.

3.3.2.1 ARI episode characteristics

The 424 ARIs included 356 URTI episodes (84%) and 68 LRTI episodes (16%). URTI episodes were observed in 68 infants (94% of all infants and 100% with ARIs) with a median of five episodes per infant (IQR 3-7.25). Thirty two (44%) had only URTI episodes, while 37 (51%) had at least one LRTI episode during their first year of life. The maximum number of URTI episodes in a single infant was 15 with a median duration of 2.5 days per episode (Infant 049; Appendix-1; Page: XIII). For all infants, the total number of days involving URTI symptoms was 3347 days (12.9% of total observation days) with a median duration of six (IQR 3-11) days per episode (Figure 3.3).

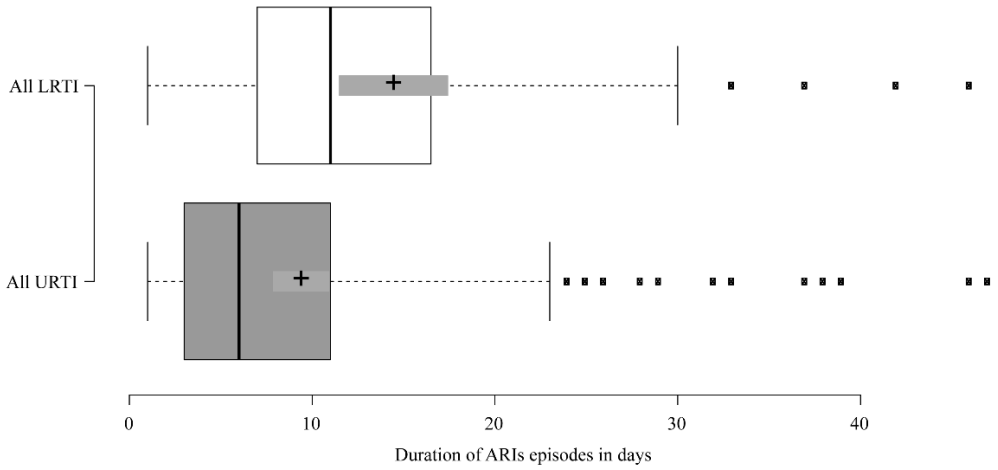


Figure 3.3 A comparison between the duration of URTI (n=356) and LRTI (n=68) episodes among our first year old cohort. The center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the IQR from the 25th and 75th percentiles. Episodes represented by dots were outliers; crosses represent sample means while the grey bars indicate the limits of the 95% CI.

Prolonged URTI episodes were also observed in our population. Eight infants experienced continuous symptoms of an URTI (2.2% of total URTI episodes) for more than six weeks, including two with continuous symptoms recorded for 102 and 192 consecutive days respectively (Infants 027 and 068; Appendix-1; Page; VII & XVIII respectively).

The 68 LRTI episodes involved 983 days (3.8% of total available observation days) and affected 37 infants (median of one LRTI episode (interquartile range (IQR) 1-2) per infant). One infant had six LRTI episodes (median 11 (IQR 11-17] days per episode; Infant 094; Appendix-1; Page XXIV). The median (IQR) duration of LRTI among infants was 11 (7-16.5) days per episode (Figure 3.3). Prolonged LRTI episodes were observed in four infants, each of whom had symptoms for more than 30 days consecutively (figure 3.3). (Infant 084; Appendix-1; Page XXI).

3.3.3 Nasal swab return and respiratory virus detection

3.3.3.1 Nasal swab return

During the first year of life, 3,446 swabs (92.1% of 3,744 expected) were obtained from the study infants. Four infants who provided weekly nasal swab samples without accompanying daily symptom data had their swabs excluded from this analysis (31 nasal swabs excluded, comprising 1, 3, 8 and 19, from each of four infants respectively). The remaining 3,415 swabs (91.2% expected) had accompanying symptom data. All 72 infants provided their first nasal swab within the first week of life. By the end of their first year of life the median number of weekly nasal swabs was 49 per infant (range 32-53 nasal swabs) (Table 3.2).

Of the 424 ARIs identified, 421(99.3%) had at least one nasal swab returned. Only three ARIs (0.7%), were un-accompanied by swabs. Overall, 596 nasal swabs were collected during days containing ARI episodes, of which 461 nasal swabs were collected during an URTI (13.49% of total swabs) and 135 during a LRTI (3.9% of total swabs).

Table 3.2 Nasal swab returns and virus detections per individual infant

SID	No. swabs available	No. swabs positive for any virus	% of positive swabs	Co-detections	% of co-detections in positive swabs	RV	IFV-A	IFV-B	PIV-1	PIV-2	PIV-3	RSV-A	RSV-B	hCoV-OC43	hCoV-NL63	hCoV-229E	hCoV-HKU1	hMPV	HAdV	WUV	KIV	hBoV
001	32	10	31.3	1	10.0	8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
002	33	10	30.3	1	10.0	8	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0
003	43	4	9.3	2	50.0	3	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1
004	53	13	24.5	1	7.7	6	0	0	0	0	1	1	1	1	0	0	0	0	1	0	2	1
005	51	15	29.4	3	20.0	8	0	0	0	1	0	1	1	1	4	0	0	0	1	0	0	1
006	52	11	21.2	0	0.0	7	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	2
010	45	19	42.2	4	21.1	14	0	0	0	0	1	0	0	0	0	0	0	0	3	2	3	2
012	35	7	20.0	0	0.0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
013	49	8	16.3	0	0.0	6	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
015	53	6	11.3	0	0.0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
016	47	14	29.8	0	0.0	12	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
017	49	12	24.5	2	16.7	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
018	51	6	11.8	0	0.0	3	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
019	29	11	37.9	3	27.3	4	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	2
020	52	11	21.2	1	9.1	10	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
021	53	12	22.6	0	0.0	10	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
023	51	23	45.1	3	13.0	20	0	0	1	0	0	2	0	1	0	0	0	0	0	2	0	0
024	51	5	9.8	0	0.0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
025	52	18	34.6	4	22.2	12	0	0	0	0	0	3	0	0	0	0	0	0	5	2	0	0
027	52	29	55.8	8	27.6	23	0	0	0	0	0	0	0	0	2	0	0	0	3	7	0	2
028	52	6	11.5	0	0.0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
029	50	15	30.0	0	0.0	13	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
030	44	14	31.8	2	14.3	8	0	0	0	0	0	0	0	0	0	0	0	0	2	0	4	2
031	52	3	5.8	0	0.0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
032	53	9	17.0	1	11.1	6	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0
034	50	11	22.0	0	0.0	8	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1
035	52	13	25.0	0	0.0	12	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
038	53	8	15.1	1	12.5	6	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
039	42	14	33.3	3	21.4	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	1
041	52	15	28.8	3	20.0	12	0	0	0	0	0	0	0	0	1	0	0	2	0	1	0	2
042	49	22	44.9	2	9.1	21	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3
043	50	16	32.0	0	0.0	12	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	0
044	30	6	20.0	0	0.0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
045	48	10	20.8	0	0.0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
046	47	21	44.7	2	9.5	14	0	0	1	0	0	0	0	0	0	0	0	0	2	0	6	0

Table 3.2 continued. Nasal swab returns and virus detections per individual infant

SID	No. swabs available	No. swabs positive for any virus	% of positive swabs	Co-detections	% of co-detections in positive swabs	RV	IFV-A	IFV-B	PIV-1	PIV-2	PIV-3	RSV-A	RSV-B	hCoV-OC43	hCoV-NL63	hCoV-229E	hCoV-HKU1	hMPV	HAdV	WUV	KIV	hBoV
048	50	2	4.0	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
049	49	10	20.4	4.0	40.0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2
050	40	7	17.5	0.0	0.0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
051	51	13	25.5	0.0	0.0	12	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
052	43	10	23.3	0.0	0.0	8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
054	49	15	30.6	2.0	13.3	13	0	0	0	0	0	1	0	0	0	0	0	0	0	2	0	1
055	41	10	24.4	0.0	0.0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
056	52	17	32.7	3.0	17.6	13	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	4
058	49	7	14.3	0.0	0.0	5	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
060	53	13	24.5	0.0	0.0	10	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2
061	39	7	17.9	1.0	14.3	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
062	46	20	43.5	1.0	5.0	18	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0
063	49	2	4.1	0.0	0.0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
064	51	18	35.3	6.0	33.3	16	0	0	0	0	0	0	0	0	1	0	0	0	2	2	3	2
065	48	17	35.4	1.0	5.9	15	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0
066	52	13	25.0	0.0	0.0	11	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
067	50	17	34.0	3.0	17.6	12	0	0	0	0	0	0	0	0	0	2	0	0	0	0	4	2
068	51	10	19.6	2.0	20.0	7	0	0	0	0	0	0	0	0	0	0	0	0	2	0	3	0
071	48	11	22.9	0.0	0.0	10	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
072	49	18	36.7	1.0	5.6	13	0	0	0	0	0	2	0	0	2	0	0	0	1	0	0	1
075	49	12	24.5	0.0	0.0	11	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
076	48	5	10.4	0.0	0.0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
077	51	6	11.8	0.0	0.0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
078	37	11	29.7	0.0	0.0	10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
079	53	8	15.1	0.0	0.0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
080	50	11	22.0	0.0	0.0	9	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
081	48	9	18.8	0.0	0.0	5	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3
084	44	3	6.8	0.0	0.0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
085	49	16	32.7	3.0	18.8	11	0	0	0	0	1	0	0	0	0	0	0	0	0	0	4	3
087	52	6	11.5	0.0	0.0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
088	46	13	28.3	2.0	15.4	10	0	0	0	0	1	0	0	0	0	0	0	0	1	2	0	1
089	47	6	12.8	0.0	0.0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
090	49	15	30.6	0.0	0.0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0
091	52	5	9.6	0.0	0.0	4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
092	48	9	18.8	2.0	22.2	9	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
094	51	16	31.4	1.0	6.3	13	0	0	0	0	0	1	0	2	0	0	0	0	1	0	0	0
095	33	14	42.4	1.0	7.1	14	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

3.3.3.2 Quality control testing

Prior to respiratory virus testing, the quality of extraction was examined by screening individual sample extracts using the quality control real-time PCR assays (described in section 2.1.4.2). Overall, 3,415 sample extracts were positive for EHV-1 with an average Ct value of 33.28. As per the ORChID quality control protocol, only sample extracts that passed the EHV-1 specific batch-to-batch criteria were eligible for subsequent respiratory virus panel screening. For samples from this cohort, 54 extracts did not meet the EHV-1 criteria (described in section 2.1.4.2) and were, therefore, re-extracted and re-tested prior to their inclusion in the analysis.

For ERV-3, which was used to test for the presence of human DNA, the average Ct value was 34.45 (range 22.86 to 43.91) cycles. There were 562 (16.4%) sample extracts that tested negative for ERV-3, all of which though were included in the analysis (for further details see chapter-4).

3.3.3.3 Respiratory virus detection in nasal swabs and infection episodes

Overall 829 (24.3%) swabs were positive for any respiratory virus. All infants provided at least two positive nasal swabs (median 11 [IQR 7-15]) per infant. The highest number of positive swabs observed in one infant was 29 (56% of provided swabs) (Table 3.2). All 17 respiratory viruses were detected in the cohort. The median (IQR) number of different viruses detected during the first year of life in each infant was three (2-4) and ranged from one to eight with the latter number observed in two infants (Table 3.2; infants 004 and 005).

The overall number of swabs positive for any RNA virus (n=80; 2.4%) other than RV was significantly lower than the number of swabs positive for any DNA virus (n=179; 5.5%) ($P<0.0001$). Of the 172 VDEs observed, the number of RNA-VDEs (68 RNA-VDEs; 39.5%) was also significantly lower than the number of DNA-VDEs (104 DNA-VDEs; 60.5%) ($P=0.0002$). The distribution of DNA-VDEs and RNA-VDEs was as follows: 29 (40.3% of infants) had both DNA and RNA-VDEs, 20 (27.7%) had only DNA-VDEs and 12 (16.7%) had only RNA-VDEs. For the remaining 11 infants, only RV-DEs were observed (Table 3.2).

3.3.3.4 Most frequently detected respiratory viruses

RVs were the most frequently detected respiratory viruses. The number of swabs positive for RV was 659 (19.1%) causing 327 rhinovirus detection episodes (RV-DEs) in 71 infants (98.6%). Only one infant (infant 048; Table 3.2) failed to have a RV detected in any of their nasal swabs. The median (IQR) number of RV-DEs in the positive infants was four (3-6) with a maximum of nine detection episodes in one infant. The median (IQR) duration of RV-shedding in all episodes was one (1-3) week and the maximum duration recorded for a single episode was nine weeks. Table 3.3 describes respiratory virus detection and VDEs among the infants.

Table 3.3 Descriptive summary of respiratory virus detection in nasal swabs, viral detection episodes (VDEs) and acute respiratory infections (ARIs) and their association with respiratory viruses

	Feature	RV	IFV-A	IFV-B	PIV-1	PIV-2	PIV-3	RSV-A	RSV-B	hCoV-OC43	hCoV-NL63	hCoV-229E	hCoV-HKU1	hMPV	HAdV	WUV	KIV	hBoV	No virus identified
Nasal swabs detection results (n=3415)	No. of infants (%)[a]	71 (98.6)	3 (4.2)	2 (2.8)	4 (5.6)	1 (1.4)	9 (12.5)	15 (20.8)	8 (11.1)	9 (12.5)	5 (6.9)	1 (1.4)	1 (1.4)	6 (8.3)	23 (31.9)	12 (16.7)	17 (23.6)	31 (43.1)	0
	No. of positive samples (%)	659 (19.1)	3 (0.09)	2 (0.06)	4 (0.12)	1 (0.03)	9 (0.26)	22 (0.64)	8 (0.23)	11 (0.32)	10 (0.29)	2 (0.06)	1 (0.03)	7 (0.2)	38 (1.11)	30 (0.88)	57 (1.67)	54 (1.58)	2586 (75.7)
	Mean number of positive samples per infant (Min-max)	9.1 (0-23)	0.04 (0-1)	0.03 (0-1)	0.06 (0-1)	0.01 (0-1)	0.13 (0-1)	0.31 (0-3)	0.11 (0-1)	0.15 (0-2)	0.14 (0-4)	0.03 (0-2)	0.01 (0-1)	0.1 (0-2)	0.53 (0-5)	0.42 (0-7)	0.79 (0-6)	0.75 (0-4)	
	Median Ct value (Min-max)	31.5 (18.1-48)	32.4 (30.2-38.2)	29.6 (29.6-29.7)	39.9 (32.8-42.5)	28.2 (28.2-28.3)	30.9 (25 - 37.6)	35.1 (25.7-42.5)	30.2 (26.8-37.3)	29.1 (26.1-36)	33.5 (29.2-36.9)	32.1 (27.6-36.6)	34.9 (34.9-34.9)	35.2 (33.7-39.2)	34.9 (24.8-41)	28.8 (18.1-37.2)	31.9 (18.3-40.1)	30.4 (16.9-42.9)	-
	Mean Ct value	32	33.6	29.7	38.8	28.3	30.3	34.6	31.1	29.8	33.2	32.1	34.9	35.4	34.3	28.3	31.1	30.5	-
VDEs in participants	No. of VDEs in infants	327	3	2	4	1	9	18	8	9	5	2	1	6	31	15	25	33	-
	No. of asymptomatic VDE (%)	110 (33.6)	1 (33.3)	1 (50)	2 (50)	0	0	5 (27.7)	1 (12.5)	3 (33.3)	0	0	0	1 (16.6)	8 (25.8)	3 (21.4)	12 (48)	12 (36.3)	
	Median VDEs per infant (min-max)	4 (1-9)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-2)	1 (1-1)	1 (1-1)	1 (1-1)	2 (2-2)	1 (1-1)	1 (1-1)	1 (1-3)	1 (1-3)	1 (1-3)	1 (1-2)	-
	Median duration of VDE (min-max)	1 (1-9)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-3)	1 (1-1)	1 (1-2)	2 (1-4)	1 (1-1)	1 (1-1)	1 (1-2)	1 (1-4)	2 (1-4)	2 (1-6)	1 (1-4)	-
	No. of months in which VDEs were observed	24	2	2	4	1	8	12	6	5	5	2	1	4	12	11	17	14	-
	Peak month[b]	August 11	September 11	August 11	October 11	January 11	October 11	October-11	March-11	April 11	August-11	July-11	August-12	July-11	July-11	October-11	November 11	July-12	-

[a] Percentage of total population, n=72

[b] Peak month was determined by calculating incidence rates (per 100 child-days).

[c] Percentage of episodes accompanied by samples

[d] Including co-detection

[e] Percentage of positive infants

Table 3.3 continued. Descriptive summary of respiratory virus detection in nasal swabs, viral detection episodes (VDEs) and acute respiratory infections (ARIs) and their association with respiratory viruses

	Feature	RV	IFV-A	IFV-B	PIV-1	PIV-2	PIV-3	RSV-A	RSV-B	hCoV-OC43	hCoV-NL63	hCoV-229E	hCoV-HKU1	hMPV	HAdV	WUV	KIV	hBoV	No virus identified
Features of ARI episodes	Number of ARIs with virus identified /total number of ARIs (n=424; %) [c,d]	239 (56.4)	2 (0.5)	1 (0.2)	2 (0.5)	1 (0.2)	8 (1.9)	12 (2.8)	7 (1.7)	5 (1.2)	2 (0.5)	6 (1.4)	0	5 (1.2)	22 (5.2)	13 (3.1)	12 (2.8)	22 (5.2)	144 (33.9)
	URTI (% of total 356 URTIs) [c,d]	204 (57.3)	1 (0.3)	1 (0.3)	1 (0.3)	1 (0.3)	7 (2)	6 (1.7)	4 (1.1)	4 (1.1)	0	6 (1.7)	0	2 (0.6)	15 (4.2)	12 (3.4)	11 (3.1)	18 (5.1)	121 (33.9)
	LRTI (%of total 68 LRTIs) [c,d]	35 (51.5)	1 (1.5)	0	1 (1.5)	0	1 (1.5)	6 (8.8)	3 (4.4)	1 (1.5)	2 (2.9)	0	0	3 (4.4)	7 (10.3)	1 (1.5)	1 (1.5)	4 (5.9)	23 (33.8)
	Median duration of ARIs in days	9	9.5	3	9.5	5	11.5	9	10	18	9.5	11.5	0	10	15.5	12	8	15.5	4
	Number of months of study in which virus was identified	23	2	1	1	1	6	8	6	4	5	2	1	2	12	10	11	12	25
	Peak month of ARIs associated with virus[e]	May-12	Jun-11	Aug-11	Mar-12	Jan-11	Aug-11	Oct-11	Mar-11	Jun-11	Aug-12	Jul-11	Aug-12	Jul-11	Jun-11	Oct-11	Dec-11	Aug-11	Sep-10
	Number of infants	67	2	1	1	1	8	10	6	5	5	1	1	4	18	9	9	21	56

[a] Percentage of total population, n=72

[b] Peak month was determined by calculating incidence rates (per 100 child-days).

[c] Percentage of episodes accompanied by samples

[d] Including co-detection

[e] Percentage of positive infants

The number of RV-DEs peaked in August-2011, with 28 RV-DEs and declined in August-2012 with only three RV-DEs (Figure 3.4) as most infants by then had reached their first birthday and left my nested cohort..

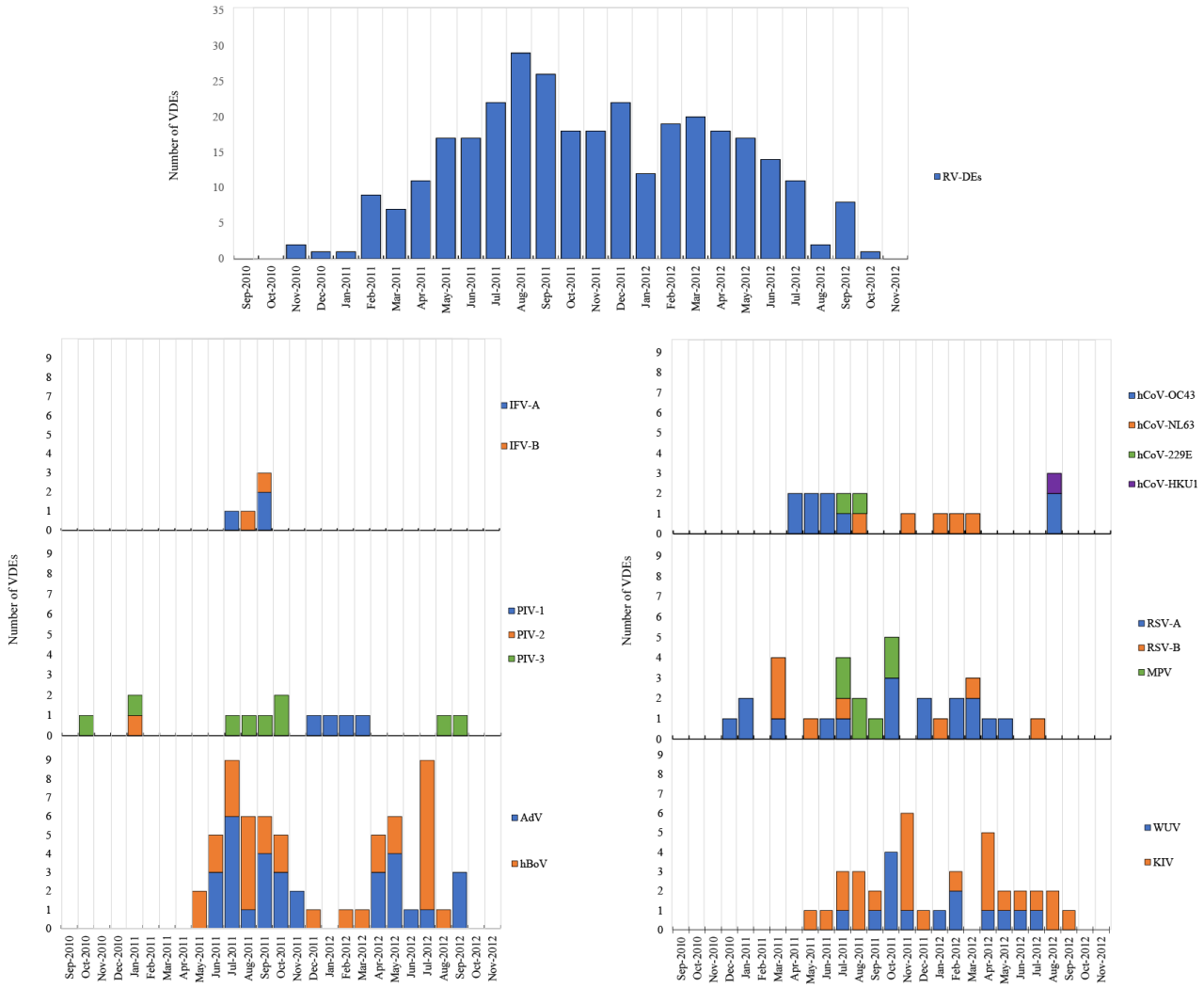


Figure 3.4 Number of infection episodes in 72 infants during two years of investigation from September-2010 to November-2012. RV-detection episodes were the most common. IFVs were observed in the first year of the study only.

After RV, the next four most commonly detected viruses were all DNA-viruses: KIV, hBoV, HAdV and WUV. Of these, KIV was the most prevalent. It was detected in 57 (1.7%) nasal swabs from 17 (23.6%) infants (Table 3.3). The median duration of KIV-DEs was two weeks and, notably, recurrent detections were observed in seven of 17 (41.2%) affected infants with a median of four (IQR 1-3) weeks between episodes.

Overall, hBoV was the second most commonly detected DNA virus and was observed in 31 (43.0%) infants. The median duration of hBoV shedding in the 31 episodes was one week with a maximum duration of four weeks observed in two infants who experienced only one hBoV-DEs each. HAdV followed as it was identified in 38 nasal swabs (1.1%) collected from 23 infants (32%) leading to 31 HAdV-DEs. The median duration of HAdV-shedding was one (IQR 1-1.5) week and the maximum duration of four consecutive weeks was observed in only one infant who experienced this sole infection episode (Infant 019; Table 3.2 and Appendix-1; Page V). Finally, WUV was detected in 30 nasal swabs (0.9%) collected from just 12 infants (16.7%). Similar to other DNA-viruses, the median duration of WUV-shedding was two weeks and the maximum duration of WUV-shedding of four weeks was observed in two infants (Table 3.3).

After RV, the second most frequently detected RNA virus in both the nasal swabs and in the infants was RSV-A, which was detected in 22 nasal swabs collected from 15 (20.8%) infants (Table 3.3). Recurrent episodes were observed for RSV-A on only one occasion 20 days after the initial detection. During this time, two intervening swabs were returned by the participating family and both were found negative for RSV-A. The Ct value in the initial RSV-A detection was (Ct = 29.18 cycles) compared to the second detection (Ct value = 34.59 cycles) (Infant 065; Table 3.2; Appendix-1; Page XVII).

hCoV-OC43 was detected in eleven nasal swabs (0.3%) leading to nine detection episodes in nine infants (12.5%) with a median shedding duration of one week. The maximum shedding duration among RNA viruses other than RVs was identified for hCoV-NL63, which was detected in ten nasal swabs (0.3%) leading to five episodes in five infants (6.9%). The longest hCoV-NL63-DE of four weeks was observed once in an infant who had a sole hCoV-NL63-DE (Infant 005; Table 3.2; Appendix-1; Page II). PIV-3 was the most commonly observed PIV virus and was detected in nine nasal swabs (0.3%) associated with nine episodes in nine infants (12.5%). Next came RSV-B detected in eight nasal (0.2%) swabs associated with eight episodes in eight infants (11.1%). For hMPV, which was detected in six

infants (8.3%), all cases were identified in the winter and spring months (Figure 3.4). PIV-1 was observed over four consecutive months from four infants (5.3%). For IFVs, IFV-A and IFV-B, few positive samples were identified during the first year of the study (July2011- September2011), and were only detected among five different infants (6.9%) (Figure 3.4; Table 3.2). hCoV-229E was detected twice in the same study infant. The duration of shedding for these two episodes was one week each with 21 interval days between the infection episodes (Infant 067; Table 3.2; Appendix-1; Page XVIII). Finally, PIV-2 and hCoV-HKU1 were detected only once each (0.03%) in a single VDE (1.3%) (Table 3.2).

3.3.3.5 Multiple detections

Multiple virus detections were observed in 80 samples (2.3% of total swabs obtained from 34 [47.2%] infants). Overall, RV was more frequently co-detected with a DNA virus than an RNA virus, while hBoV was the most common DNA virus co-detected with another respiratory virus (Table 3.4).

Table 3.4 Respiratory virus detection in nasal swab samples from 72 subjects, including multiple detections for each respiratory virus

Virus	Sole	Co-detection		Average Ct value			Swabs with multiple viruses									
		no.	%	Average	Av. Sole	Av. Co-detection	RV	PIV-1	PIV-3	RSV-A	RSV-B	hCoV-NL63	HAdV	WUV	KIV	hBoV
RV	590	69	10.5	31.6	32.1	31.4	0	1	2	2	0	2	18	14	19	17
KIV	36	21	36.8	32.0	32.2	29.4	19	0	1	0	0	0	0	0	0	2
hBoV	31	23	42.6	30.4	31.6	28.5	17	0	0	1	0	0	1	2	2	0
HAdV	19	19	50.0	34.9	33.5	35.0	18	0	0	0	0	0	0	0	0	1
WUV	13	17	56.7	28.9	27.5	28.8	14	0	0	2	0	1	0	0	0	2
RSV-A	16	6	27.3	35.2	35.1	33.2	2	0	0	0	1	0	0	2	0	1
hCoV-OC43	11	0	0.0	29.1	29.8	0.0	0	0	0	0	0	0	0	0	0	0
hCoV-NL63	7	3	30.0	33.5	33.0	33.5	2	0	0	0	0	0	0	1	0	0
PIV-3	6	3	33.3	30.9	29.7	31.4	2	0	0	0	0	0	0	0	1	0
RSV-B	7	1	12.5	30.2	31.1	31.1	0	0	0	1	0	0	0	0	0	0
hMPV	7	0	0.0	35.3	35.4	0.0	0	0	0	0	0	0	0	0	0	0
PIV-1	3	1	25.0	40.0	39.0	38.5	1	0	0	0	0	0	0	0	0	0
IFV-A	3	0	0.0	32.4	33.6	0.0	0	0	0	0	0	0	0	0	0	0
IFV-B	2	0	0.0	29.7	29.7	0.0	0	0	0	0	0	0	0	0	0	0
hCoV-229E	2	0	0.0	32.2	32.2	0.0	0	0	0	0	0	0	0	0	0	0
PIV-2	1	0	0.0	28.3	28.3	0.0	0	0	0	0	0	0	0	0	0	0
hCoV-HKU1	1	0	0.0	34.9	34.9	0.0	0	0	0	0	0	0	0	0	0	0

Figure 3.5 illustrates different VDEs where multiple viruses were identified and compares their percentages with single-virus VDEs. Although RV was the most frequently detected virus, more than 80% of RV-DEs were singletons (i.e. no other virus was detected). DNA-viruses were more often co-detected with RV compared to RNA-viruses. Moreover, 80% of WUV-DEs were associated with at least one other virus, and this occurred commonly with other DNA-viruses too, namely KIV (64% of total KIV-DEs), HAdV (54.8% of total HAdV-DEs) and hBoV (48.5% of total hBoV-DEs). In contrast, RSV-B was the only virus that was not accompanied by RV co-detection.

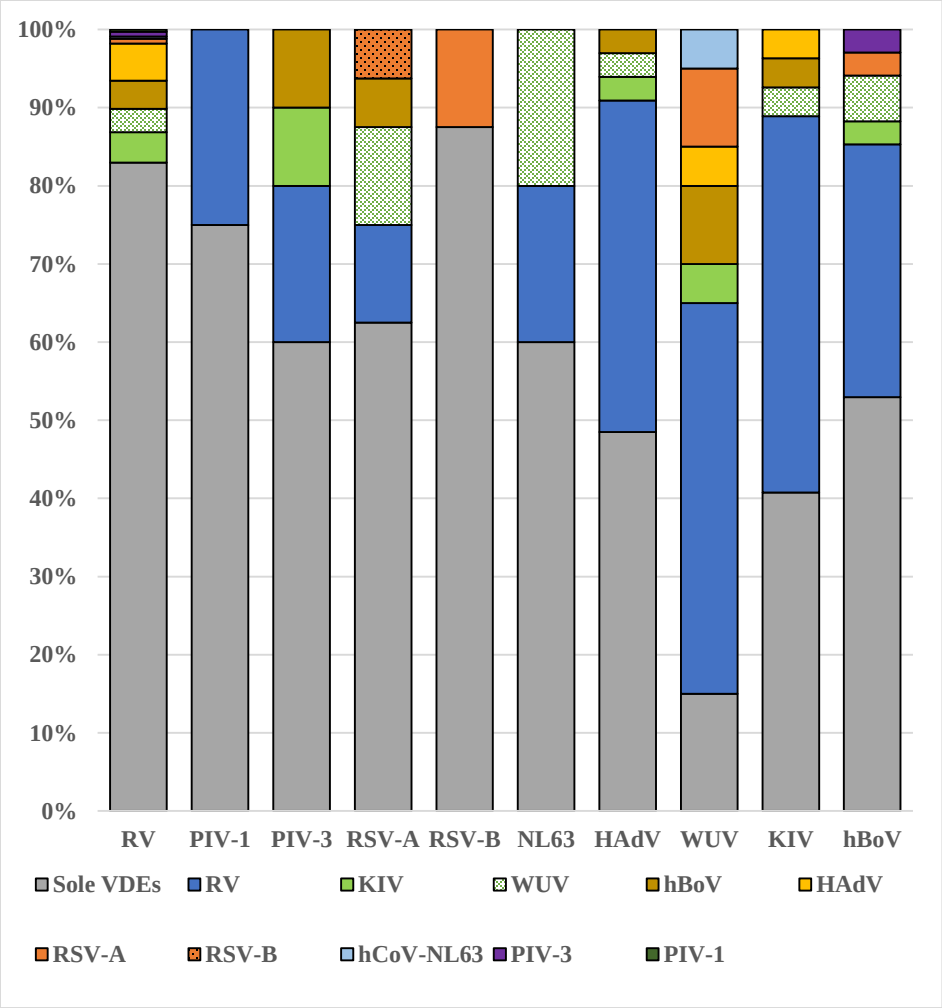


Figure 3.5 The percentage of multiple virus detection episodes for ten respiratory viruses. The denominator used is the overall number of VDEs

3.3.3.6 Respiratory virus Ct values

Overall, the median RV Ct value for the 656 RV-positive samples was 31.6 cycles (range 18.1-48.1 cycles), which was comparable to KIV, hBoV, WUV and the remaining RNA viruses. In contrast, PIV-1 and RSV-A provided relatively high Ct values in nasal swab samples (Table 3.4). HAdV positive samples provided Ct values that were significantly higher than all other DNA viruses and most RNA viruses (P= 0.01 compared to each of RV, WUV, KIV, WUV and the remaining grouped RNA viruses). The median Ct value obtained from the 38 samples positive for HAdV was 34.9 (range 24.9-41.0) cycles.

3.3.4 Respiratory virus infection episodes and ARIs

Of the 421 ARI episodes accompanied by samples, 220 (52.3%) were associated with one respiratory virus, 47 (11.2 %) were associated with two, 11 (2.7 %) were associated with three and three (0.7%) were associated with four respiratory viruses. No virus was detected in the remaining 141 (33.5%) ARI episodes. RV was the most commonly observed virus and was associated with 239 (56.7%) ARI episodes accompanied by samples. Table 3.3 describes the main features of ARI episodes associated with respiratory virus detection. The median (IQR) duration of all ARI episodes associated with viruses was nine (4-12) days, which was significantly longer than when no viruses were detected (median (1-36) days: $P < 0.001$; Mann Whitney U test). In general, ARI episodes associated with a sole virus were of shorter duration than episodes associated with more than one virus. For URTIs, the median duration of ARI episodes associated with RV alone (eight days) was significantly shorter than those associated with RV and other co-detected viruses (13 days; $P < 0.001$; Mann Whitney U test). Similarly, the median duration of LRTIs with RV alone (8.5 days) was significantly shorter than LRTIs associated with RV co-detected with other viruses (20 days; $P < 0.01$; Mann Whitney U test). For respiratory viruses other than RV, sole detection was also associated with shorter duration of URTI symptoms (n=28, median six days) than when multiple viruses were detected (n=3, median ten days) though numbers were too small to allow a robust comparison. Similarly, LRTI episodes associated with sole respiratory virus detections (n=2, median eleven days) and those associated with multiple viruses (other than RV; n=9, median nine days) were too small in number for any conclusions to be drawn. Figure 3.6 further compares the duration of URTI and LRTI episodes when RV or other viruses were detected and when no respiratory virus was detected.

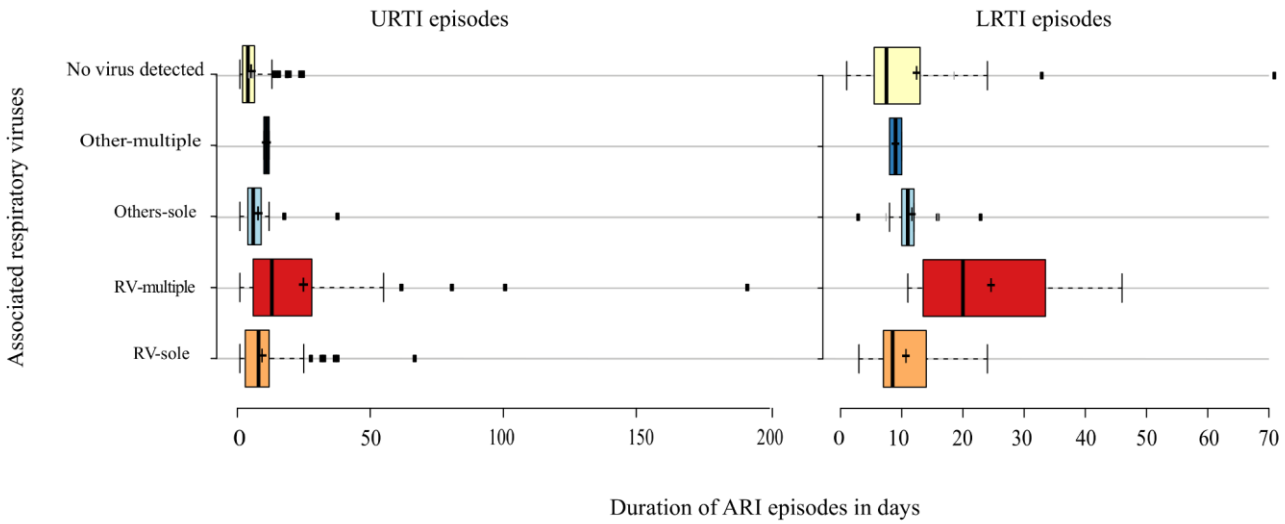


Figure 3.6 Comparison between the duration of URTI (left) and LRTI (right) episodes among our one year old cohort according to the respiratory viruses detected. The centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the IQR from the 25th and 75th percentiles. Episodes represented by dots were outliers; crosses represent sample means.

3.3.5 ARIs with virus detected and demographic data

Among the possible confounding factors only age and season had a significant impact upon the number of ARI episodes that were associated with virus detection (Table 3.5). The number of ARIs associated with viruses increased significantly in the second half of the first year of life (n=198; 72.5% of total ARIs in this age group) compared to the first six months of life (n=82; 52.2% of total ARI in this age group) (crude OR 2.22; 95% CI 1.46-3.36). The number of days involving ARIs during the last six months of infancy (3026 days) was also significantly greater than the numbers of days of ARI symptoms during the first 6 months of life (1359 days; $P < 0.001$; Mann Whitney U test) (Table 3.5; Figure 3.7).

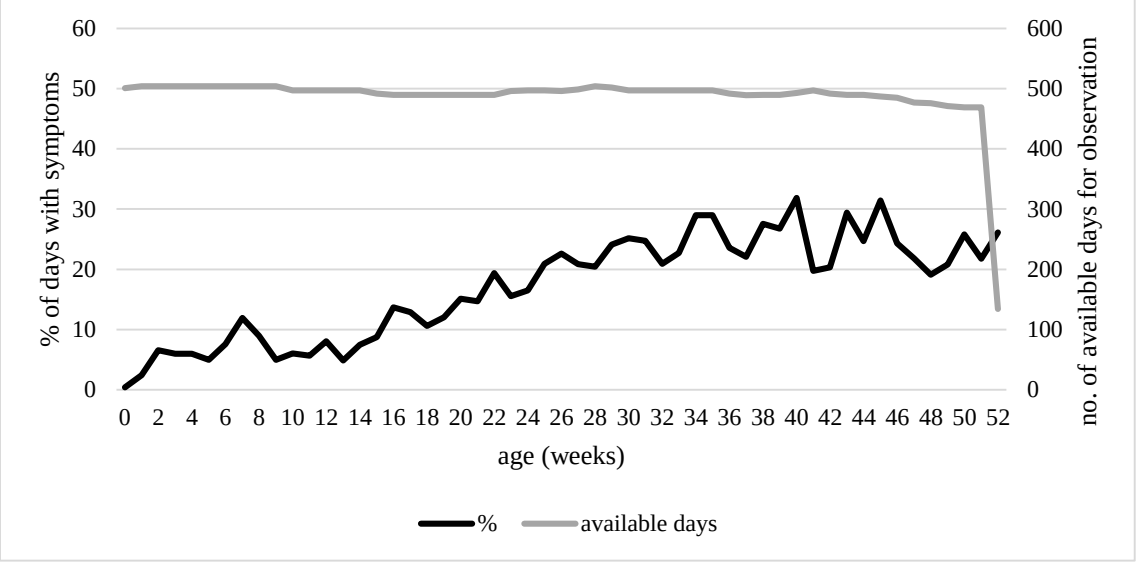


Figure 3.7 the number of days available for observation during the 52 weeks of the first year of life (grey) and the percentage of days with symptoms (black). The percentage of days with ARI symptoms increased by age.

Winter months were more likely to contain higher numbers of ARIs associated with viruses than other seasons (adjusted OR 2.4; 95% CI 1.29-4.69; $P < 0.006$). The association between the remaining confounding factors and ARI episodes with virus was limited. Table 3.5 provides a comprehensive univariate and multivariate analyses for the demographic data and the study outcomes.

Table 3.5 ARI episodes identified during the first year of life and their association with the cohort characteristics

Variable		Total ARIs		ARIs with virus associated		
		No of infants with ARIs (% of total)	**No. of ARI episodes (%)**	**No of episodes (%)**	**Univariate OR (95%); P value**	**Multivariate[a] OR (95%); P value**
Age quarter (mths)	0-3	30 (14.9)	52 (12.3)	26 (50)	1	1
	3-6	55 (27.4)	99 (23.3)	56 (56.5)	1.44 (0.69-2.9); 0.32	1.7 (0.79-3.85); 0.167
	6-9	56 (27.9)	132 (31.1)	96 (72.7)	**3.08 (1.49-6.37); 0.002**	**3.1 (1.24-8.06); 0.016**
	9-12	60 (29.8)	141 (33.3)	102 (72.3)	**2.78 (1.36-5.66); 0.005**	**4.38 (1.64-11.71); 0.003**
Gender	Male	34 (50)	195 (45.99)	132 (67.7)	1	1
	Female	34 (50)	229 (54.01)	148 (64.6)	0.79 (0.47-1.35); 0.405	0.68 (0.39-1.19); 0.182
Exposure to smoking	Mother	1 (1.5)	5 (1.2)	4 (80)	2.06 (0.15-27.25); 0.582	1.66 (0.12-23.0); 0.704
	Father	8 (12.1)	46 (11.1)	29 (63.04)	0.80 (0.35-1.84); 0.605	0.54 (0.22-1.37); 0.199
	No exposure	57 (86.4)	362 (87.7)	239 (66.02)	1	1
Mother IFV vaccine status	Had IFV vaccine	50 (74.6)	328 (77.7)	218 (66.4)	1.04 (0.56-1.95); 0.887	1.2 (0.63-2.28); 0.568
	No IFV vaccine	17 (25.4)	94 (22.3)	61 (64.9)	1	1
Number of children in the household[b]	No children	43 (63.2)	261 (61.6)	168 (64.4)	1	1
	One child	18 (26.4)	113 (26.7)	80 (70.8)	1.28 (0.69-2.34); 0.423	1.61 (0.8-3.2); 0.18
	≥ two children	7 (10.3)	50 (11.8)	32 (64)	0.99 (0.43-2.23); 0.983	1.04 (0.44-2.4); 0.92
Feeding	Exclusively breastfed (BF)	40 (36.4)	78 (18.4)	41 (52.6)	1	1
	BF and other milk	66 (60)	332 (78.3)	232 (69.8)	**2.09 (1.21-3.61); 0.008**	1.06 (0.49-2.2); 0.87
	Solids	4 (3.64)	14 (3.3)	7 (50)	0.68 (0.17-2.62); 0.17	0.29 (0.06-1.38); 0.121
Childcare status	Formal	23 (23.9)	80 (18.9)	53 (66.3))	1.005 (0.55-1.83); 0.98	0.69 (0.33-1.43); 0.323
	Informal	9 (9.4)	26 (6.1)	15 (57.7)	0.782 (0.29-2.10); 0.627	0.43 (0.15-1.26); 0.129
	Formal and informal	2 (2.1)	2 (0.47)	2 (100)	Not included[w]	Not included[w]
	No childcare	62 (64.6)	316 (74.5)	210 (66.5)	1	1
Season	Spring	50 (23.9)	97 (22.9)	56 (57.7)	1	1
	Summer	45 (21.5)	73 (17.2)	47 (64.4)	1.26 (0.65-2.47); 0.491	1.2 (0.59-2.53); 0.569
	Autumn	55 (26.3)	124 (29.2)	79 (63.7)	1.23 (0.691-2.21); 0.474	1.4 (0.74-2.64); 0.298
	Winter	59 (28.2)	130 (30.7)	98 (75.4)	**2.23 (1.22-4.08); 0.009**	**2.4 (1.29-4.69); 0.006**

[a] Adjusted for all variables in the table.

[b] other than the participating infant.

[w] Small number of observations, so the univariate OR and 95% CI were omitted.

Values in **bold** indicate statistical significance.

3.3.6 Asymptomatic VDEs

Overall, 147 VDEs (29.4%) were unaccompanied by symptoms. These asymptomatic VDEs were associated with twelve viruses (Table 3.3). More than two-thirds of the symptom-free VDEs (69%) were RV-DEs, including 110 RV-DEs (34% of total RV-DEs). Table 3.6 summarises the number of asymptomatic VDEs identified for different viruses. Almost half of the episodes associated with IFV (2/5) and KIV (12/25) were asymptomatic, but the numbers of episodes for the former in particular are very small. For the twelve respiratory viruses, Ct values obtained from asymptomatic VDEs were comparable to Ct values from symptomatic VDEs ($P \geq 0.05=$ for all viruses). Further, median virus shedding duration for the VDEs associated with symptoms were comparable to the median shedding duration in VDEs not associated with symptoms (one vs one week; $P \geq 0.05$ for all viruses). As shown in Table 3.6, while the number of asymptomatic VDEs remained roughly constant throughout the first year of life, their proportion fell as the overall number of VDEs increased with age. Asymptomatic VDEs were also less likely during the winter months compared to other seasons and when multiple rather than sole viruses were detected. In other words, to turn these results around, increasing age in the second half of infancy, the winter season and detecting multiple respiratory viruses are independent risk factors for ARI symptoms. There was also weak evidence that not being exclusively breast fed and those born higher in the birth order were less likely to have asymptomatic VDEs, though numbers of siblings and household size did not appear to have an effect and there were signals that possible confounding factors were influencing the results in both directions.

Table 3.6 Asymptomatic VDEs identified during the first year of life and their association with the cohort characteristics

Variable		number of subjects (% of all subjects)	No. of VDEs (%)	Asymptomatic VDEs		
				No. (%)	Univariate OR (95%); P value	*Multivariate OR (95%); P value
Age quarter (months)	0-3	44 (18.9)	64 (14.8)	35 (54.7)	1	1
	3-6	57 (24.5)	87 (20.1)	38 (43.7)	0.57 (0.27-1.23); 0.157	0.7 (0.3-1.66); 0.425
	6-9	67 (28.8)	155 (35.9)	44 (28.6)	**0.25 (0.12-0.52); 0.001**	0.54 (0.19-1.56); 0.262
	9-12	65 (27.9)	126 (29.2)	30 (23.8)	**0.18 (0.08-0.38); 0.001**	**0.31 (0.09-0.96); 0.043**
Gender	Male	35 (48.6)	217 (50.2)	77 (35.7)	1	1
	Female	37 (51.4)	215 (49.8)	70 (32.6)	0.96 (0.49-1.88); 0.92	1.34 (0.64-2.81); 0.427
Exposure to smoking	Mother	1 (1.4)	7 (1.7)	3 (42.8)	2.2 (0.17-29.3); 0.54	2.69 (0.16-44.9); 0.49
	Father	8 (11.4)	54 (12.9)	20 (37.0)	1.2 (0.48-3.49); 0.608	1.7 (0.55-5.26); 0.351
	No exposure	61 (87.1)	358 (85.4)	117 (32.7)	1	1
Mother IFV vaccine status	Had IFV vaccine	53 (74.7)	327 (76.9)	107 (32.8)	0.9 (0.44-1.9); 0.86	0.74 (0.32-1.72); 0.495
	No IFV vaccine	18 (25.3)	98 (23.1)	34 (34.7)	1	1
Participant infant order (mother)	First born	49 (68.1)	276 (63.9)	98 (35.6)	1	1
	Second	14 (19.4)	98 (22.7)	34 (34.7)	0.97 (0.43-2.2); 0.95	0.21 (0.03-1.27); 0.091
	Third	7 (9.7)	44 (10.2)	13 (29.5)	0.67 (0.22-2.1); 0.49	0.05 (0.001-2.29); 0.130
	Other	2 (2.8)	14 (3.2)	2 (14.3)	0.2 (0.02-2.1)	0.02 (0002-2.13); 0.103
Number of children in the household [a]	No children in the household	46 (63.9)	257 (59.5)	89 (34.8)	1	1
	One other child	18 (25)	122 (28.2)	44 (36.4)	1.06 (0.49-2.2); 0.866	0.73 (0.31-1.74); 0.48
	≥ two other children	8 (11.1)	53 (12.3)	14 (26.4)	0.57 (0.19-1.71) 0.323	0.39 (0.11-1.3); 0.137
Household size [b]	3	49 (68.1)	238 (55.1)	79 (33.3)	1	1
	4	14 (19.4)	107 (24.8)	39 (36.4)	1.24 (0.55-2.78); 0.59	0.86 (0.33-2.24); 0.77
	5	7 (9.7)	69 (15.9)	25 (36.2)	1.001 (0.38-2.61); 0.998	0.68 (0.22-2.11); 0.506
	≥ 6	2 (2.8)	18 (4.2)	4 (22.2)	0.52 (0.08-3.06); 0.473	0.62 (0.09-4.1); 0.627
Feeding	Exclusively breastfed	46 (38.7)	91 (21.1)	50 (54.9)	1	1
	Other milk	70 (58.8)	335 (77.6)	94 (28.1)	**0.24 (0.13-0.44); 0.001**	0.41 (0.16-1.01); 0.053
	Solids	3 (2.5)	6 (1.4)	3 (50)	0.57 (0.07-4.81); 0.612	1.03 (0.1-9.8); 0.979
Childcare status	Formal	19 (19)	67 (15.7)	18 (27.3)	0.48 (0.22-1.07); 0.075	0.99 (0.39-2.47); 0.983
	Informal	10 (10)	24 (5.6)	8 (33.3)	0.78 (0.24-2.49); 0.675	1.61 (0.44-5.8); 0.466
	Formal and informal	4 (4)	10 (2.3)	5 (50)	1.78 (0.29-10.66); 0.524	3.2 (0.39-26.2); 0.278
	No childcare	67 (67)	326 (76.3)	112 (34.3)	1	1

Table 3.6 continued. Asymptomatic VDEs identified during the first year of life and their association with the cohort characteristics

Variable		number of subjects (% of all subjects)	No. of VDEs (%)	Asymptomatic VDEs		
				No. (%)	Univariate OR (95%); P value	*Multivariate OR (95%); P value
Season	Spring	53 (23.1)	101 (23.4)	40 (39.6)	1	1
	Summer	56 (24.4)	81 (18.8)	33 (40.7)	1.17 (0.59-2.34); 0.63	0.83 (0.39-1.79); 0.648
	Autumn	58 (25.3)	113 (26.2)	37 (33.0)	0.75 (0.39-1.43); 0.39	0.56 (0.27-1.15); 0.12
	Winter	62 (27.1)	137 (31.7)	37 (27.0)	**0.45 (0.23-0.85); 0.015**	**0.34 (0.16-0.7); 0.003**
Number of respiratory viruses	One virus	72 (6739)	377 (87.3)	139 (36.9)	**1**	1
	≥ 2 viruses	34 (32.1)	55 (12.7)	8 (14.5)	**0.27 (0.11-0.64); 0.003**	**0.29 (0.12-0.82); 0.019**

Adjusted for all variables in the table.
[a] Other than the participating infant.
[b] Including the participating infant.
Values in **bold** indicate statistical significance.

3.4 Discussion

This analysis of a subset of infants participating in the ORChID study provides important data on respiratory viruses detected during the first year of life, including their shedding characteristics and association with ARI symptoms.

3.4.1 Prevalence of different respiratory viruses during the first year of life

In otherwise healthy infants, RVs were the most frequently detected viruses. This finding is broadly consistent with other infant cohort studies. Comparisons though are difficult because of varying study designs, including differences in study populations, sampling frequency, collection techniques and study duration.

The number and proportion of IFV detections in this sub-set of the ORChID community-cohort was low with only five infection episodes (3 IFV-A, 2 IFV-B) observed. Symptoms were associated with their detection in three of these cases. Similar low rates were also observed for many of the other established respiratory pathogens (eg. hMPV and PIV viruses) with the exception of HAdV (discussed below) and RSV A. This however is not to say that these viruses are not important viruses in the community (Nair et al., 2010), but simply in cohort studies of this nature they are observed less frequently. Overall, the rates observed differ from the higher proportions observed in hospital-based studies, and are likely due to more severe ARIs leading to hospital presentations, and hence over-representation in hospital cohorts. Similar observations have been made in other community-based birth and infant cohort studies. For example, in an Australian community-based study investigating the cost of influenza-like illnesses (ILIs) and economic burden in Australian children attending child care, a low prevalence of IFVs was observed despite using similar nasal swab and sample transport techniques to ORChID and the study being conducted during IFV season (Yin et al., 2013). Likewise, other community-based studies involving infants, older children and those attending day care, have found that RV and to a lesser extent RSV have predominated with low detection rates for the other RNA viruses, including influenza (Budge et al., 2014; Fairchok et al., 2010; Kusel et al., 2006; Lambert et al., 2007; Legg et al., 2005; Martin et al., 2013; van der Gugten et al., 2013; van der Zalm et al., 2009). Nevertheless, the rate of RSV detection we observed was lower than expected (Simoes, 1999; Woensel et al., 2003). This might be because of the lower sensitivity of nasal swabs for detecting mild RSV cases where viral loads are likely to be low and is an observation that has been reported elsewhere (Meerhoff et al., 2010). It also probably reflects the nature of the cohort (many from single child, non-smoking families, high breast feeding rates and low child care attendance) and that we only investigated the first year of life. RSV rates are likely to

increase in the second year of life and by their second birthday almost 90% of children should have had at least one RSV infection (Simoes, 1999). Further data will be available after the completion of the ORChID study.

An alternative explanation for the low prevalence of respiratory viruses other than RV detected in this cohort could be that by taking nasal swabs and mailing them into the laboratory there may have been some loss of sensitivity in detecting these viruses. However, on the whole this would seem unlikely based upon our own experience and studies conducted by others. Direct comparisons between health care worker collected specimens and those performed by parents and mailed into the laboratory showed no decrease in sensitivity (van der Zalm et al., 2006). Similarly, there was no loss in detection yields between samples immediately frozen and those sent by regular mail over long distances (O'Grady et al., 2011). Furthermore, stability of viruses at room temperature and in the mail was examined in a pilot study in the laboratory. Briefly, five influenza-A positive NPAs with different concentrations were used to inoculate a total of 45 Virocult swabs. Each strain was inoculated onto nine swabs which were then treated in nine different ways including being immediately frozen, left on the laboratory bench for 1, 3, 5 and 7 days, or otherwise sent by post and received back within 1, 3 5 and 7 days prior to freezing and testing. Compared to the immediately frozen swabs, there was no substantial change to the Ct values of the influenza-A detection, irrespective if they sat on the bench or mailed, or over time; all average Ct values were within 3 cycles showing that any decrease in viral load was within 1 log. Frequent and regular sampling may also help overcome any mild loss of sensitivity, especially for HAdV where adding oropharyngeal swabs can increase detection yields (Hammitt et al., 2011; Meerhoff et al., 2010).

It was also interesting that after RV, DNA viruses were the next four most commonly detected viruses (KIV, hBoV, HAdV and WUV). These were the only four DNA viruses investigated. While HAdV is a well-recognised respiratory pathogen, the clinical significance of the human polyomaviruses KIV and WUV, and of hBoV in childhood remains controversial (Babakir-Mina et al., 2013; Bialasiewicz et al., 2007; Payungporn et al., 2008; Schildgen et al., 2008), The Ct values observed for HAdV real-time PCR were typically very high compared with almost all other viruses. Such high values are indicative of low viral loads and raise questions over whether such low loads are indeed consistent with disease. On the other hand, HAdVs exhibit much sequence diversity and thus the observed Ct values could be due to technical problems with the PCR assay itself, which in light of the discussion in the previous paragraph might also impact adversely upon assay sensitivity. This potential issue is explored in chapter-5.

3.4.2 Prolonged viral shedding?

Previous studies have suggested that DNA viruses are shed for longer periods than RNA viruses (Gangell et al., 2014; Martin et al., 2010), which is in general agreement with our observations. Prolonged VDEs of three or more consecutive weeks were observed mainly in DNA viruses: KIV, WUV, HAdV, and hBoV, although this also occurred on occasions with some RNA viruses, namely hCoV-NL63, RSV-A and RV. However, given the high prevalence of RV infections in our study, combined with the known diversity in RV and HAdV genotypes, I hypothesised that the longer duration of shedding observed within RV was unlikely to be caused by a single virus in each case, and that genotype analysis would reveal diverse RV strains (Jartti et al., 2008; van der Zalm et al., 2011). For instance, a prolonged RV-DE was identified in infant 023 (see Appendix-1; Page VI). This RV-DE continued for nine weeks (63 observation days) with only one RV-negative interval swab. Symptoms were first reported on day 37 following the initial detection and continued until the last day of detection (day 63). During the nine weeks of RV detection no other virus was detected. RV-genotyping is important not only to further understand the patterns of the prolonged RV-DEs, but also to investigate the clinical role of different RV-genotypes. Hence, the application of RV genotyping to our RV-positive samples could help address questions relating to the clinical significance of individual RV strains, and forms the basis of studies conducted in chapter-6 of this thesis.

3.4.3 VDEs and ARIs

Overall for our cohort, 65% of ARIs were associated with at least one respiratory virus. This proportion is similar to rates observed in an earlier Australian community-based cohort of infants at high risk for asthma (69%), but lower than the 79-85% reported for infant cohort studies from overseas (Kusel et al., 2006; Legg et al., 2005; Regamey et al., 2008). Differences in methodologies and ARI definitions used in these studies may partially account for this observation. For instance, the ORChID study employed daily symptom diaries and weekly parent-collected specimens without needing health care workers to contact the family. This strategy, as well as the broader definition of ARI episodes used, may have increased the total number of captured ARI episodes, including those with milder symptoms, which serve as the denominator (Regamey et al., 2008). While Kusel et al also used symptom diaries to collect clinical data, health care workers still needed to visit the family and the mean number of captured ARI episodes was 4.1 compared to a mean of 6.29 ARI episodes identified in our study (Kusel et al., 2006). However, the method of data collection by its own should not eliminate other important factors such as geographic location and demographic features of the study cohorts. Three participating infants did not report any ARIs during their first year of

life, despite viruses being detected in 14 of the provided swabs (infants 015, 044 and 048; Tables: 3.2 and 3.3). A broader definition of ARI did not change these outcomes significantly (data not shown), a finding that suggests an ARI-free year for these infants. Similar cases have been described by another cohort study in which 22% of the study population (n=88) did not experience any ARI during their first year of life, though the documentation of ARIs relied upon the family contacting research staff and thus some episodes may have been missed (Legg et al., 2005; van der Zalm et al., 2006).

Another interesting observation was detecting different viruses at different time-points during the same ARI episode. This was noted during both brief and prolonged ARIs. An example of this was during a 46-day ARI episode observed for infant 010, from whom three different viruses were detected in each of the six swabs provided during this episode (infant 010; Appendix-1-; Page III); starting with a sole hBoV detection, followed by a four week RV-DE, which itself preceded a two week HAdV-DE. Similar multiple VDEs during single ARIs were observed for infant 019 (Appendix-1; Page V), Infant 027 (Appendix-1; Page VII), and infant 062 (Appendix-1; Page XVI). It is likely that these multiple VDEs underpin what many parents believe to be a single "ARI" episode. The numbers here are too small to provide any meaningful data, however it is envisaged that such questions will be answered upon completion of the broader ORChID project.

Similar to above, we also observed numerous single ARIs that had multiple viruses detected at the same time point (i.e. co-detections; Tables 3.3 and 3.5). Co-detections are often observed in hospital cohorts and in some other studies where they can be associated with more severe disease (Greer et al., 2009; Lauinger et al., 2013; Schildgen et al., 2008). Here we found that co-detections are also very common in the community and that they too were more likely to be associated with symptoms (Jartti et al., 2008). They also pose a challenge when trying to determine which viruses may be responsible for causing the presenting illness. This is a finding that is being explored further in the larger ORChID cohort (chapter-7 further discusses this point).

Both age and season had a significant influence over the frequency of recorded ARI episodes. ARIs were less likely in the first six months of life, probably because of protective maternal antibodies from the placenta, breast feeding and to a lesser likelihood of being enrolled in day care. While for season, the increase in ARI episodes during winter could be explained by greater exposure to circulating respiratory viruses, which survive better in the conditions of lower temperature and humidity levels encountered at these times. In addition, the cooler and dry winter environment can dry out nasal mucous membranes impairing local defence barriers against viruses, lower levels of

sunlight lead to declining vitamin D levels, potentially further impairing innate and adaptive immunity. Finally, winter is associated with increased indoor or household crowding, further increasing the risk of respiratory virus transmission.

3.4.4 Asymptomatic VDEs as nascent infections before symptoms develop

A further factor that complicates the symptom data was that respiratory viruses were often detected immediately prior to the onset of an ARI episode, but were not always detected a week later during the ARI episode. An example of the latter was a single detection of PIV-3 that was observed two days prior to an 18 day ARI episode for infant 010 (Appendix-1; Page III). Although none of the three subsequent swabs collected during this ARI were positive for any respiratory virus, this PIV-3 detection was still considered associated with the ARI. The subsequent failure to detect PIV-3 for this infant may have been caused by a rapid decline in viral load beyond the sensitivity of the assay, and thus may indicate early clearance of infection. If so, this highlights the usefulness of the weekly sampling strategy used in this study to maximise the probability of capturing respiratory virus associated ARIs. Alternatively, it is also possible that the PIV-3 detection in this case was unrelated to the subsequent ARI, and thus could potentially overestimate virus-associated ARIs.

3.4.5 Questions over parental-collected nasal swabs and symptom data

While swab and diary return rates were high overall, analysis of the real-time PCR and symptom data did raise questions regarding the *quality* of collection for some infants, despite the discussion outlined in section 3.4.1. For the nasal swabs collection, infant 048 is one particular example. This infant did not have any recorded ARI symptoms and was negative for RV during their first year of life. While plausible, this was unusual within the characteristics of this cohort. In addition to infant 048, there were 11 other infants who were negative for all respiratory viruses (other than RV) and had lower proportions of RV detected compared to other infants. These findings raised further questions regarding the quality of nasal swab sample collection and sample transportation and became the subject of further analysis described in chapter-4.

3.5 Conclusion and directions

This study described respiratory virus shedding during the first year of life, including those associated with ARIs. The high sample and data return rates obtained from participating infants by the end of their first year of life provide a strong basis to better understand the patterns of viral-associated ARIs in this age group. It was observed that RV was the most frequently detected virus in ARIs in otherwise healthy infants. Ct values were not associated with symptoms in this setting

however and further analysis is needed to confirm this preliminary finding. The rate of ERV-3 negative samples and further observations obtained from nasal swab samples provided by some infants raised questions over the quality of sample collection. Further, PCR results obtained from HAdV screening and emerging information of HAdV sequence variation raised concerns about the suitability of the assay used for this longitudinal, multiple season study. These technical aspects are covered in the following chapters (chapter-4 and 5). Finally, given the overall high rates of RV-DEs described in this chapter, further investigations became warranted to study the nature of RV-DEs. In chapter-6, I investigate the RV genotypes contributing to these episodes, whether prolonged shedding is associated with genotype or strain substitutions, and their association with symptoms.

3.6 References

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Chapter 4

The quality of nasal swab samples:

Importance of optimal collection and transportation for pragmatic community-based studies.

Publications based on this chapter

- Alsaleh, A. N., Whiley, D. M., Bialasiewicz, S., Lambert, S. B., Ware, R. S., Nissen, M. D., Sloots, T. P., et al. (2014). Nasal swab samples and real-time polymerase chain reaction assays in community-based, longitudinal studies of respiratory viruses: the importance of sample integrity and quality control. BMC infectious diseases, 14(1), 15. doi:10.1186/1471-2334-14-15

4.1 Overview

Recently introduced molecular-based viral diagnostic techniques have much improved sensitivity compared with previous classical culture and phenotypic-based methods and have also led to the discovery of new respiratory viruses (Beck and Henrickson, 2010). However, contemporary studies employing these new techniques have often used convenience samples obtained from patients admitted to hospital or attending Emergency Department clinics (Arden et al., 2006; Calvo et al., 2010; Sloots et al., 2006). Similarly, community-based studies have relied upon clinic or home visits by trained healthcare workers to collect specimens during an ARI episode. For community-based studies the latter can impose restrictions upon busy families and may lead to biased disease estimates and specimen availability (Jartti et al., 2008; Legg et al., 2005; van der Zalm et al., 2006). In addition, the cost and feasibility of using healthcare workers may impinge upon study feasibility, particularly when large longitudinal, community-based cohort studies, involving frequent specimen collections, are planned. To help address these limitations, we and others have begun testing parent-collected, anterior nasal swab specimens that have been transported to the research laboratory using the standard mail (Heikkinen et al., 2002; Meerhoff et al., 2010; van der Zalm et al., 2011, 2009). This approach is considered to be safe, convenient and cost-effective (Lambert et al., 2008a).

Importantly, when using highly sensitive PCR assays, the detection of respiratory viruses is roughly similar for both anterior nasal swab specimens and samples collected by the more traditional NPA technique (Lambert et al., 2008b; Waris et al., 2007). Building on this information, later studies have also shown that PCR testing for respiratory viruses provided similar results from parent-collected anterior nasal swab specimens and from either nasopharyngeal swabs or NPAs collected by healthcare professionals (Lambert et al., 2008a; Meerhoff et al., 2010). Other studies examining sample transport have also shown that mailing swabs at ambient temperature has limited or no impact upon respiratory virus detection by PCR (Akmatov et al., 2011; O'Grady et al., 2011; van der Zalm et al., 2011), although the need to further investigate the effects of transporting samples for extended periods and at higher temperatures was highlighted in one study (O'Grady et al., 2011).

As described in the preceding chapters, the ORChID project is a prospective community-based, dynamic, longitudinal cohort study, which seeks to describe the nature and timing of respiratory viruses detected in Australian children during the first two years of life (Lambert et al., 2012). The study commenced in late 2010 and involves parents collecting and mailing nasal swabs weekly to the research laboratory for PCR-based respiratory virus screening. During the first year of the study

mould was seen in some samples reaching the laboratory and we became concerned about the impact of this contaminant upon sample integrity. Therefore, in this study, we undertook a broader investigation of sample quality, examining collection and transportation and how these impact upon respiratory virus detection. Our objectives were first to determine the quality of specimen collection by testing for the presence of human DNA (ERV-3) and then to investigate sample quality by analysing PCR performance when visible mould is present in samples reaching the laboratory.

The hypotheses were:

1- ERV-3 as a marker for human DNA can be used to inform the quality of nasal collection.
2- Visible mould in the arriving nasal swabs are due to prolonged times in reaching the laboratory and other environmental factors.
3- Visible mould may impact upon the performance of real-time PCR.

4.2 Material and methods

Nucleic acids were extracted from nasal swabs collected as part of ORChID and tested as outlined in chapter-2. Additional methods relating to this chapter (and not detailed in chapter-2) are provided below.

4.2.1 Fungal testing

During the initial phases of the study, mould was observed on some nasal swabs at the time of their arrival at the laboratory. In light of this observation, before extraction all swabs were inspected visually for mould and were assigned a semi-quantitative score according to a sliding scale (0 to 3), whereby 0 = no mould observed, 1 = low, 2 = medium, and 3 = high levels of visible mould present (Figure 4.1).

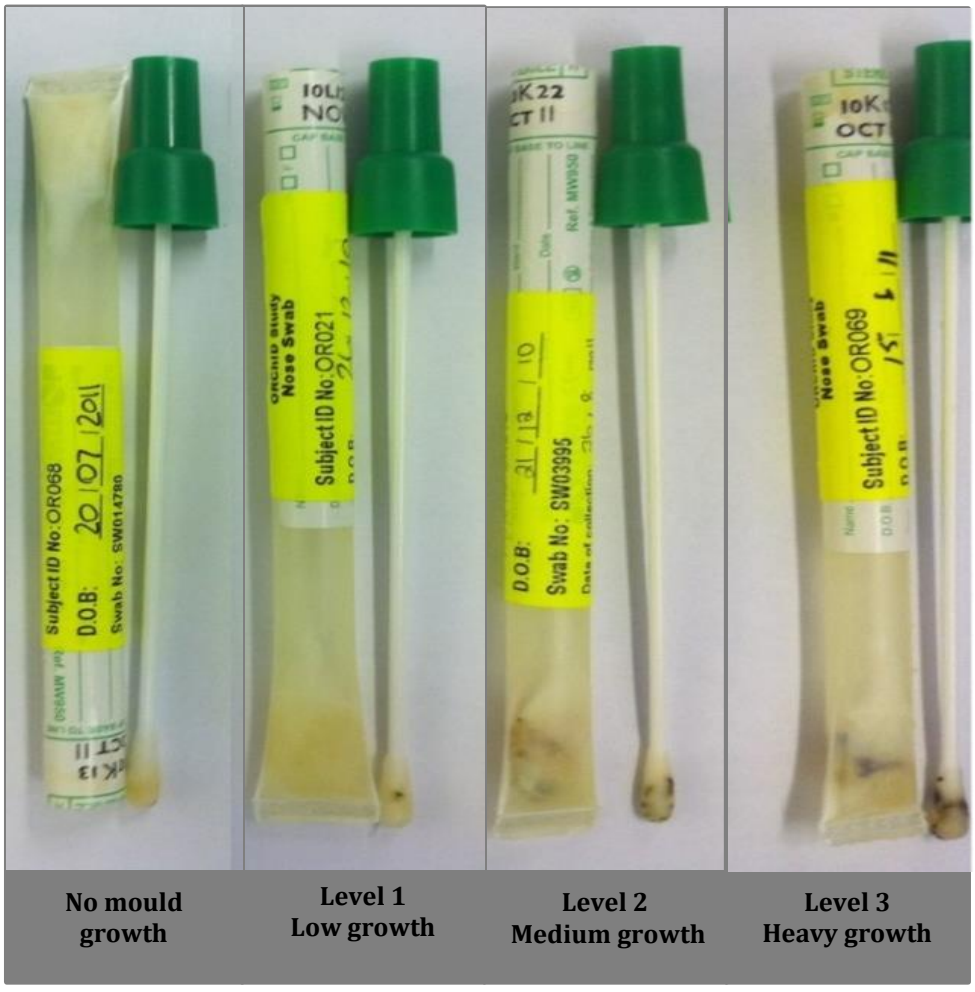

Figure 4.1 Mould growth was observed in some swabs reaching the laboratory. The level of mould growth was assigned a semi-quantitative sliding scale.

DNA sequencing was used to identify the types of fungi present on a subset of swabs exhibiting varying degrees of visible mould growth (ten swabs where no mould was seen, and 20 each where low, medium and high levels of mould contamination were present respectively).

PCR amplification of a highly conserved fungal internal transcribed spacer (ITS) region was performed using 10 pmoles of forward and reverse primers (ITS1 forward primer TCCGTAGGTGAACCTGCGG and ITS4-reverse primer TCCTCCGCTTATTGATATGC (Pryce et al., 2006)**,** 25µL of Qiagen SYBR® master mix (Qiagen, Australia) and 5µL of template in a 50µL reaction mix. Amplification was performed using the following conditions: 95°C for 15-minutes followed by 45 cycles of 95°C for 30-seconds, 50°C for 30-seconds and 72°C for 60-seconds and a melting step of 60-95°C at the end of the thermal cycling during which florescence data were acquired continuously. An aliquot of each PCR product was examined using 2% agarose gel electrophoresis and the remaining PCR product was sent to the Australian Genome Research Facility (The University of Queensland, Brisbane) for automated sequencing.

4.2.2 Exclusion criteria

For this study, samples that failed EHV-1 criteria (as mentioned in chapter-2; section 2.1.4.2) or were not inspected for mould growth were excluded from the analysis.

4.2.3 Data analysis

The association between variables of interest and binary outcomes was investigated using mixed effects logistic regression models, with participants included as a random intercept to account for possible correlated outcomes within each infant. The association with continuous outcomes was investigated using mixed-effects linear regression. When examining the association of mould level with sample quality and respiratory virus detection we conducted both univariate and multivariate analyses, with multivariate analyses adjusting for the potential confounders of the child's age, sex, relationship of collector to participant (e.g. father, mother or others), the season the specimen was collected, and time from specimen collection to being frozen in the laboratory. Analyses were conducted using Stata statistical software v.11.0 (StataCorp, College Station, TX, USA).

4.3 Results

4.3.1 Sample numbers

Between September 2010 and July 2012, 152 infants were recruited into the study. All participants lived within the greater Brisbane metropolitan area and none were from rural communities. One-hundred and twenty-five recruits remained active study participants up until the date of this analysis. Of the 27 withdrawals, four had moved out of the study area, two others were later deemed ineligible, ten withdrew for personal reasons and eleven were unable to fulfil sampling requirements. For the remaining families, swab return rates were >90% yielding almost 35,000 child-days of observation. In total, 4,933 weekly nasal swab specimens (~510 nasal swabs/month) were batched in 56 (96 well) racks, extracted and tested. The median time from collection to swab arrival in the laboratory was two days (interquartile range 2-4 days); however 10.9% of swabs were received more than seven days after their collection.

4.3.2 Excluded samples

During EHV-1 extraction and inhibition testing, 42 (0.81%) DNA extracts failed to achieve the specified EHV-1criteria and were excluded. The initial 1,525 samples were not inspected for mould growth during the early stages of the study and therefore were also excluded from further analysis.

4.3.3 ERV-3 detection

Of the remaining 3,366 samples, there were 2,718 (80.7%) samples positive for ERV-3 with PCR amplification Ct values ranging from 23-45 (median 36) cycles. Overall, ERV-3 was not detected in 649 (19.2%) samples. During the first eight months of batching and screening conducted in the laboratory, the number of ERV-3 negative samples ranged from 11 to 25 in each of the 56 extraction runs with a median of 17 negative samples per run (Figure 4.2).

However, following a cluster of samples that were negative for ERV-3 (Figure 4.2; batches 41, 43, 44) study nurses contacted parents and reminded them of the optimal swab collection technique they had been shown at enrolment of their infant. After this feedback the numbers of ERV-3 negative samples declined.

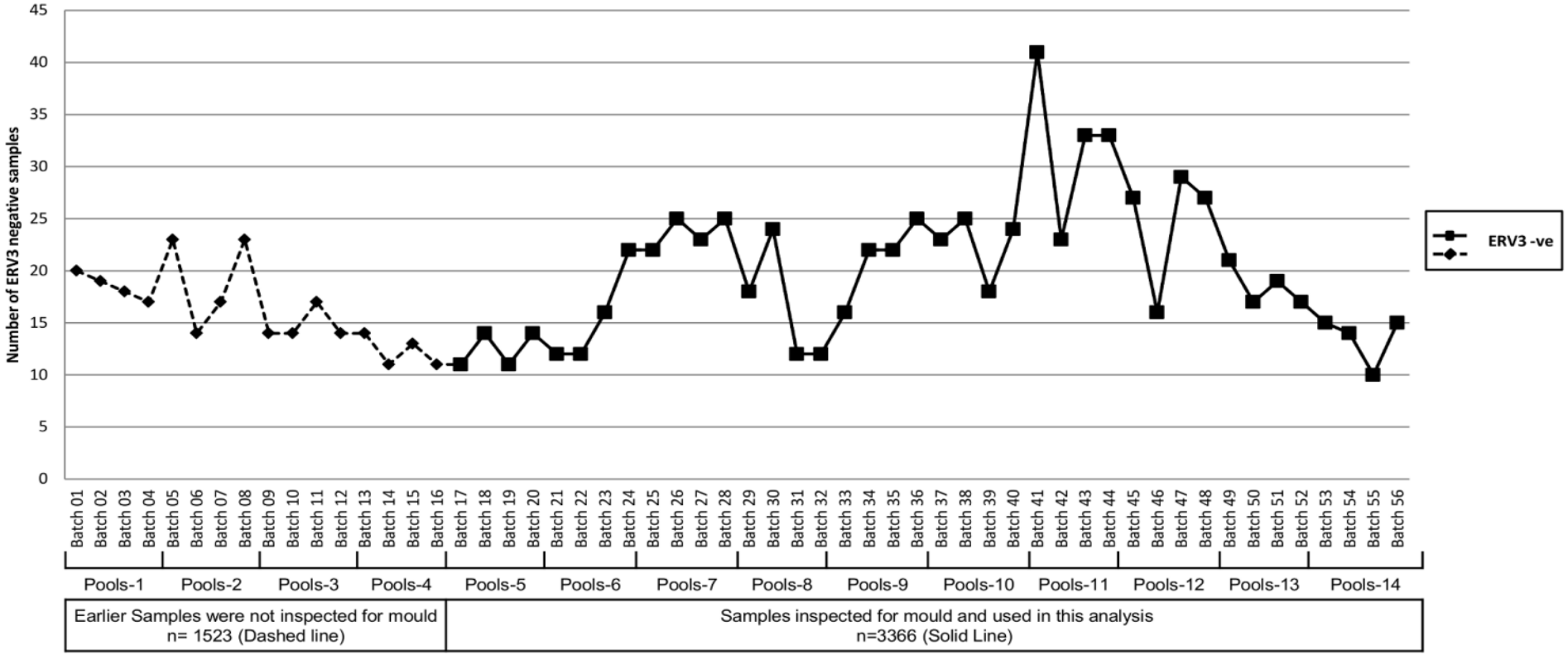

Figure 4.2 Number of samples negative for ERV-3 during screening of each sample extraction batch (92 samples per batch). A total of 56 extraction batches were performed in the first 20-months of the study. The quality of each batch was tested using EHV-1/ERV-3 PCR, following which every four batches were pooled and pools were screened for respiratory viruses other than RV. The batches included in the analysis are indicated by the solid line.

4.3.4 Respiratory viruses detected

The respiratory virus detections for the infant cohort that I followed have been detailed already in chapter-3. Of the 3,366 samples from this particular study at least one respiratory virus was detected in 885 (26.2%) samples. Dual or multiple virus detections were observed in 105 (2.14%) samples. RV was the most frequent virus detected, being present in almost 20% of nasal swab samples, followed by hBoV, KIV, HAdV and RSV-A (Table 4.1).

Table 4.1 Results for respiratory virus screening from 3366 parent collected nasal swab specimens between July 2011 and July 2012 and fulfilling the EHV-1 criteria

Virus	No. of infants	No. of samples	% of all samples
RV	105	726	21.57
hBoV	26	46	1.37
KIV	17	41	1.22
HAdV	23	30	0.89
RSV-A	26	30	0.89
WUV	13	28	0.83
hCoV-NL63	12	16	0.48
IFV-B	11	11	0.33
hCoV-229E	3	6	0.18
PIV-1	6	6	0.18
HMPV	5	5	0.15
PIV-3	3	3	0.09
hCoV-HKU1	3	3	0.09
IFV-A	2	2	0.06
RSV-B	2	2	0.06
hCoV-OC43	1	1	0.03
PIV-2	0	0	0

RV, rhinovirus; **HAdV**, human adenovirus; **hBoV,** human bocavirus; **hCoV**, human coronavirus; **HMPV**, human metapneumovirus; **IFV**, influenza virus; **PIV**, parainfluenza virus; **KIV**, KI-polyomavirus **RSV,** respiratory syncytial virus **WUV**, WU- polyomavirus;

4.3.5 Mould

Of 3,366 swab samples visually inspected for mould, 99 (2.9%) had high, 252 (7.5%) medium and 411 (12.2%) had low levels present, while 2,604 swabs (77.4%) had no visible signs of mould. The mean (SD) time from collection until being frozen at the laboratory for samples with no observed mould was 2.9 (3.0) days. In comparison for low level mould it was 4.9 (3.6) days (crude mean difference compared with no mould; = 1.7; 95% CI 1.4 – 2.1 days), for medium level mould it was 7.4 (4.9) days (3.9; 95%CI 3.4 – 4.3), and for high level mould 11.4 (10.7) days (7.1; 95%CI 6.4 – 7.8). The mean difference in time from collection until being frozen between each mould group and the no mould group was statistically significant (P<0.001 for each comparison). A significant association was also observed between mould and season. In specimens collected in summer, mould was observed in 28.2% of swabs. In comparison mould detection rates were 31.0% in spring (crude OR 1.08; 95% CI 0.87 – 1.34), 15.8% in autumn (OR 0.47; 95%CI 0.37 – 0.59) and 13.7% in winter (OR 0.40; 95%CI 0.29 – 0.53). However, when considering samples that contained mould, there was no statistically significant association between season and level of mould.

Fungal identification was achieved for 48 of 70 swabs subjected to PCR and DNA sequencing (Table 4.2). A diverse range of species was observed with *Epicoccum nigrum* and *Cladosporium cladosporioides* the most prevalent.

Table 4.2. Species detected in samples with different levels of fungal growth

Species.	**Number of swabs contaminated by this species.**	**Number of swabs and degree of contamination. (high; medium; low; no visible mould)**
Epicoccum nigrum	15	(7,2,4,2)
Cladosporium cladosporioides	7	(3,3,1,0)
Aureobasidium pullulan	4	(1,1,2,0)
Cryptococcus flavescens	3	(1,2,0,0)
Alternaria alternata	2	(1,1,0,0)
Alternaria tenuissima	1	(0,0,1,0)
Aspergillus westerdijkiae	1	(0,0,1,0)
Candida parapsilosis	1	(0,1,0,0)
Cladosporium silenes	1	(0,0,0,1)
Cladosporium tenuissimum	1	(0,1,0,0)
Cladosporium uredinicola	1	(1,0,0,0)
Cochliobolus lunatus	1	(0,1,0,0)
Curvularia brachyspora	1	(0,1,0,0)
Curvularia trifolii	1	(0,1,0,0)
Leptosphaerulina australis	1	(0,1,0,0)
Paraphaeosphaeria sp	1	(1,0,0,0)
Penicillium fellutanum	1	(0,0,1,0)
Penicillium oxalicum	1	(0,1,0,0)
Penicillium polonicum	1	(0,0,0,1)
Penicillium spinulosum	1	(0,0,1,0)
Phoma herbarum	1	(0,0,1,0)
Rhodotorula slooffiae	1	(0,1,0,0)
Total	**48**	

4.3.6 ERV-3, visible mould and respiratory virus detection

Of the 2,718 samples that were ERV-3 positive, 810 (37.2%) had at least one respiratory virus detected by PCR. In contrast, the respiratory virus detection rate in ERV-3 negative samples was significantly lower (75/649, 11.5%; crude OR 0.35; 95% CI 0.27-0.44) when ERV-3 was absent in swab specimens. We also observed that among ERV-3 positive swabs, the average ERV-3 Ct value for samples positive for any respiratory virus (32.8 cycles) was significantly lower (indicating greater ERV-3 load) than the average Ct value (35.4 cycles) in samples negative for all viruses (crude difference = 2.6, 95% CI 2.3 – 2.9; Figure 4.3).

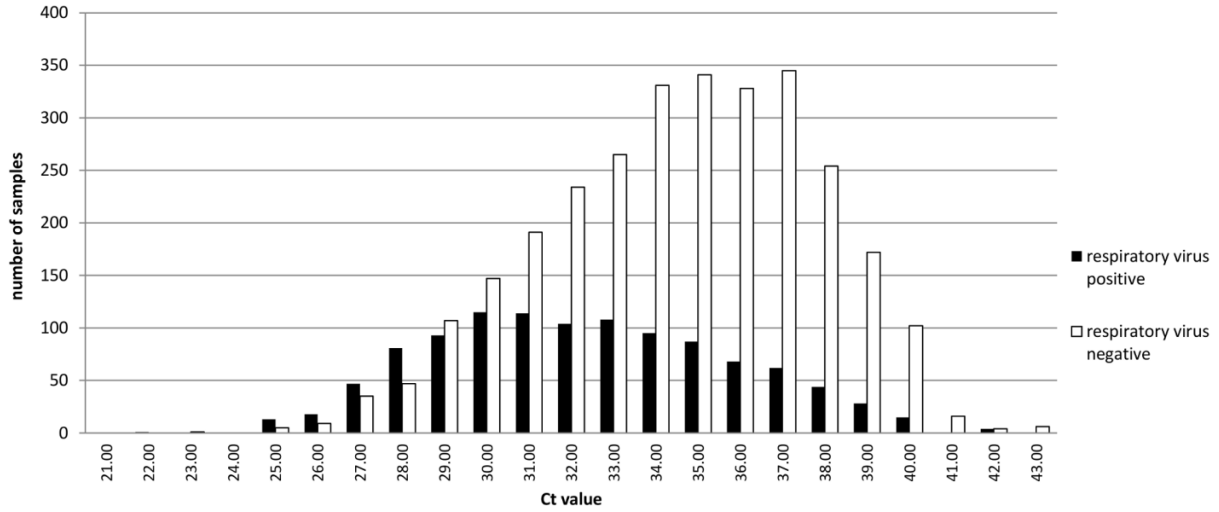

Figure 4.3 Comparison between average ERV-3 cycle threshold (Ct) values in respiratory virus positive (dark bars) versus negative (light bars) samples. In ERV-3-positive samples, the average ERV-3-Ct values (32.8) in samples positive for any virus was significantly lower than the average ERV-3-Ct values (35.4) in samples negative for all viruses (difference = 2.6, 95% confidence interval 2.3-2.9).

Moreover, there was a significant difference in ERV-3 Ct values (P=0.001) in samples that had a single respiratory virus detection (average = 33.01 cycles) compared with samples that had multiple respiratory viruses detected (average = 31.27 cycles).

Of the 762 samples with visible mould, 529 (69.4%) were positive for ERV-3, which was significantly lower than rates in samples without visible mould (84.0%; crude OR 0.35, 95% CI 0.28-0.43). The proportion of samples with visible mould and positive respiratory virus testing (178/762; 23.4%) was significantly lower than proportion of samples without mould (707/2606; 27.1%; crude OR 0.70, 95% CI 0.57-0.86).

Table 4.3 examines the association between ERV-3 and respiratory virus detection and potential explanatory and confounding variables. ERV-3 positive sample rates increased with age, varied by season and declined with increasing mould levels and time taken for samples to reach the laboratory and to be frozen. Similarly, respiratory virus detection rates increased with age, specimen collection outside the summer months, and time taken to reach the laboratory, while decreasing as visible mould levels in samples reaching the laboratory increased.

Table 4.3 ERV-3 and respiratory virus positive samples detected by polymerase chain reaction assays in 3366 parent-collected nasal swab specimens

Variable		No. samples (%)	ERV-3 Positive			Respiratory virus positive		
			No. samples (%)	Univariate OR (95% CI); P value	*Multivariate OR (95%); P value	No. samples (%)	Univariate OR (95% CI); P value	*Multivariate OR (95%); P value
Age (months)	< 6	1293 (38.4)	995 (77.0)	1	1	208 (16.1)	1	1
	6-<12	1295 (38.5)	1061 (81.9)	1.20 (0.94-1.53); 0.15	1.28 (0.98-1.68); 0.07	411 (31.7)	**2.59 (2.07-3.24); <0.001**	**2.38 (1.89-3.01); <0.001**
	≥12	778 (23.1)	662 (85.1)	**1.49 (1.06-2.10); 0.02**	**1.93 (1.27-2.93); 0.002**	266 (34.2)	**2.98 (2.26-3.92); <0.001**	**2.16 (1.57-2.99); <0.001**
Sex	Male	1647 (48.9)	1335 (81.1)	1	1	461 (28.1)	1	1
	Female	1719 (51.06)	1383 (80.4)	0.81 (0.54-1.22); 0.32	0.87 (0.58-1.29); 0.48	424 (24.7)	0.82 (0.60-1.12); 0.21	0.83 (0.61-1.12); 0.23
Collector	Mother	2845 (84.5)	2307 (81.1)	1	1	766 (26.9)	1	1
	Father	441 (13.1)	342 (77.6)	0.91 (0.66-1.27); 0.60	0.87 (0.62-1.22); 0.42	109 (24.7)	0.94 (0.70-1.26); 0.67	0.88 (0.65-1.19); 0.41
	Research staff	45 (1.3)	40 (88.9)	2.71 (1.00-7.36); 0.05	1.76 (0.65-4.81); 0.27	3 (6.7)	**0.24 (0.07-0.79); 0.02**	0.36 (0.11-1.21); 0.10
	Other	35 (1.0)	29 (82.9)	1.31 (0.49-3.51); 0.59	1.39 (0.46-4.16); 0.56	7 (20.0)	0.72 (0.30-1.74); 0.47	0.87 (0.35-2.13); 0.76
Season	Summer	926 (27.5)	729 (78.7)	1	1	178 (19.2)	1	1
	Autumn	1059 (31.5)	802 (75.7)	0.90 (0.71-1.13); 0.37	**0.74 (0.58-0.96); 0.02**	304 (28.7)	**1.99 (1.59-2.49); <0.001**	**1.74 (1.38-2.20) ;<0.001**
	Winter	541 (16.1)	482 (89.1)	**2.63 (1.87-3.70); <0.001**	**2.41 (1.67-3.49); <0.001**	198 (36.6)	**3.06 (2.36-3.97); <0.001**	**2.63 (2.01-3.45); <0.001**
	Spring	840 (25.0)	705 (83.9)	**1.39 (1.07-1.79); 0.01**	**1.50 (1.13-1.99); 0.005**	205 (24.4)	1.27 (1.00-1.61); 0.05	**1.43 (1.11-1.84); 0.005**
Mould	None	2604 (77.4)	2189 (84.1)	1	1	707 (27.2)	1	1
	Low	411 (12.2)	308 (74.9)	**0.47 (0.36-0.62); <0.001**	**0.69 (0.52-0.93); 0.01**	97 (23.6)	**0.73 (0.56-0.95); 0.02**	0.81 (0.61-1.07); 0.14
	Medium	252 (7.5)	163 (64.7)	**0.27 (0.20-0.37); <0.001**	**0.47 (0.33-0.66); <0.001**	60 (23.8)	**0.70 (0.50-0.96) ; 0.03**	0.70 (0.49-0.99); 0.05
	High	99 (2.9)	58 (58.6)	**0.20 (0.13-0.33); <0.001**	**0.40 (0.24-0.66); <0.001**	21 (21.2)	**0.57 (0.34-0.96); 0.04**	**0.53 (0.31-0.93); 0.03**
Time to reach Laboratory (days)	0-3	2281 (67.8)	1983 (86.9)	1	1	587 (25.7)	1	1
	4- 7	723 (21.5)	513 (71.0)	**0.32 (0.25-0.40); <0.001**	**0.39 (0.30-0.50); <0.001**	187 (25.9)	0.96 (0.78-1.18); 0.69	1.03 (0.82-1.29) ;0.80
	>7	362 (10.8)	222 (61.3)	**0.17 (0.13-0.24); <0.001**	**0.24 (0.17-0.34); <0.001**	111 (30.7)	1.16 (0.89-1.52); 0.28	**1.42 (1.05-1.94); 0.02**

[a] Adjusted for all variables in the table.

[b] Values in **bold** indicate statistical significance.

4.4 Discussion

The ORChID project is an ongoing comprehensive community-based study using PCR assays to detect respiratory viruses in anterior nasal swab specimens taken weekly by parents from their infants throughout the first two years of life. This requires parents following a standardised protocol of obtaining swabs regularly and mailing them promptly to our laboratory. However, we have observed that suboptimal sample collection and prolonged sample transport, as determined by ERV-3 detection and presence of visible mould in swab samples reaching the laboratory negatively affect sample quality and impact on respiratory virus detection.

The data from the first 20-months of our longitudinal study indicate that respiratory virus detection is associated with the overall DNA ERV-3 load in nasal swab specimens. Swabs negative for ERV-3, presumably from sub-optimal collection and not from the presence of inhibitors, had reduced respiratory virus detection rates compared with samples containing ERV-3. Furthermore, in those specimens positive for ERV-3, a higher ERV-3 load was associated with a higher likelihood of respiratory virus detection. Overall, this shows the importance of measuring human DNA as a marker for cellular component (epithelial or inflammatory) swab samples, which if tested and monitored in real time, can identify problems associated with collection that can be quickly addressed. This is illustrated in the current study when a sudden increase in ERV-3 negative samples was observed.

We were also concerned at finding mould on some samples, which occurred despite the commercial swab tubes containing antifungal agents. Most fungal species identified in the swabs were saprophytic, and the most common fungus found, *Epicoccum nigrum,* is a known contaminant of clinical specimens (Domsch et al., 1980). The relationship between fungal air spora counts and meteorological conditions is complex and impacts at the species level (Rutherford et al., 1997). In Brisbane, *Cladosporium and Alternaria* airspora are detected commonly throughout the year, but as with *Epicoccum* sp their levels peak during the warmer, humid months. Other factors, such as rainfall and wind speed, can also influence fungal air spora composition (Rees, 1964; Rutherford et al., 1997). In our study, mould was associated mainly with longer time intervals between swab collection and arrival at the laboratory. However, this was especially evident during the warm, humid spring and summer months, which led me to speculate that fungal contamination occurred during sample collection and was influenced by the aforementioned environmental factors. Unfortunately, we could not explore this further as it was beyond the scope of the present study. In

addition, while mould growth proved to be an issue in the subtropical climate of Brisbane, this may be less of a problem in more temperate climates with lower temperatures and humidity levels.
As a result of these findings, study nurses began regularly reminding parents about sample collection protocols and prompt mailing of swabs after their collection. Following which, there was a decline in ERV-3 negative sample rates towards baseline levels and a decrease in the number of late swabs and consequently a decrease in mould observed in the swabs. Of interest however, was that respiratory virus detection rates were not affected by prolonged transport times, but in fact appeared to increase with time taken to reach the laboratory. While the observed increase was unexpected and may have occurred simply by chance, it is plausible that viral nucleic acids were protected to some extent by being encapsulated within the viral capsid, and by using viral transport medium in the swabs.

Fungi were found to be associated with fewer ERV-3 detections and, at high levels, significantly reduced respiratory virus detections. At least three points emerge from this study. First, although commercial swabs may contain antimicrobial agents, the risk of fungal and potentially bacterial contamination may still arise when they are exposed to warmer temperatures for increasingly long periods of time. Second, the times between swab collection and laboratory arrival should be monitored and feedback provided if delays occur. Finally, if delays are expected, swabs should be placed in the household refrigerator until mailed to the laboratory (O'Grady et al., 2011).

4.5 Conclusion

We found that ERV-3 as a marker for human DNA and by inference, respiratory epithelial and inflammatory cells, was also an important indicator of sample quality for our study. For community-based investigations similar to our own, which rely upon self or parent-collected specimens, real-time sample processing and ERV-3 detection can facilitate rapid interventions to maintain sample quality and to optimise respiratory virus detection. Indeed, this may have broader implications as nasal swabs are beginning to replace the traditional, but more invasive nasopharyngeal swab or NPA samples in hospitals and clinics (Lambert et al., 2008a), especially more recently following the 2009 influenza pandemic. Thus, if studies in community health centres show similar results to our own, comparable ERV-3 testing strategies could be used by diagnostic laboratories to improve or monitor sample collection quality for optimal respiratory virus detection. Finally, the potential problem of visible mould contamination of swabs taken during community-based studies can be minimised by ensuring samples are transported promptly to the laboratory.

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Chapter 5

Laboratory issues impacting upon PCR-based detection of respiratory viruses in longitudinal studies

Publications based on this chapter

- Alsaleh, A. N., Grimwood, K., Sloots, T. P., & Whiley, D. M. (2014). A retrospective performance evaluation of an adenovirus real-time PCR assay. Journal of Medical Virology, 86(5), 795–801. doi:10.1002/jmv.23844

5.1 Introduction

In the previous studies that utilised nasal swabs for sample collection, it was noted that HAdV Ct values were typically much higher than those of the other viruses (Echavarria et al., 2006; Lambert et al., 2008a), which agree with the results obtained from this PhD study cohort (see chapter-3 discussion). However, the high levels of variation observed in the gene target of the selected HAdV real-time PCR assay selected for the ORChID study, as well as the age of the assay raised further concerns over how it might perform in a longitudinal study spanning several years. Having previously identified collection and transport issues, in this chapter we explored whether specific laboratory protocols selected for this study may have influenced the findings above.

HAdVs (Genus *Mastadenovirus*, Family *Adenoviridae*), which were first isolated from the respiratory tract by tissue culture in 1953 (Rowe et al., 1953), are known to cause a wide range of human disease, including respiratory, gastrointestinal, ocular, urinary and central nervous system infections (Lynch et al., 2011). Although HAdV infections are typically mild and self-limiting, they can be severe in immunocompromised (especially transplant) patients (La Rosa et al., 2001; Lion, 2014; Zahradnik et al., 1980) in neonates and previously healthy infants on rare occasions (Henquell et al., 2009; Mistchenko et al., 1998; Ronchi et al., 2014) and when a new variant emerges (Louie et al., 2008). HAdVs can also cause localised outbreaks in small and crowded populations, such as in boarding schools and military facilities (Caldwell et al., 1974; Yu et al., 2013).

NAATs, such as PCR assays, provide high levels of sensitivity compared with cell culture and serology. With reduced turnaround time, NAATs are also superior to other viral detection methods for routine clinical diagnostic laboratory testing and in large epidemiological studies. PCR has been used to detect HAdV in clinical samples since the 1990s (Allard et al. 1990; Pring-Akerblom and Adrian 1994; Echavarria et al. 1999). However, designing a diagnostic PCR assay that is comprehensive for all human HAdV types can be difficult. HAdVs are sub-grouped into seven species (A to G) that include more than 60 different types of HAdVs (Matsushima et al., 2013; Robinson et al., 2011). Notably, there are considerable genetic differences between HAdV types, and these differences are likely to impact upon the performance of diagnostic PCR assays if not accounted for, relying as they do upon highly conserved target sequences.

In 2003, the first real-time PCR method was developed for pan-HAdV detection (Heim et al., 2003) and since then this assay (called HAdV-PCR hereafter) has been used widely (Lambert et al. 2008a;

Kwofie et al. 2012; Tsou et al. 2012; Ayoub et al. 2013). The original description of the method considered the extent of HAdV genetic variation known at the time, and importantly, showed that the assay was able to detect a broad range of HAdV types despite known mismatches being present (Heim et al., 2003). However, in view of the negative impact that genetic variation may have upon PCR assay performance (Whiley & Sloots 2005; Whiley & Sloots 2006), I was concerned by the (i) potential for false-negative results and (ii) delayed Ct values arising from the appearance of new HAdV types or variants. To address this, an *in-silico* analysis of recent HAdV sequence data was undertaken initially and the stability of the target sequence determined. Two new HAdV PCR assays were then designed and used to retest respiratory samples collected from the ORChID study.

My hypotheses were:

1- Sequence variation associated with changes in HAdV strains alters real-time PCR performance either qualitatively of quantitatively.
2- High level of variation may exist in the HAdV hexon gene targeted by the HAdV-PCR oligonucleotides.

5.2 Methods

5.2.1 Sequence analysis of HAdV-PCR oligonucleotides

To investigate the extent of sequence variation in the HAdV-PCR primer and probe targets, HAdV hexon gene sequences from the GenBank database were downloaded and analysed. Briefly, these comprised representative sequences of all 68 known human HAdV types, excluding genotypes 57 and 60 as sequence data for these HAdV-PCR targets were unavailable. Sequences were aligned using BioEdit software (version 7.0.4.1) and the number and location of mismatches in each oligonucleotide target identified (Figures 5.1-5.3).

5.2.2 Assay design

Based on the above sequence data, two additional assays (defined as Mod1-PCR and Mod2-PCR) were designed. For Mod1-PCR assay, the same sequence targets as used for the HAdV-PCR were used, except that degenerate bases were incorporated at appropriate positions to accommodate sequence mismatches; three forward primers, three reverse primers and two probes were designed subsequently (Table 5.1). The Mod2-PCR assay was designed with the aid of Primer Express (Applied Biosystems, version 2.0; Foster City, USA) and targeted sequences flanking those of the above HAdV-PCR and Mod1-PCR methods. Similar to the Mod1-PCR, multiple oligonucleotides with degenerate bases in various positions were used to accommodate the observed sequence variation; two forward primers, two reverse primers and one probe (Table 5.1).

Table 5.1 Oligonucleotides used for the Mod1-PCR and Mod2-PCR methods

Designation	Oligonucleotide sequence (5`-3`)	Position±
Mod1-PCR assay		
Mod1-P1	TCGG**R**GTACCT**S**AG**T**CCGGGTCTGGTGCA	18401-18430
Mod1-P2	TCGGAGTACCTGAG**C**CC**S**GG**K**CTGGTGCA	
Mod1-F1	GCC**S**CA**R**TGGGCATACATGCACATC	18362-18387
Mod1-F2	GCC**G**CAGTGG**K**C**K**TACATGCACATC	
Mod1-F3	GCC**C**CAGTGG**K**C**K**TACATGCACATC	
Mod1-R1	GCCACTGTGGGGTTTCTAAA**Y**TT	18471-18494
Mod1-R2	GCCAC**S**GTGGGGTT**Y**CTAAACTT	
Mod1-R3	GC**T**ACGGT**R**GG**A**TTTCTAAACTT	
Mod2-PCR assay		
Mod2-P1	CTGGTGCAGTT**Y**GCCCG**Y**GC**M**AC	18422-18446
Mod2-F1	ACATGCACATCTCGGGCCAGGA	18376-18398
Mod2-F2	ACATGCACATC**G**C**C**GG**R**CAGGA	
Mod2-R1	CGGTC**S**GTGGTCACATC**R**TGGGT	18498-18521
Mod2-R2	CGGTCGGTGGTCAC**R**TCGTG**S**GT	

±Position according to HAdV-D type 16, GenBank accession number JN860680

5.2.3 Real-time PCR reaction mix and cycling conditions

The real-time PCR reactions for all three assays were performed using the Qiagen Quantitect Probe PCR kit (Qiagen; Doncaster, Australia). The reaction mixes consisted of a total reaction volume of 20.0µL, including 10.0µL of Qiagen Quantitect Probe mix (Qiagen; Doncaster, Australia) and 2.0µL of sample extract or control. For the HAdV-PCR, 8.0 pmoles of the previously described forward and reverse primers, and 2.0 pmoles of probe were used (Heim et al., 2003). For the Mod1-PCR, 2.6 pmoles each of the three forward primers (mod1-F1, F2 and F3; Table 5.1), 2.6 pmoles each of the three reverse primers (mod1-R1, R2 and R3; Table 5.1), and 2.0 pmoles of each probe (Mod1-P1 and P2; Table 5.1) were employed. Similarly, the Mod2-PCR, comprised 4.0 pmoles each of the two forward primers (mod2-F1 and F2; Table 5.1), 4.0 pmoles each of the two reverse primers (mod2-R1 and R2; Table 5.1), and 2.0 pmoles of the probe (Mod2-P1; Table 5.1). All three methods were cycled on the Rotorgene-Q (Qiagen; Doncaster, Australia). Cycling was performed under the following conditions: initial activation at 95°C for 15 min and 45 cycles of 95°C for 15s and 60°C for 1 min.

5.2.4 HAdV cultures

The performance of the three PCR methods was assessed initially using eight HAdV isolates of various types (Table 5.2). Briefly, ten-fold dilutions of DNA extracted from each isolate were tested in all three methods and the detection limits and associated Ct value data compared.

5.2.5 Community-based respiratory samples

Assay performance was also investigated using 8,800 nasal swab samples collected between September 2010 and April 2012 as part of the ORChID study. Briefly, samples were pooled and tested as per above, except that only the HAdV-PCR and Mod1-PCR assays were used, the Bioline SensiMix II Probe Kit (Bioline; Sydney, Australia) was used instead of the Qiagen Quantitect Probe mix and cycling was performed on an ABI7500 (Applied Biosystems; Melbourne, Australia). The decision not to use the Mod2-PCR assay for this testing was based on the similarity of results obtained when using the Mod1-PCR and Mod2-PCR methods to test the above virus cultures and clinical samples (Table 5.2).

5.2.6 Performance of assay using clinical samples

The performance of the HAdV-PCR was investigated retrospectively by retesting clinical samples using both the Mod1-PCR and Mod2-PCR assays. All respiratory specimens were provided by the Central Laboratory, Pathology Queensland (Herston, Queensland) and collected during the year

2012. These included 79 samples that had tested positive previously by the HAdV-PCR assay at Pathology Queensland as well as an additional 700 de-identified respiratory specimens. The latter 700 samples were pooled (ten samples per pool) for testing. All samples from pools providing positive results by any method were then tested individually by all three assays (HAdV-PCR, Mod1-PCR and Mod2-PCR) in parallel and the results compared.

5.2.7 HAdV-PCR sequence target analysis

DNA sequencing was used to investigate sequence variation in the HAdV-PCR targets in all control isolates, as well as ORChID and clinical samples providing discrepancies between the three PCR methods. Briefly, a sequence of 489 bases (from HAdV-E type 4; GenBank accession number EF371058) was amplified by PCR using primers flanking the above assay targets (ACTCTGAACAGCATCGTGGGT, this study; and CAGCACGCCGCGGATGTCAAAGT; Allard et al. 1990). The 25µL reaction mix comprised the following: 12.5µL of QuantiTect SYBR Green PCR Master Mix (Qiagen-Australia) and 5.0µL of sample extract and 0.4µM of each primer. The amplification was performed under the following conditions: an activation step of 95ºC for 15-minutes, 45 cycles of (1) a denaturation step at 95ºC for 30-seconds and (2) annealing and elongation steps at 50ºC for 30-seconds and 72ºC for 60-seconds then one melting step at 60-95ºC for 60-seconds. PCR target sequence amplicons underwent melting curve analysis and gel electrophoresis. For gel electrophoresis, an aliquot of approximately 8µL of each PCR product was examined using 2% agarose gel electrophoresis. Once a positive result was obtained, the remaining PCR product was purified using QIAquick purification kit (Qiagen, Australia) following the manufacturer instructions. The purified DNA was submitted to the Australian Genome Research Facility (The University of Queensland, Brisbane) for automated sequencing.

5.2.8 HAdV genotyping

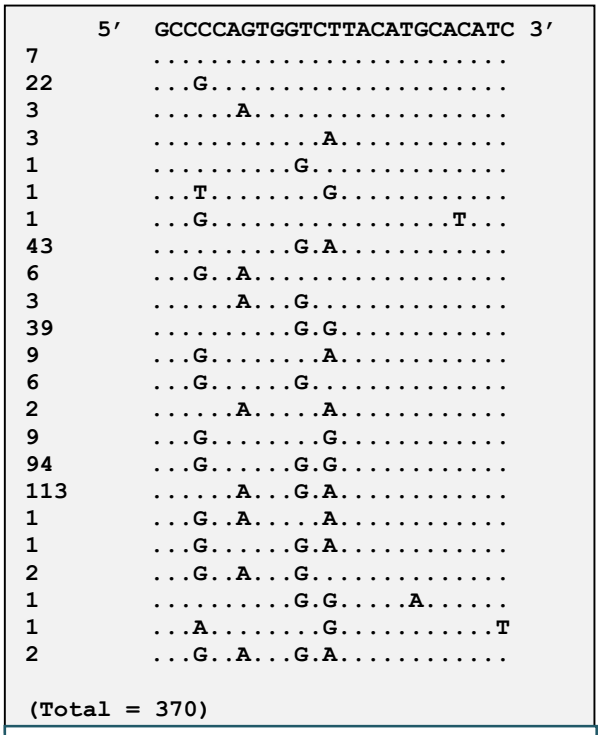
HAdV genotyping was performed to determine the genotypes of the discrepant clinical samples and to further investigate circulating HAdV genotypes in the local population. Twenty randomly-selected HAdV-positive clinical samples (from the 2012 Pathology Queensland sample set above) were also subject to HAdV genotyping. The hyper-variable region-7 (HVR7) in the hexon gene was amplified using previously designed primers (the sequence of the forward primer was: 5`-CTGATGTACTACAACAGCACTGGCAACATGGG-3` and the reverse primer sequence was: 5`-GCGTTGCGGTGGTGGTTAAATGGGTTTACGTTGTCCAT-3`) (Sarantis et al., 2004). The 25µL reaction mix comprised the following: 12.5µL of QuantiTect SYBR Green PCR Master Mix (Qiagen-Australia) and 5.0µL of sample extract and 0.4µM of each primer. The amplification was

performed under the following conditions: an activation step of 95ºC for 15-minutes, 45 cycles of (1) a denaturation step at 95ºC for 30-seconds and (2) annealing and elongation steps at 50ºC for 30-seconds and 72ºC for 60-seconds, then one melting step at 60-95ºC for 60-seconds. The acquisition of the PCR product signal occurred during the annealing and elongation step and the melting step. PCR results interpretation was performed using both melting curve analysis and gel electrophoresis. PCR product purification and sequencing was performed as described in section 5.2.7.

5.3 Results

5.3.1 Sequence analysis of HAdV-PCR oligonucleotide targets

Overall, 334 (90%) of the 370 HAdV hexon gene sequences available on the GenBank database had at least two mismatches with the HAdV-PCR forward primer and 215 (58%) sequences had at least three mismatches (Figure 5.1).



```
     5′  GCCCCAGTGGTCTTACATGCACATC 3′
7        .........................
22       ...G.....................
3        ......A..................
3        ...........A............
1        ..........G.............
1        ...T........G...........
1        ...G................T...
43       ..........G.A...........
6        ...G..A.................
3        ......A...G.............
39       ..........G.G...........
9        ...G........A...........
6        ...G......G.............
2        ......A.....A...........
9        ...G........G...........
94       ...G......G.G...........
113      ......A...G.A...........
1        ...G..A.....A...........
1        ...G......G.A...........
2        ...G..A...G.............
1        ..........G.G.....A.....
1        ...A........G...........T
2        ...G..A...G.A...........

(Total = 370)
```

Figure 5.1 Sequence alignment of the HAdV-PCR forward primer with 370 HAdV sequences from the Genbank database. Dots indicate a match whereas capitalised bases indicate mismatches with the primer sequence

Likewise, 254 (69%) of the sequences had at least two mismatches with the HAdV-PCR reverse primer (Figure 5.2). The HAdV-PCR probe was the most conserved with 304 (82%) sequences having either one or no mismatches (Figure 5.3). In general, the highest level of variation was observed amongst HAdV subspecies B1 and B2 (data not shown). This variation involved predominantly the forward primer, with three mismatches observed typically.

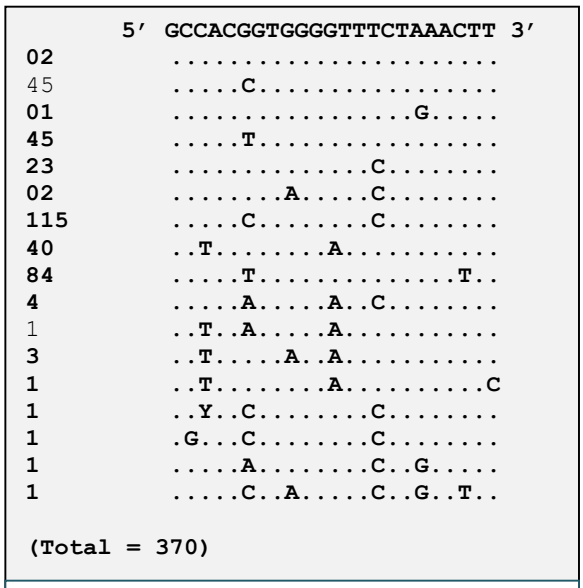


Figure 5.2 Sequence alignment of the HAdV-PCR reverse primer with 370 HAdV sequences from the GenBank database. Dots indicate sequence identity whereas capitalised bases indicate mismatches with the primer sequence

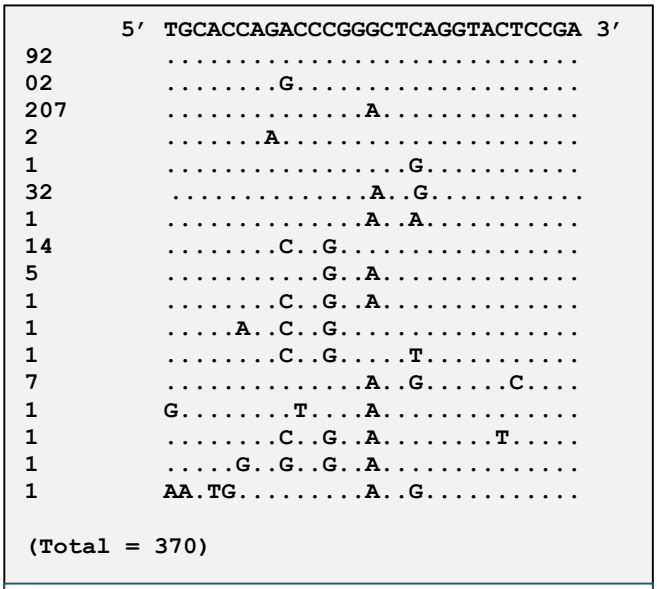


Figure 5.3 Sequence alignment of the HAdV-PCR probe with 370 HAdV sequences from the GenBank database. Dots indicate sequence identity whereas capitalised bases indicate mismatches with the probe sequence

5.3.2 Evaluation of HAdV assays using culture isolates

Testing ten-fold dilutions of the control isolates revealed similar detection limits between the three assays (within one-tenfold dilution) for all isolates (data not shown). However, significant differences in Ct values were observed between assays for some isolates (Table 5.2). Notably, isolates 1 and 2 (HAdV types 34 and 11, both B species) showed significantly lower Ct values in the Mod1-PCR and Mod2-PCR assays by ten and five cycles respectively compared with the HAdV-PCR (Table 5.2). In contrast, Ct values for all other control isolates (3 C, 1 E, 1 F and 1 B species) differed by only one to two cycles between assays. DNA sequencing of the HAdV-PCR targets for the control isolates (Table 5.2) revealed mismatches for primer and probe targets for all isolates. However, the greatest number of mismatches were observed for control isolates 1 and 2 (HAdV types 34 and 11 (both B species), with three mismatches present in both the forward and reverse primers of each isolate.

Table 5.2. Cycle threshold (Ct) values for eight control isolates and 13 selected positive clinical samples for adenovirus (HAdV) as tested by the HAdV-PCR, Mod1-PCR and Mod2-PCR assays. Sequence alignment of the HAdV-PCR oligonucleotides with target sequences are also provided where available. Dots indicate matching bases

Sample info.	HAdV type (species)	Ct values			Mismatches between HAdV-PCR oligonucleotides and HAdV genotypes		
		HAdV -PCR	Mod1-PCR	Mod2-PCR	F-Primer (5'–3') GCCCCAGTGGTCTTACATGCACAT	Probe (5'–3') TCGGAGTACCTGAGCCCGGGTCTGGTGCA	R-Primer (5'–3') GCCACGGTGGGGTTTCTAAACTT
Control isolates (2000)							
1	34 (B2)	26.72	16.21	15.24	......A...G.A..........	..............T..............	..T.....A..A..........
2	11 (B1)	21.04	16.2	15.22	......A...G.A..........	..............T..............	..T........A...A.......
3	04 (E)	21.81	22.55	20.43	..........G.G..........	..............T..............	.....C........C........
4	14 (B2)	20.29	20.51	19.22	..........G.A..........	..............T..............	..T........A..........
5	40 (F)	17.51	18.35	18.78	...G..A................	....................C........	................G.....
6	06 (C)	21.25	21.69	20.88	...G...................	................C..G........	.....C.................
7	02 (C)	25.24	25.51	24.94	...G...................	................C..G........	.....C.................
8	05 (C)	20.63	20.78	20.56	...G...................	................C..G........	.....C.................
Clinical samples (2012)							
1	03 (B1)[a]	24.19	20.2	18.39	......A...G.A..........	..............T..............	.....T.............T..
2	03 (B1)[a]	16.99	12.17	11.65	......A...G.A..........	..............T..............	.....T.............T..
3	n/a	38.09	nd	nd	n/a	n/a	n/a
4	n/a	35.41	nd	33.47	n/a	n/a	n/a
5	n/a	35.35	37.16	nd	n/a	n/a	n/a
6	02 (C) [a]	36.33	36.7	nd	n/a	n/a	n/a
7	02 (C)[a]	nd	38.52	33.95	n/a	n/a	n/a
8	02 (C)[a]	nd	36.23	32.6	n/a	n/a	n/a
9	01 (C)[a]	nd	38.9	35.57	n/a	n/a	n/a
10	n/a	nd	37.97	35.49	n/a	n/a	n/a
11	03 (B1)[a]	19.29	13.51	12.88	......A...G.A..........	..............T..............	.....T.............T..
12	n/a	41.69	34.76	33.05	n/a	n/a	n/a
13	03 (B1)[a]	41.91	33.49	32.75	n/a	n/a	n/a

nd = not detected; n/a = not available; [a]based on genotyping of the hyper variable region 7 in the HAdV hexon gene.

5.3.3 Community-based respiratory samples

The testing of the 8,800 nasal swab samples provided almost identical results for both the HAdV-PCR and Mod1-PCR assays. Fifty-three samples were positive by both methods with similar Ct values (i.e. <3 cycles difference between assays) for 51 samples with Ct values ranging from 23.1 to 41 cycles (mean 34 cycles) in the Mod1-PCR. A further two samples were positive by both methods, but provided earlier Ct values in the Mod1-PCR (34 and 35.2 cycles respectively) compared to the HAdV-PCR (39.1 and 41.4 cycles respectively).

5.3.4 Clinical samples

For the 79 HAdV-positive specimens from Pathology Queensland, the three methods were positive. Similar Ct values (ie. <3 cycles difference between assays) resulted for 67 samples (12 to 38.8 cycles in the HAdV-PCR; mean 27 cycles). A further two samples were positive by all three methods, but produced earlier Ct values (>3 cycles difference) in both the Mod1-PCR and Mod2-PCR assays (samples 1 and 2; Table 5.2). One sample was positive by the HAdV-PCR only (sample 3; Table 5.2), one sample positive by both the HAdV-PCR and Mod2-PCR assays only (sample 4; Table 5.2), two samples were positive by HAdV-PCR and Mod1-PCR only (samples 5 and 6; Table 5.2) and there were two samples that were negative by all three methods. Of note, was that there were four additional samples negative by the HAdV-PCR, but positive by both the Mod1-PCR and Mod2-PCR assays (samples 7 to 10; Table 5.2). To further investigate the latter, all four samples were retested in duplicate in the HAdV-PCR; two samples provided positive results in both replicates, and two samples were positive in one of the two replicates.

The additional testing of 70 respiratory pools (representing 700 respiratory virus extracts) identified ten positive pools in all methods and an additional two pools positive by the Mod2-PCR only (total of 12 positive pools for this method). The 120 individual samples from these twelve pools were subsequently tested with all three assays and twelve samples were found positive by all three methods (ie. one positive sample/pool). Of the twelve positive samples, nine provided similar Ct values in all three methods (23.7 to 34.8 cycles in the HAdV-PCR; mean 29.8 cycles). Three samples provided earlier Ct values in the Mod1-PCR and Mod2-PCR assays compared to the HAdV-PCR (samples 11 to 13; Table 5.2).

DNA sequencing of the HAdV-PCR targets was attempted for all thirteen clinical samples providing discordant results (samples 1 to 13; Table 5.2). Of these, the HAdV-PCR sequence

targets could only be sequenced for three samples (samples 1, 2 and 11; Table 5.2) with the highest viral load (based on the PCR Ct values).

5.3.5 Additional HAdV genotyping

HVR7 genotyping was successful in eight of the 13 samples providing discordant typing results (Table 5.2). Similar to the results of the control isolates, samples 1, 2 and 11 had a total of five mismatches in the primers, were members of species B1, and correlated with the late Ct values observed in the HAdV-PCR. HVR7 genotyping of the additional 20 randomly selected HAdV-positive clinical samples revealed four types from three species; nine comprised HAdV type-1 (species C), eight had HAdV type-2 (species C), two were typed as HAdV type-3 (species B) and only one sample was typed as an HAdV type-4 (species E).

5.4 Discussion

The concerns over the HAdV-PCR assay performance were instigated because of the higher Ct values obtained from the study cohort and the age of the assay. These two factors raised the possibility of new HAdV genotypes or their variants being present and leading to either delayed Ct values or false-negative results

The *in-silico* sequence analysis showed a high level of variation in the HAdV-PCR oligonucleotide targets, particularly for the primers, which validate the initial concerns. However, the variation observed was similar to that described originally (Heim et al. 2003). In addition, the experimental data showed that despite considerable variation in the HAdV-PCR targets, few false-negative results were observed in the HAdV-PCR. The samples that were negative by HAdV-PCR, but positive by either Mod1-PCR or Mod2-PCR had typically low viral loads (as indicated by their respective high Ct values; clinical samples 7 – 10; Table 5.2), and so low template load may explain these results. This explanation is supported by observing that these samples provided positive results upon repeat testing in the HAdV-PCR assay. In addition, other samples positive by HAdV-PCR at high Ct values were negative by one or both of the other two methods (clinical samples 3-6; Table 5.2).

Overall, these results suggest an unexpected tolerance of the HAdV-PCR assay for mismatches, further confirming Heim et al.'s original data. The explanation for this tolerance is likely to be due to variation being largely absent from the extreme 3' end of the primers (Figures 5.1 and 5.2), which is the critical region for primer hybridisation and optimal assay performance. It should be noted however that false-positive results in the HAdV-PCR could explain the failure of the Mod1-PCR and Mod2-PCR assays to confirm the HAdV-PCR results, particularly for sample 3 (Table 5.2). The most logical way to explore the potential for false-positive results here would be to use DNA sequencing. However, we did not attempt this as the real-time Ct values involved were very high, making this very difficult. The main problem though is that failure to obtain a clear HAdV sequence could lead us to incorrectly conclude HAdV was absent, whereas a small amount of specific HAdV-PCR product might still be present, but mixed with the various non-specific products, which occur typically towards the end of PCR cycling.

While the results indicate the HAdV-PCR remains suitable for routine detection of HAdVs, the data suggest that variation does have some impact upon amplification, insofar as Ct values were delayed. For example, control isolates 1 and 2 (HAdV types 34 and 11) had three mismatches in both the

forward and reverse primers that led to significant delays in Ct values (five to ten cycles) compared to the Mod1-PCR or Mod2-PCR methods. Similar issues were observed for the clinical samples. While not affecting *qualitative* detection, these results do suggest however that the HAdV-PCR assay would be unsuitable for *quantitative* purposes and that the Mod1-PCR or Mod2-PCR assays may be more suitable for such use.

For the ORChID study, this means that a small proportion of the late Ct values may have arisen as a result of sequence variation impacting on PCR amplification or PCR-product detection efficiency. Reassuringly, this means that most of the ORChID results were accurate and the late Ct values were not an artefact of the PCR, but instead indicate HAdVs are typically present at lower loads in nasal swab specimens. This observation reaffirms the findings of the previous studies (Echavarria et al., 2006; Lambert et al., 2008b) and implies the importance of assessing the quality of sample collection to further eliminate possible factors that may impact upon HAdV Ct values (as discussed in chapter-4). The fact that we have now confirmed that the HAdV Ct values and hence viral loads are indeed typically lower than the other viruses raises further questions over the role of these viruses in the community and whether nasal swabs alone are necessarily the best specimens for detecting these viruses (Hammitt et al., 2011) (and is discussed in more detail in chapter-7).

In light of these findings I elected to replace the HAdV-PCR assay with the Mod1-PCR so as to optimise screening of the ORChID samples for this virus in my studies.

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Chapter 6

Molecular epidemiology of human rhinoviruses in an infant cohort

6.1 Background

Across all age groups, RVs are the most frequently detected viruses in respiratory secretions and this includes their presence in both symptomatic and asymptomatic individuals. Traditionally, RVs were considered to consist of ~100 serotypes, divided into either A or B species based on their phylogeny and recognised solely as URTI pathogens since they were thought to replicate principally in the cooler temperatures of the upper airways. However, temperature preferences vary between RV types and some replicate at the higher temperatures of the lower airway (Jacobs et al., 2013). Indeed, earlier studies reported the ability of RVs to infect and replicate in the LRT and provided experimental support for clinical studies reporting an association between RVs and LRTIs, especially for acute exacerbations of some chronic pulmonary disorders, such as asthma and chronic obstructive pulmonary disease (COPD) (Johnston et al., 1995; Papadopoulos et al., 1999). More recent data show that RVs are associated with increased risk of subsequent wheezing illnesses and asthma following severe LRTI associated with wheeze during early childhood (Calışkan et al., 2013; Jackson, 2010; Jackson et al., 2008; Lemanske et al., 2005).

This recognition of an expanded role for RVs during infancy has been facilitated by the recent introduction of molecular methods for their diagnosis and characterisation, which now allow a more comprehensive description of RV infection rates. These techniques led to the discovery of a new divergent group of RV sequences that has since been categorised as a novel RV species: RV-C (Arden et al., 2006; Kistler et al., 2007). In addition to diagnosing and identifying novel types, these molecular-based techniques have resulted in changes in the classification system of RV types and provided new insight into characterising RV-infections, such as those associated with upper and lower airway involvement, prolonged-shedding, co-detection and subclinical infections (Bruce et al., 1990; McIntyre et al., 2013; Mori and Clewley, 1994; Simmonds et al., 2010). However, we still have a limited understanding of these particular disease patterns, particularly as they relate to RV infections in the community. Thus, carefully conducted longitudinal studies of otherwise-healthy infants are necessary to further understand the types of illnesses, including the shedding characteristics, associated with these viruses.

6.1.1 RV genome structure

The RVs are non-enveloped viruses possessing a single-stranded, positive sense RNA genome that is approximately 7.2 kb in length. The length of the single open reading frame (ORF) is approximately 6.5 kb. The ORF regions in RV-A and RV-B have more Adenine and Uracil (A+U) positions compared to RV-C (Arden and Mackay, 2011; Gama et al., 1989; Lu et al., 2008) and in all species the ORF is bracketed by two untranslated regions (UTR). The 5`UTR comprises approximately 650 nucleotides and starts with a small viral protein (VPg) that serves as a primer for the genome replication. The 5`UTR functions, by way of several structural sequences, as a regulator for the replication and translation for the viral genome. The 3`UTR, in contrast, is shorter and consists of approximately 50 nucleotides that end with a polyA chain and serves as a regulator of transcription (Arden and Mackay, 2010; Jacobs et al., 2013; Palmenberg et al., 2009; Paul et al., 1998). The ORF can be divided into three regions (P1, P2 and P3). The first encodes four structural proteins (viral proteins 4, 2, 3 and 1, which together form the virion's capsid), while the remaining regions of the ORF encode seven non-structural proteins that are essential for infection and replication, including protease, RNA-dependant polymerase, VPg and other proteins.

6.1.2 RV classification

The three RV species; *Rhinovirus A, Rhinovirus B* and *Rhinovirus C* are members of the genus *Enterovirus*, family *Picornavirdae*. These three species encompass more than 160 serotypes and genotypes (hereafter called types). The two well established species, RV-A and B have been sub-classified using various approaches according to their serological profiles, tissue tropism, antiviral susceptibility and phylogeny, as well as their cell entry mechanisms and cellular receptors. All RV-B and most RV-A types use the intracellular adhesion molecule-1 (major) receptor for cell entry, while a minor subset of RV-A types use a low density lipoprotein receptor (Bochkov et al., 2011). By 1987, more than 100 RV-A and B serotypes were recorded (Hamparian et al., 1987). Later efforts for RV-A and RV-B type classification employed molecular methods, which in the early 1990s targeted the 5` UTR or VP4/VP2 gene regions in the RV genome (Bruce et al., 1990; Mori and Clewley, 1994). For genotype nomenclature, species letter and type number are now used respecting the historical naming system (Liggett et al., 2014). For RV-C, type classification methods are slightly different. The discovery of this species was achieved using purely molecular techniques (Arden et al., 2006). Since its discovery in 2006, growing RV-C *in vitro* has not been possible using standard cell lines and a system for *in vitro* RV-C replication was not available until 2011. Only two RV-C isolates were successfully propagated, initially in sinus organ culture of nasal

epithelial cells (Bochkov et al., 2011). Thus, typing of RV-C has relied upon genomic comparisons (Simmonds et al., 2010).

Full genomic analyses of all known sequences of RV-A and RV-B and selected sequences of RV-C was achieved in 2009 by sequencing the previously described serotypes and ten additional field samples (Palmenberg et al., 2009). The analysis revealed that ten of twelve possible recombination events in the RV genome involve the 5`UTR. Additional studies have revealed that most 5`UTR sequences obtained from species C are genetically similar to some species A sequences (McIntyre et al., 2010). These data highlight a limitation of using the 5`UTR for classification purposes. Due to the considerable heterogeneity in the capsid genes (VP1 and VP4/VP2) and their resulting phylogenetic clustering, VP1 and VP4/VP2 sequencing has now gained wider acceptance for RV genotyping in a system similar to that used for the assignment of new and existing *Enterovirus* types. A threshold of 10% divergence was suggested when using the VP4/VP2 genes, while when using the VP1 gene, a threshold of 13% divergence was proposed (Simmonds et al., 2010).

6.1.3 RV diagnosis

RVs can be detected from most respiratory specimens at high titre, including nasopharyngeal swabs or aspirates, nasal washes and nose/throat swabs (for URTIs) and BAL or bronchial aspirates (for LRTIs) (Versalovic et al., 2011). RV detection methods are similar to the detection methods used for other respiratory viruses and therefore can encounter similar challenges (discussed in chapter 1; section 1.4). Due to the high diversity of RV types and the lack of available serological data and universal reagents, both cell culture and serodiagnosis/antigen detection-based methods have proven to be insensitive, costly and also time consuming for routine RV detection. The gold standard detection technique is now conventional and real-time RT-PCR methods. The majority of RV PCR assays use 5`UTR for the detection target, particularly the ‘Gama’ assay site - being one of the most highly conserved regions in all RV genomes [OL26 and OL27], (Gama et al., 1989). This method has also been used for classification purposes in cases where primary typing methods (eg. VP1 or VP4/VP2) have failed to provide typing data (Arden et al., 2010; Bruce et al., 1990). Despite the ‘Gama’ assay site being highly conserved, the high level of heterogeneity in RV types has still created challenges for developing a comprehensive real-time PCR assay since a real-time PCR requires an additional conserved site for an oligoprobe (i.e. in addition to the primer targets). In 2008, the first comprehensive RV real-time PCR detection method was finally described (Lu et al., 2008).

6.1.4 Burden of RV infections during infancy and early childhood

Depending upon severity, RVs are among the most commonly detected viruses during infancy and early childhood ARIs. Previous serological and virological analyses performed in children younger than two years of age have found that approximately 90% had already acquired at least one RV infection (Blomqvist et al., 2002). For young children, RV infections are associated with a variety of clinical presentations in both the URT and LRT. For example, RVs are associated with URTIs including the common cold, sinusitis (Alho, 2005) and AOM (Chonmaitree et al., 2008), while RVs are also associated with LTRIs including bronchiolitis (Calvo et al., 2010; Mansbach et al., 2012; McErlean et al., 2008) and pneumonia (Daleno et al., 2013; Juvén et al., 2000). Key studies examining RV disease are summarised below.

6.1.4.1 RVs and URTIs

RVs were the most frequently detected virus in respiratory secretions collected from both hospitalised infants and children, including those at high risk of atopy from previous community-cohort studies (Kusel et al., 2006; Miller et al., 2011). The viral aetiology of the common cold has been the subject of many studies worldwide. In one study in which 194 young children with the common cold were investigated, RVs were detected in 71% (Ruohola et al., 2009). RVs were also detected in up to 41% of middle ear fluid samples collected from infants and children with AOM (Blomqvist et al., 2002).

6.1.4.2 RVs and LRTIs

The significant role of RVs in young children hospitalised due to LRTIs has been recognised recently. In the US, RV infections were detected in five hospitalisations per 1000 children under five years of age (Miller et al., 2007). Moreover, fatal RV infections were reported from an outbreak in two orphanages in Vietnam during 2012. Seven of the twelve children who were hospitalised for severe ARI were younger than six months of age and none survived. Their samples were tested for eleven different respiratory viruses, including RV, which was the only virus detected in five of these infants, while in the remaining two, RSV and HAdV were also detected (Hai et al., 2012). In an investigation that included 643 children hospitalised with community-acquired pneumonia, all three RV-species were identified in 76.3% of the 198 RV-positive samples with a predominance of RV-A across all age groups in this particular study, followed by RV-C and rarely RV-B (Daleno et al., 2013).

RVs were detected in 17-26% of infants hospitalised with severe bronchiolitis (Calvo et al., 2010; Mansbach et al., 2012) and it has been suggested that such individuals are at increased risk of receiving an asthma diagnosis later in childhood. (James et al., 2012). In a long-term follow up of post-bronchiolitis cases at seven years of age, asthma was more common in infants with a prior history of RV-induced bronchiolitis (52%) than those with previous RSV-induced bronchiolitis (15%) (Jartti and Korppi, 2011). There are two possible explanations for these clinical observations. The first is that severe RV LRTI in young infants may damage epithelial cells during a critical phase in airway development leading to a sequence of events resulting in airflow limitation and wheezing. Alternatively, severe RV infection may occur primarily in those who are genetically predisposed to wheezing, amplifying this process and resulting in asthma symptoms from early childhood (Calvo et al., 2007).

6.1.4.3 RVs, asthma and asthma exacerbation

The relationship between RV-infections and asthma exacerbation is well recognised. Approximately two-thirds of viral asthma exacerbation episodes are associated with RVs. This was investigated at RV-species level, and in one study RV-C type 10 (RV-C10) was detected in 23% of children hospitalised with an asthma exacerbation (Arden et al., 2010). In contrast, for non-hospitalised asthmatic children, RV-A types were associated with longer symptom duration when compared to RV-C types. The latter were also detected as singletons (i.e. the only virus detected) more frequently (Arden and Mackay, 2010). This indicates that although RV-A and RV-C may play a role in asthma exacerbation and wheezing, their infection characteristics may lead to different clinical outcomes. As above, the frequent wheezing events caused by RV infections imply they may have a role in either unmasking or feature in the causal pathways leading to asthma in children at high risk of atopy, especially if they were sensitised previously by aeroallergens, as explained by the ‘dual hit’ hypothesis (Calışkan et al., 2013; Holt and Sly, 2012; Jackson et al., 2008; Lemanske et al., 2005).

6.1.4.4 RVs and other chronic diseases

RV infections are also associated with worsening of other chronic pulmonary disorders, such as cystic fibrosis (CF) at all ages (Kieninger et al., 2013; Wat et al., 2008) and COPD in adults (Mallia et al., 2011). For CF, RVs have been identified as the predominant agent during virus-associated exacerbations in children (Burns et al., 2012; Wat et al., 2008). The prevalence of RV in CF children from a community-based study was as high as 73% (Emerson et al., 2013) and they were associated with more severe and protracted respiratory symptoms than in healthy controls. Interestingly, the load of RV was significantly higher in BAL samples collected from CF children

than from asthmatic children and heathy controls, while local anti-inflammatory and anti-viral mediators in the BAL fluid were negatively associated with RV load, suggesting impaired RV clearance mechanisms in CF patients. (Kieninger et al., 2013).

The severity of RV-infections is linked to several host-related factors, such as their age, maturity of their innate and adaptive immune responses, and presence of any chronic underlying diseases (e.g. cardiorespiratory, neuromuscular or immunodeficiency disorders) as well as co-detection with other respiratory viruses and possibly the RV-species associated with infection (Lee et al., 2012; Miller et al., 2011). In contrast with the recent study in Italian children with pneumonia (Daleno et al., 2013), RV-C and not RV-A was found to be the predominant RV species associated with ARIs in hospitalised children (Cox et al., 2013; Lauinger et al., 2013; Linsuwanon et al., 2009; Renwick et al., 2007). Interestingly, community-based studies have also found that RV-A was the dominant species associated with milder ARIs not requiring admission to hospital (Lee et al., 2012).

Age may be an important factor in determining the severity of symptoms caused by a RV infection. For example, one study has shown that members of the RV-C species were more likely to cause severe symptoms in children younger than three years of age (Lauinger et al., 2013), while another noted roughly equal proportions of RV-A and RV-C during infancy, but RV-A dominated after one year of age (Daleno et al., 2013). Further, the season in which a RV-infection occurs may also play a role in the severity of the illness. In the northern hemisphere, there is a clear seasonal pattern for RV-infections and symptom severity. Infections that occur in summer months were more likely to be associated with milder respiratory symptoms compared to autumn and winter months (Lee et al., 2012). Whether these differences in severity are from an inoculum effect related to more crowding indoors during winter, drying of the nasal mucous membranes from breathing cool, low humidified air or low vitamin D levels affecting anti-viral defences is unknown (Bryson et al., 2014).

6.1.5 Nature of RV infections detected in the post-molecular era

The advent of molecular methods, which helped to generate renewed interest in RVs, has led to a better understanding of RV-infections, including observing RVs frequently in asymptomatic individuals, prolonged detection after symptom disappearance and co-detection with other respiratory viruses. These are summarised below.

6.1.5.1 Asymptomatic RV infections

Asymptomatic RV infections were recognised after the increased use of PCR assays for viral detection, and were found to be relatively common in infants. For example, RVs were detected in 38% of 988 nasopharyngeal samples collected from 433 healthy children participating in a randomised controlled trial of a pneumococcal conjugate vaccine in the Netherlands and who had undergone serial testing between the ages of six to 24 months (van den Bergh et al., 2012). A previous community-based study in 'high-risk' infants of atopic parents from Western Australia showed that 11.4% of 456 samples collected during asymptomatic periods were positive for RV (Kusel et al., 2006), while another from Wisconsin in the US (the COAST study) that investigated 285 infants who were also at 'high-risk' of asthma reported higher RV-detection rate (35%) during asymptomatic periods (Jartti et al., 2008). Variations in study design could help explain these differences. Although these two birth cohort studies investigated RV-infections during the first year of life, the former obtained respiratory samples only during two asymptomatic periods (one in winter and one in summer) (Kusel et al., 2006), while the latter collected samples during five routine scheduled visits throughout the first year of life (Jartti et al., 2008). This single variation in design suggests that in infants at high risk of asthma, the likelihood of detecting asymptomatic RV-infections can increase by simply escalating the frequency of sample collection. Overall, and to place this within a clinical context, the detection of RV in asymptomatic infants can be due to any one of the following circumstances: (i) symptoms are present, but go unrecognised, (ii) a nascent or incubating infection that leads to subsequent symptoms, (iii) a recent infection that has resolved, or (iv) a genuine subclinical infection.

6.1.5.2 Prolonged detection

RV shedding is typically considered to last between 11-21 days (Gern et al., 2000; Lu et al., 2008), although RV-B types can have slower replication and longer periods of viral shedding (Nakagome et al., 2014). Nevertheless, prolonged detection of RVs for more than four consecutive weeks has been reported by studies using PCR-based detection methods in clinical samples obtained from both healthy and immunocompromised children (Jartti et al., 2004; Kling et al., 2005). The median duration of RV-shedding in young immunocompromised patients was four-times longer than in immunocompetent healthy children (Peltola et al., 2013). However, without using genotyping techniques, little is known about possible type replacement during prolonged RV-detection periods. Indeed, prolonged detection of RV is now thought to be caused by strain replacement or overlapping RV infections rather than persistent shedding of the same type. A small study by van der Zalm and colleagues (2011) has demonstrated that in 19 otherwise healthy children, the perception of prolonged shedding was instead due to a series of sometimes overlapping infections

by different types. However, the median age of these children was 3.6 years and no details about the virus exposure status of these children were provided (i.e. childcare or school attendance). Longitudinal and more comprehensive data to support these assumptions in otherwise healthy infants are still lacking.

6.1.5.3 Co-detection

Another aspect of RV-infections that became apparent after the introduction of molecular methods was the frequent co-detections of RV with other respiratory viruses. In fact, it was due to the common co-detection of RV and other respiratory viruses that the clinical role of RV was questioned (Jacobs et al., 2013). A previous study examined the association between RVs and 16 other respiratory viruses in 1257 clinical specimens from different seasons. RVs were statistically less likely to be associated with many other respiratory viruses (Greer et al., 2009). Together these studies confirm that RVs have a leading role in respiratory illness rather than being mere passengers as has been suggested previously (Arden and Mackay, 2010; Mackay, 2008) and that the observed high rates of RV co-detections are more likely to be caused by the high rates of RV-infections in general. Likewise, previous studies have also shown that mixed RV/RV co-detections also occur (Lee et al., 2007; Renwick et al., 2007). Although the rate of RV/RV co-detection was relatively low in samples obtained from hospitalised patients (2/101 clinical samples) (Lee et al., 2007), the possibility of RV/RV co-detection in RV positive individuals in the community is unknown.

6.1.6 Study objectives

This chapter aims to describe RV infections in infants living within the community, including their incidence, prevalence, shedding, co-detection and seasonality, as well as catalogue basic clinical features of infected infants during the first year of life.

My hypotheses were:

1- RV-C infections in infants are more likely to be associated with symptoms than the other two RV species.
2- Prolonged detection of RV in otherwise healthy infants is from type replacement and overlapping RV infections rather than by persistent infection of a single type.

To achieve these aims, RV-positive samples from the cohort described in chapter-3 underwent VP4/VP2 typing to define their RV-genotype.

6.2 Methods

6.2.1 Study cohort and sample population

The same infant cohort described in chapter-3 (section 3.2.1) was used for this analysis. RV-genotypes were investigated in RV-positive nasal swabs collected from infants (n=3446) during their first year of life and from their parents (n=134) during the initial visit undertaken shortly after the birth of the study infants (chapter-2, section; 2.1.2.1). To investigate the clinical impact upon infants infected with different RVs, a sub-set of 3415 child swabs, which were accompanied by comprehensive symptom data were analysed (chapter-3; section: 3.3.3.1).

6.2.2 RV-TDE and ARI definitions

A new RV type detection episode (RV-TDE) was defined when a new RV-type was first detected or when the same RV-type was detected more than 14 days after a previous detection with the same genotype. During the 14 RV-free days, two negative intervening nasal swabs should be available. If one of these interval samples was missing, 30 days were required to define a new infection episode. Each untypeable RV detection was defined as a new RV-DE. ARI episodes were defined as described in chapter-3 (section 3.2.1).

6.2.3 RV screening and principal VP4/VP2 genotyping assay

Nucleic acid purification and RV screening were performed as per the ORChID study protocol (chapter-2; sections 2.1.4.2 and 2.1.4.3). The initial RV-typing workflow used in this project is illustrated in Figure 6.1.

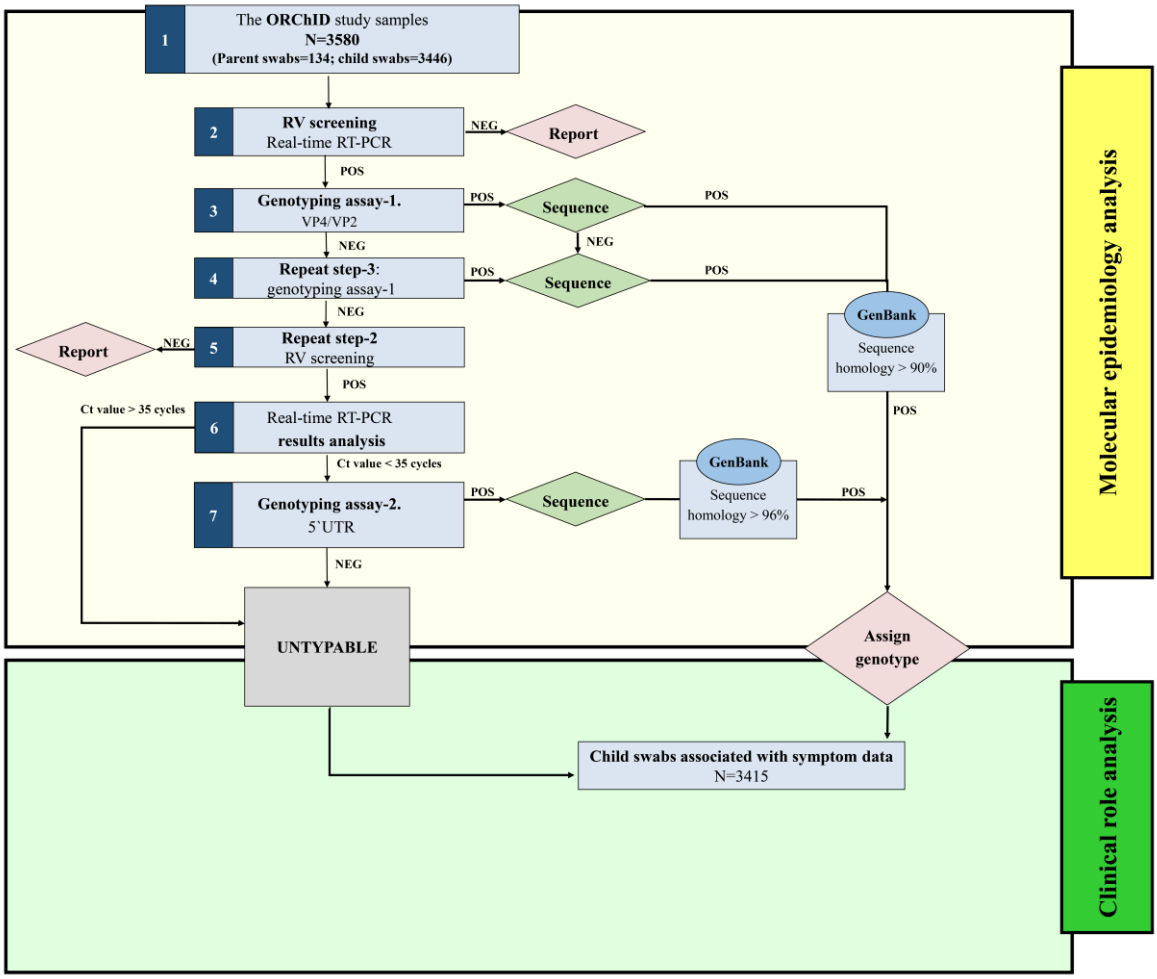

Figure 6.1 The workflow of RV-typing and description of two sample populations according to the availability of symptom data. The first sample population (yellow background) was used to study the molecular epidemiology of RV types and the second (green background) was used to analyse the clinical picture in patients with these types.

A previously described nested PCR assay was used for VP4/VP2 typing (Wisdom et al., 2009). The primers for all reaction rounds are listed in Table 6.1; Assay-1. The 20µL reaction mix for the first round, a RT step, comprised the following: 10µL SensiFast No Rox OneStep Mix (Bioline-Australia), 2µL of template, 0.4µL of RNase inhibitor enzyme, 0.2 RT-Taq polymerase enzyme and 0.6µM of each VP4/VP2 forward and reverse primers. The amplification was performed in the following conditions: a RT step at 45ºC for 20-minutes followed by an RT inactivation/Taq activation step of 94ºC for 2-minutes, 35 cycles of (1) a denaturation step at 94 ºC for 18-seconds and (2) two annealing and elongation steps at (a) 50ºC for 21-seconds and (b) 72ºC for 90 seconds, then a final elongation step of 72ºC for 7-minutes. A 2ul aliquot of the RT-PCR product resulting from first round RT-PCR was used as a template for the second round of PCR that comprised a 20µL reaction mix containing the following: 4 µL MyTaq HS DNA polymerase reaction buffer (Bioline, Australia), 0.1µL of MyTaq HS DNA polymerase and 1.75 mM of $MgCl_2$. The second round PCR was performed under the following cycling conditions: a polymerase activation step of

94ºC for 1-minutes, 35 cycles of (1) a denaturation step at 94 ºC for 18-seconds and (2) annealing and elongation steps at 50ºC for 21-seconds and 72ºC for 90-seconds, then a final elongation step of 72ºC for 7-minutes. An 8µL aliquot of each amplification reaction was examined by electrophoresis through a 2% agarose gel. The remaining PCR product from all positive reactions was purified using a QIAquick purification kit (Qiagen, Australia) by following the manufacturer instructions.

Table 6.1 Conventional RT-PCR and PCR assays used for RV-genotyping

Assay name; round	Primer name	Primer sequence	Genome target	Type of assay	References
Assay-1: VP4/VP2[a]					
Round 1 (outer primer pair)	VP4/VP2-OF[b] VP4/VP2-OR[b]	CCGGCCCCTGAATGYGGCTAA ACATRTTYTSNCCAAANAYDCCCAT	VP4/VP2	Nested RT-PCR	(Wisdom et al., 2009)
Round 2 (inner primer pair)	VP4/VP2-IF[b] VP4/VP2-IR[b]	ACCRACTACTTTGGGTGTCCGTG TCWGGHARYTTCCAMCACCANCC			
Assay-2: 5`UTR[c]	5`UTR-F 5`UTR-R	GCACTTCTGTTTCCCCC CGGACACCCAAAGTAG	5`UTR	RT-PCR	(Gama et al., 1989)

[a] VP: viral protein.
[b] OF; outer forward primer, OR; outer reverse primer, IF: inner forward primer, IR: inner reverse primer.
[c] UTR: untranslated region

Troubleshooting steps: Where VP4/VP2 amplification failed, the assay was repeated (Figure 6.1). For samples that produced more than one PCR product band when electrophoresed, each band was extracted from the gel and purified using QIAquick gel extraction kit (Qiagen, Australia) following the manufacturer instructions. The purified DNA for each band was sent to the Australian Genome Research Facility (The University of Queensland, Brisbane) for automated Sanger sequencing. Sequence data were compared to the Genbank database using BLASTn and analysed using Geneious version 5.5.7 (Biomatters Ltd). For VP4/VP2 genotyping, a nucleotide sequence identity of 90% or greater was required to assign the virus as a variant of a known RV genotype (McIntyre et al., 2013; Simmonds et al., 2010). To visualize the RV types, infer species and observe relationships, a neighbour-joining phylogenetic tree was constructed by MEGA version 5.2 using the generated VP4/VP2 sequences. The number of bootstrap replications was 2000 and p-distance was used for branch length. To root the tree, *Enterovirus* C (EV-C) sequences were used.

6.2.4 Alternative RV-typing RT-PCR assay (5`UTR)

Previous studies have shown that a small number of samples may fail to genotype using the VP4/VP2 method (Lee et al., 2007) and a 5'UTR typing method was established for such samples. Here, the 5`UTR assay was performed using previously designed primers (listed in Table 6.1; Assay-2) and reaction conditions. The 20µL reaction mix included the same components used in the first round of the VP4/VP2 reaction, while the amplification process was performed using slightly

different conditions as in the following: a RT step at 45ºC for 20-minutes followed by an activation step of 94ºC for 2-minutes, 40 cycles of (1) a denaturation step at 94ºC for 20-seconds, (2) two annealing and elongation steps at (a) 55ºC for 20-seconds and (b) 72ºC for 50-seconds, then a final elongation step of 72ºC for 10-minutes. The post-PCR sequencing and sequence analysis steps were performed as mentioned above. For 5`UTR, a sequence identity of 96% or greater was required for assignation (Lee et al., 2007).

6.2.5 Outcomes of interest and confounders

The outcomes of interest for the analyses performed in this chapter included subcategories of all RV-species; RV-A, RV-B, RV-C and episodes caused by untypeable samples. For all outcomes, twelve confounding variables were included. These were the age quarter of which the outcomes of interest occur, gender, exposure to smoking, maternal vaccination status, the participating infant's order in the family, number of siblings in the household, feeding status, childcare, season, co-detection with other respiratory viruses and the association between the outcome of interest and symptoms. The variable exposure to smoking included three categories, depending on parental smoking (mother or father) and the absence of this exposure. For feeding status, the measurement of outcome was compared in three cases; (i) when the infant was exclusively breastfed, (ii) when other milk was introduced and (iii) when solids were introduced. For childcare status the occurrence of the outcome of interest was examined for any infant who attended (i) formal childcare, (ii) informal childcare, (iii) both formal and informal childcare and (iv) no childcare. Informal childcare was defined as care arranged by a parent and provided by an unpaid carer (family member or friends).

6.2.6 Statistical analysis

The association between variables of interest and outcomes was investigated using mixed effects logistic regression models, with infants included as a random intercept to account for the possibility of correlated outcomes within each infant. Both univariate and multivariate analyses were conducted, with the multivariate analysis adjusted for age, gender, number of siblings in the house and season. Analyses were conducted using Stata statistical software v.11.0 (StataCorp, College Station, TX, USA).

6.3 Results

6.3.1 RV detection and the study cohort

In total, 667/3580 (18.6%) nasal swabs collected from participating infants and parents were positive for RV detection. These comprised, 659/3446 (19.1%) child nasal swabs collected during their first year of life and 8/134 (5.9%) parental nasal swabs that were collected at the beginning of the study and shortly after the birth of the participating infant. The distribution of RV-positive swabs was described in chapter-3 (section 3.3.3.3) with six additional RV-positive swabs included here that were part of the 31 nasal swabs not associated with symptoms data and were, therefore, excluded from the original analysis in chapter-3. These six RV-positives included four additional samples for infant 019 and one each from infant 044 and 055.

6.3.2 Genotyping

Of the 667 RV-positive samples selected for sequencing, 475 (71.1%) were sequenced successfully using the VP4/VP2 region. These included 471 samples collected from infants and four from parents. The remaining 193 samples that failed the VP4/VP2 sequencing (189 child and four parent samples) underwent further investigations as mentioned below.

In the 471 typable samples, 99 different sequences related to the genus *Enterovirus* were identified during the 26 months of investigation. Of these, 87 were assigned to previously defined RV-types. Ten other types did not meet sequence homology criteria and were unable to be assigned to a specific RV-type (hereafter named; unclassified VP4/VP2 sequences). Four other types were related to other *Enterovirus* species.

Unclassifiable VP4/VP2 sequences were identified from 60 samples collected from 31 infants. Eight sequences were most closely related to VP4/VP2 sequences from species C (C/seq-1 to C/seq-8) while the remaining two sequences were closely related to species A (A/seq-1 and A/seq-2). Figure 6.2 illustrates the phylogeny of these VP4/VP2 sequences and the closely related types.

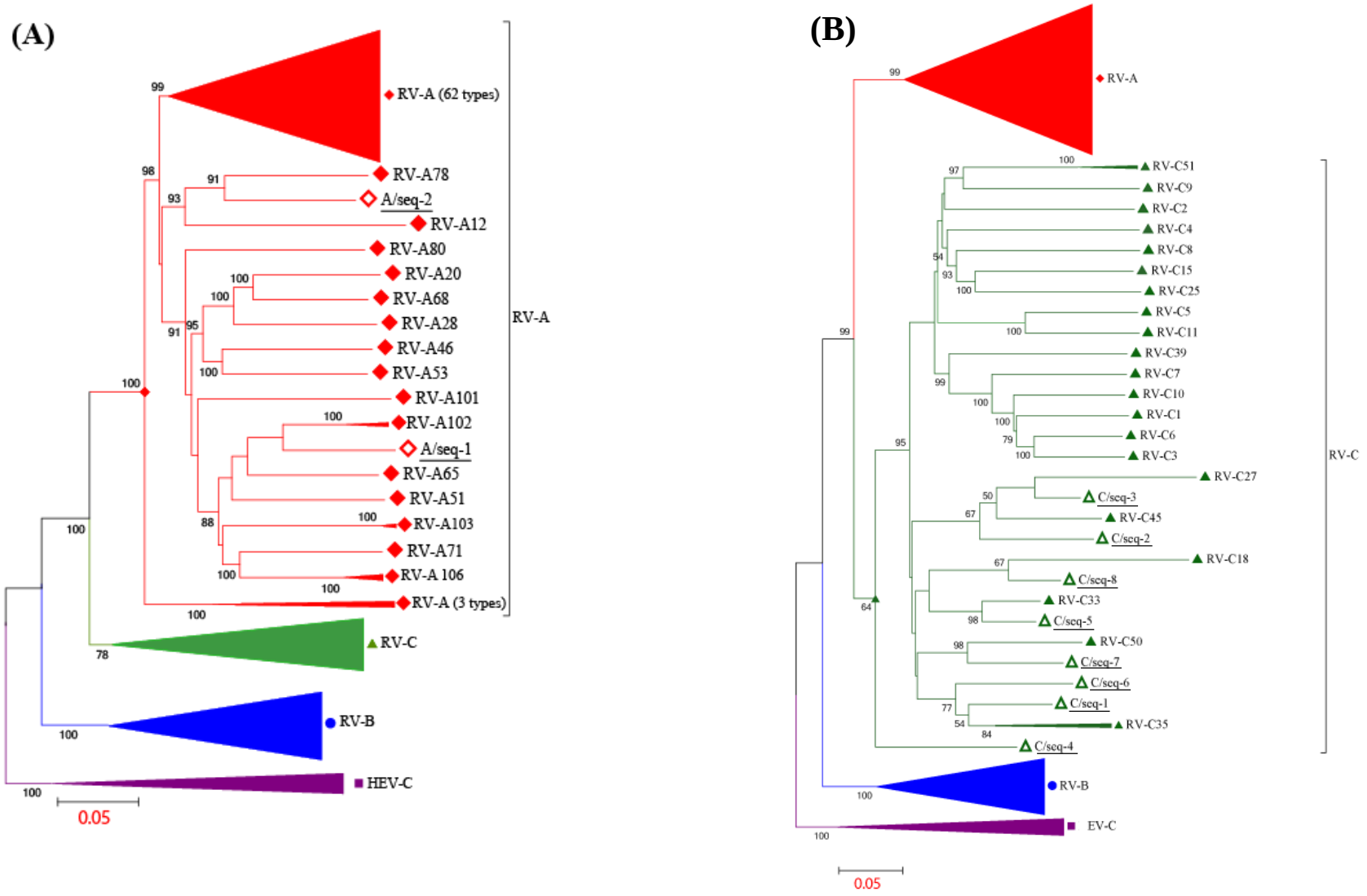

Figure 6.2 Two neighbour-joining trees to show the phylogeny of the ten unclassified VP4/VP2 sequences (Appendix-1). The two trees were constructed based upon representative full-length VP4/VP2 sequences. The trees are rooted using sequences from EV-C. Relevant nodes are labelled with bootstrap values (%). Multiple sequences from the same RV-species are shown as triangles, the height and depth corresponding to the number and divergence of the sequences used respectively. (A) Illustrates two unclassified sequences that were closely related to RV-A (in bold; hollow diamonds). (B) Illustrates eight unclassified sequences were identified as RV-C (B. Underlined; hollow diamonds).

For types C/seq-1 and C/seq-6, which were closely related to RV-C35, the pairwise identity of the two sequences was 83.4%, which is below the required level by 6.6%. For sequence C/seq-3, the highest identity value of 91% was obtained from a relatively low coverage of 84%, while higher coverages consistently produced identities below 90%. The ten unclassifiableVP4/VP2 sequences are listed in Appendix 6-1. Four other *Enterovirus* species were identifed, a sole detection of echovirus-7 was identified in one infant nasal swab sample, while the remaining three were co-detected with RV. To investigate the multiple detections, each of the six PCR products was sequenced. For the three samples, sequencing of the smaller size bands provided uninterpretable sequence data, while sequencing the larger size bands indicated one EV-D68 and two human coxackievirus-B3 viruses.

RV-A was the most commonly detected species in infant swabs (n=210; 31.9 % of the total RV-positive infant swabs) followed closely by RV-C (n=199; 30.2%) and then by RV-B (n=61; 9.3%). There was almost identical degree of types diversity observed in RV-A and RV-C whereas 44 distinct RV-A types and 43 RV-C types were identified. For RV-B, only 7 types identified (Table 6.2).

Table 6.2 Features of RV-species identified in the first year of life in 71 infants

Feature	**A**	**B**	**C**	**UT samples**
Number of types detected	44	7	43	-
Most frequently detected type in nasal swabs	A/seq-2	B/84	C/02	-
Most frequently detected type in infants	A/seq-2	B/84	C/02	-
Number of positive swabs (% of 659 positive swabs)[a]	210 (31.9)	61 (9.3)	199 (30.2)	189 (28.7)
Number of positive infants (% of 71 positive infants) [a]	59 (83.1)	29 (40.8)	64 (90.1)	64 (90.1)
Number of RV-TDEs (% of 520 total DEs) [a]	127 (24.4)	38 (7.3)	164 (31.5)	189 (36.3)
Median duration of RV-TDEs. (min-max)	1 (1-5)	1 (1-5)	1 (1-3)	-
Number of months in which RV-TDEs were identified	23	16	20	21
Peak month	October-2011	May-2011	May-2012	August & December-2011
Number of RV-TDE associated with ARIs (% of 520 total RV-TDEs)	86 (16.5)	13 (2.5)	115 (22.1)	102 (19.6)
Multiple viruses RV-TDEs (% of RV-TDEs).	25 (19.6)	4 (10.5)	20 (12.2)	14 (7.4)
KIV	5	2	4	2
RSV-A	1	1	0	1
hBoV	5	0	6	4
HAdV	7	1	6	3
HCoV-NL63	0	0	1	1
WUV	4	0	3	2
PIV-1	1	0	0	0
PIV-3	2	0	0	1

[a] Including the detected human echovirus

The median number of different RV-types identified among positive infants, including the unclassified RV-sequences, was five types per infant with a maximum of ten different types, which were identified in two infants each (infants 010 and 094). Overall, 520 RV-TDEs were identified during the first year of life, including those that were caused by untypeable samples, with a median incidence of seven RV-TDEs per infant (IQR 5-9) (Table 6.2) and a maximum of 14 RV-TDEs in infant 042 (Table 6.3).

Table 6.3 Descriptive analysis of different RV-genotypes identified during the first year of life in the 71 infants who tested positive for RV

RV type	No. positive infants (% in 71 positive participants)	No. of positive samples (% of typeable samples (459)	% in samples from same species; (A=210; B=61; C=172)	No. of TDEs. (% of 520 TDEs.)	No. multiple virus TDEs. (% of TDEs)	No. symptomatic TDEs (% of TDEs)	No. symptomatic and multiple TDEs.
A/seq-2[a]	13 (18.3)	31 (6.8)	14.8	13 (2.5)	5 (38.5)	11 (84.6)	4
A/78	9 (12.7)	25 (5.4)	11.9	11 (2.1)	4 (36.3)	9 (81.8)	3
A/63	8 (11.3)	16 (3.5)	7.6	8 (1.5)	1 (12.5)	7 (87.5)	1
A/22	8 (11.3)	12 (2.6)	5.7	8 (1.5)	0 (0)	5 (62.5)	0
A/59	7 (9.9)	10 (2.2)	4.8	7 (1.3)	2 (28.6)	4 (57.1)	2
A/19	6 (8.5)	8 (1.7)	3.8	7 (1.3)	1 (14.3)	5 (71.4)	1
A/24	6 (8.5)	8 (1.7)	3.8	6 (1.2)	1 (16.7)	3 (50)	1
A/80	5 (7)	8 (1.7)	3.8	5 (1)	0 (0)	5 (100)	0
A/12	4 (5.6)	7 (1.5)	3.3	4 (0.8)	0 (0)	3 (75)	0
A/58	4 (5.6)	6 (1.3)	2.9	4 (0.8)	1 (25)	3 (75)	1
A/01	4 (5.6)	5 (1.1)	2.4	4 (0.8)	1 (25)	1 (25)	0
A/60	4 (5.6)	5 (1.1)	2.4	4 (0.8)	0 (0)	2 (50)	0
A/85	3 (4.2)	5 (1.1)	2.4	3 (0.6)	1 (33.3)	2 (66.7)	1
A/20	2 (2.8)	4 (0.9)	1.9	2 (0.4)	0 (0)	2 (100)	0
A/45	3 (4.2)	4 (0.9)	1.9	3 (0.6)	0 (0)	2 (66.7)	0
A/56	3 (4.2)	4 (0.9)	1.9	3 (0.6)	0 (0)	1 (33.3)	0
A/103	2 (2.8)	3 (0.7)	1.4	2 (0.4)	1 (50)	1 (50)	1
A/33	1 (1.4)	3 (0.7)	1.4	1 (0.2)	0 (0)	1 (100)	0
A/36	2 (2.8)	3 (0.7)	1.4	2 (0.4)	0 (0)	2 (100)	0
A/38	1 (1.4)	3 (0.7)	1.4	1 (0.2)	0 (0)	0 (0)	0
A/55	1 (1.4)	3 (0.7)	1.4	1 (0.2)	0 (0)	0 (0)	0
A/68	2 (2.8)	3 (0.7)	1.4	2 (0.4)	1 (50)	2 (100)	1
A/89	3 (4.2)	3 (0.7)	1.4	3 (0.6)	0 (0)	2 (66.7)	0
A/90	3 (4.2)	3 (0.7)	1.4	3 (0.6)	0 (0)	1 (33.3)	0
A/21	1 (1.4)	2 (0.4)	1.0	1 (0.2)	0 (0)	0 (0)	0

[a] Unclassified sequence from RV-A

Table 6.3 Continued. Descriptive analysis of different RV-genotypes identified during the first year of life in 71 infants

RV type	No. positive infants (% in 71 positive participants)	No. of positive samples (% of typeable samples (459)	% in samples from same species; (A=210; B=61; C=172)	No. of TDEs. (% of 520 TDEs.)	No. multiple virus TDEs. (% of TDEs)	No. symptomatic TDEs (% of TDEs)	No. symptomatic and multiple TDEs.
A/47	2 (2.8)	2 (0.4)	1.0	2 (0.4)	0 (0)	2 (100)	0
A/49	1 (1.4)	2 (0.4)	1.0	1 (0.2)	0 (0)	0 (0)	0
A/54	1 (1.4)	2 (0.4)	1.0	1 (0.2)	0 (0)	0 (0)	0
A/07	1 (1.4)	2 (0.4)	1.0	1 (0.2)	0 (0)	1 (100)	0
A/71	1 (1.4)	2 (0.4)	1.0	1 (0.2)	0 (0)	0 (0)	0
A/81	1 (1.4)	2 (0.4)	1.0	1 (0.2)	1 (100)	0 (0)	0
A/09	1 (1.4)	2 (0.4)	1.0	1 (0.2)	0 (0)	1 (100)	0
A/seq-1	1 (1.4)	2 (0.4)	1.0	1 (0.2)	0 (0)	0 (0)	0
A/101	1 (1.4)	1 (0.2)	0.5	1 (0.2)	0 (0)	1 (100)	0
A/18	1 (1.4)	1 (0.2)	0.5	1 (0.2)	1 (100)	1 (100)	1
A/28	1 (1.4)	1 (0.2)	0.5	1 (0.2)	0 (0)	1 (100)	0
A/31	1 (1.4)	1 (0.2)	0.5	1 (0.2)	0 (0)	1 (100)	0
A/32	1 (1.4)	1 (0.2)	0.5	1 (0.2)	1 (100)	1 (100)	1
A/39	1 (1.4)	1 (0.2)	0.5	1 (0.2)	1 (100)	1 (100)	1
A/40	1 (1.4)	1 (0.2)	0.5	1 (0.2)	1 (100)	1 (100)	1
A/46	1 (1.4)	1 (0.2)	0.5	1 (0.2)	1 (100)	1 (100)	1
A/82	1 (1.4)	1 (0.2)	0.5	1 (0.2)	0 (0)	0 (0)	0
A/94	1 (1.4)	1 (0.2)	0.5	1 (0.2)	0 (0)	0 (0)	0
B/84	8 (11.3)	20 (4.4)	32.8	8 (1.5)	1 (12.5)	4 (50)	0
B/06	7 (9.9)	14 (3.1)	23.0	8 (1.5)	1 (12.5)	2 (25)	0
B/35	7 (9.9)	8 (1.7)	13.1	7 (1.3)	0 (0)	1 (14.3)	0
B/52	6 (8.5)	9 (2)	14.8	6 (1.2)	0 (0)	1 (16.7)	0
B/42	5 (7)	6 (1.3)	9.8	5 (1)	1 (20)	3 (60)	0
B/26	2 (2.8)	2 (0.4)	3.3	2 (0.4)	1 (50)	1 (50)	1
B/14	1 (1.4)	2 (0.4)	3.3	1 (0.2)	0 (0)	1 (100)	0
C/02	18 (25.4)	28 (6.1)	16.3	19 (3.7)	2 (10.5)	14 (73.7)	1
C/36	12 (16.9)	18 (3.9)	10.5	14 (2.7)	1 (7.1)	8 (57.1)	1
C/10	8 (11.3)	11 (2.4)	6.4	9 (1.7)	1 (11.1)	7 (77.8)	0
C/15	8 (11.3)	11 (2.4)	6.4	9 (1.7)	1 (11.1)	8 (88.9)	1
C/06	10 (14.1)	13 (2.8)	7.6	10 (1.9)	3 (30)	8 (80)	3
C/40	8 (11.3)	10 (2.2)	5.8	8 (1.5)	2 (25)	6 (75)	1
C/seq-4[b]	9 (12.7)	9 (2)	5.2	9 (1.7)	0 (0)	6 (66.7)	0
C/43	7 (9.9)	10 (2.2)	5.8	8 (1.5)	3 (37.5)	5 (62.5)	2
C/20	6 (8.5)	7 (1.5)	4.1	6 (1.2)	1 (16.7)	3 (50)	1

[b] Unclassified sequence from RV-C

Table 6.3 Continued. Descriptive analysis of different RV-genotypes identified during the first year of life in 71 infants

HRV type	No. positive infants (% in 71 positive participants)	No. of positive samples (% of typeable samples (459)	% in samples from same species; (A=210; B=61; C=172)	No. of TDEs. (% of 520 TDEs.)	No. multiple virus TDEs. (% of TDEs)	No. symptomatic TDEs (% of TDEs)	No. symptomatic and multiple TDEs.
C/05	4 (5.6)	6 (1.3)	3.5	4 (0.8)	0 (0)	3 (75)	0
C/12	4 (5.6)	5 (1.1)	2.9	4 (0.8)	0 (0)	3 (75)	0
C/14	3 (4.2)	5 (1.1)	2.9	3 (0.6)	0 (0)	1 (33.3)	0
C/seq-2[b]	4 (5.6)	5 (1.1)	2.9	4 (0.8)	1 (25)	2 (50)	0
C/42	4 (5.6)	4 (0.9)	2.3	4 (0.8)	0 (0)	1 (25)	0
C/51	4 (5.6)	4 (0.9)	2.3	4 (0.8)	0 (0)	4 (100)	0
C/seq-6[b]	4 (5.6)	4 (0.9)	2.3	4 (0.8)	0 (0)	4 (100)	0
C/13	2 (2.8)	3 (0.7)	1.7	2 (0.4)	0 (0)	2 (100)	0
C/17	3 (4.2)	3 (0.7)	1.7	3 (0.6)	0 (0)	3 (100)	0
C/19	3 (4.2)	3 (0.7)	1.7	3 (0.6)	0 (0)	2 (66.7)	0
C/38	3 (4.2)	3 (0.7)	1.7	3 (0.6)	0 (0)	2 (66.7)	0
C/seq-1[b]	3 (4.2)	3 (0.7)	1.7	3 (0.6)	0 (0)	2 (66.7)	0
C/01	1 (1.4)	2 (0.4)	1.2	1 (0.2)	0 (0)	1 (100)	0
C/11	2 (2.8)	2 (0.4)	1.2	2 (0.4)	0 (0)	2 (100)	0
C/39	1 (1.4)	1 (0.2)	0.6	1 (0.2)	0 (0)	1 (100)	0
C/41	1 (1.4)	1 (0.2)	0.6	1 (0.2)	0 (0)	1 (100)	0
C/48	1 (1.4)	1 (0.2)	0.6	1 (0.2)	0 (0)	0 (0)	0
C/seq-5 [b]	1 (1.4)	1 (0.2)	0.6	1 (0.2)	1 (100)	1 (100)	1
C/seq-7 [b]	1 (1.4)	1 (0.2)	0.6	1 (0.2)	0 (0)	1 (100)	0

[b] Unclassified sequence from RV-C

RV-A type detection episodes (RVA-TDEs) were detected in all months, except for September-2012, while RVC-TDEs and RVB-TDEs were absent in four and eight months respectively (Figure 6.3). There were two peaks of RV-TDEs; the first was in August-2011 and the second was observed in May-2012. However, at a species level this double-peak model was not obvious for either RVA-TDEs or RVB-TDEs (Figure 6.3).

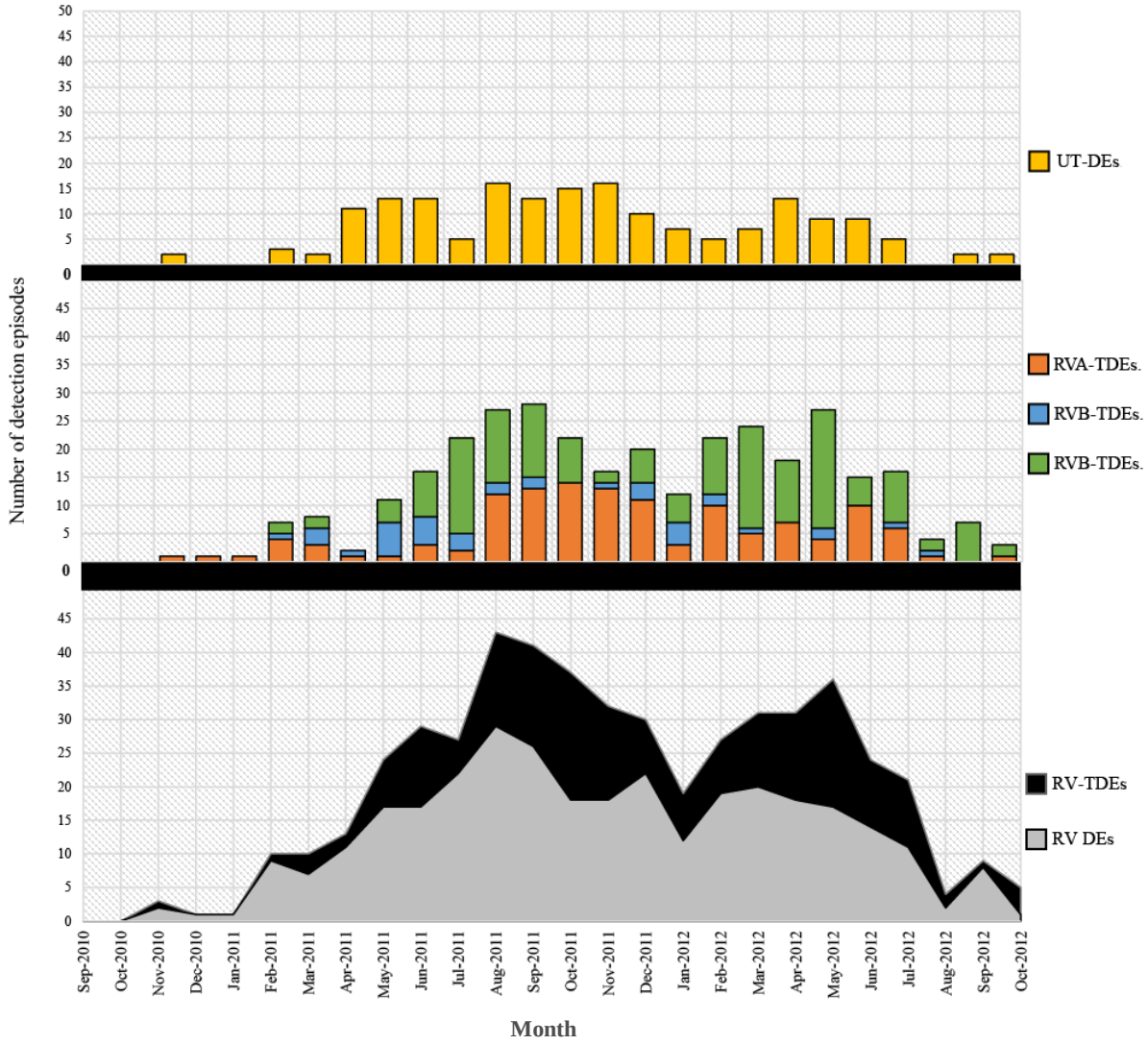

Figure 6.3 A comparison between the overall number of RV-detection episodes before typing (RV-DEs) and RV-type detection episodes after typing (RV-TDEs) (bottom). Number of RV-type detection episodes by species A (RVA-TDEs, orange), B (RVB-TDEs, blue) and C (RVC-TDEs, green) (middle) and the number of detection episodes that were untypeable (UT-DEs, top) during the 26 months of investigation.

For RV-A, the most frequently detected sequence was the unclassified type RV-A/seq-2, which was found to be closely related to the second most frequently detected type, RV-A/78. The latter was identified in 21 nasal swab samples (4.4% of typed samples) in nine infants (12.7% of positive infants) leading to eleven RV-TDEs, 81.8% of which were associated with symptoms (Table 6.3). The median duration of shedding in RV-A genotypes was one week (IQR 1-2 weeks). The maximum duration of shedding in RV-A types was observed in RV-A/seq-2 with five consecutive weeks in only one infant. Among RVA-TDE, 23 (18.1% of total RVA-TDEs) included another respiratory virus and two

detection episodes included two additional respiratory viruses (Table 6.2). RV-A/seq-2 was the most common type to be detected with other respiratory viruses, where five detection episodes (38.5% of total RV-A/seq-2 TDEs) contained another virus followed by RV-A/78 with four co-detections (Table 6.3).

For RV-C, RV-C/02 was identified in 18 infants (25.4% of positive infants) causing 19 rhinovirus C-type detection episodes (RVC-TDEs) (3.7% of total RV-TDEs), which is the highest detection rate among all identified RV-types. The median duration of shedding for RV-C types was one week and the maximum shedding duration of three weeks was observed in three detection episodes caused by three types; these were RV-C/5, RV-C/14 and RV-C/15. For RVC-TDEs, 20 episodes (12.2% of total RVC-TDEs) contained another virus and there was no episode that contained more than one other respiratory virus. For these, only one episode was accompanied by an RNA virus, which was hCoV-NL-63, while the remaining 19 episodes were accompanied by DNA viruses (Table 6.2). For RV-C/02, the most frequently detected amongst the RV-C species, only two episodes were accompanied by another virus, which was hBoV in both. Both RV-C/43 and RV-C/06 were the most common RV-C types to be detected with another respiratory virus, where each of three detection episodes from each type were accompanied by another respiratory virus (37.5% and 30% of total detection episodes respectively) (Table 6.3).

For RV-B, the most frequently observed type was RV-B/84, which was detected in 20 nasal swabs (4.4% of total typeable swabs) collected from eight participants (11.3% of total positive participants) and causing eight RVB-TDEs with a median incidence in the first year of life of one episode per positive participant. The median duration of shedding for RV-B types was two weeks and the maximum of five weeks was observed in RV-B/84. Four RVB-TDEs (10.5% of total RVB-TDEs) were accompanied by another virus. These were KIV in two episodes (RV-B/06 and RVB-84), HAdV in one RV-B/26 detection episode and RSV-A in one RV-B/42 detection episode.

6.3.3 RV-species in infants

To investigate the association between RV-species and clinical outcome, 3415 nasal swabs with accompanying clinical data were included in this analysis (Figure 6.1). This excludes a total of 31 nasal swabs, two RVA-TDEs and two RVB-TDEs. The success of VP4/VP2 typing differed between infants. Only six had 100% typing rates (infants; 094,092,039,081,063 and 091). (Table 6.4).

Table 6.4 Distribution of RV-species and number of types and RV-TDEs in infants

SID	Available swabs	Number of RV-positive samples (%)	Total number of types	RV-A positive samples (%of RV-typeable samples)	Number of species A-types	Number of RVA-TDEs	RV-B positive samples (%of RV-typeable samples)	Number of species B-types	Number of RVB-TDEs	RV-C positive samples (%of RV-typeable samples)	Number of species C-types	Number of RVC-TDEs	UT samples (% of RV-posivies)
094	51	13 (25.5)	10	5 (38.5)	3	3	0 (0)	0	0	7 (53.8)	7	7	0 (0)
027	52	23 (44.2)	9	10 (50)	4	5	6 (30)	3	3	2 (10)	2	2	3 (13)
046	47	14 (29.8)	9	3 (27.3)	3	3	0 (0)	0	0	6 (54.5)	6	6	3 (21.4)
065	48	15 (31.3)	8	7 (53.8)	4	4	3 (23.1)	2	2	2 (15.4)	2	2	2 (13.3)
067	50	12 (24)	8	4 (36.4)	2	2	0 (0)	0	0	6 (54.5)	6	6	1 (8.3)
072	49	13 (26.5)	8	7 (58.3)	4	4	2 (16.7)	1	1	3 (25)	3	3	1 (7.7)
095	33	14 (42.4)	8	6 (60)	4	4	1 (10)	1	1	3 (30)	3	3	4 (28.6)
042	49	21 (42.9)	7	6 (37.5)	3	3	5 (31.3)	2	3	2 (12.5)	2	3	5 (23.8)
078	36	10 (27.8)	7	3 (37.5)	2	2	0 (0)	0	0	5 (62.5)	5	5	2 (20)
088	46	10 (21.7)	7	4 (44.4)	3	3	1 (11.1)	1	1	3 (33.3)	3	3	1 (10)
001	32	8 (25)	6	4 (57.1)	3	3	1 (14.3)	1	1	2 (28.6)	2	2	1 (12.5)
016	47	12 (25.5)	6	2 (28.6)	2	2	2 (28.6)	2	2	2 (28.6)	2	2	5 (41.7)
017	49	10 (20.4)	6	4 (50)	2	2	0 (0)	0	0	4 (50)	4	4	2 (20)
023	51	20 (39.2)	6	6 (37.5)	2	2	3 (18.8)	1	1	3 (18.8)	3	5	4 (20)
025	52	12 (23.1)	6	5 (71.4)	4	4	0 (0)	0	0	2 (28.6)	2	2	5 (41.7)
051	51	12 (23.5)	6	1 (14.3)	1	1	2 (28.6)	2	2	3 (42.9)	3	3	5 (41.7)
054	49	13 (26.5)	6	4 (50)	3	3	0 (0)	0	0	3 (37.5)	3	3	5 (38.5)
055	44	9 (20.5)	6	3 (42.9)	2	2	1 (14.3)	1	1	3 (42.9)	3	3	2 (22.2)
060	53	10 (18.9)	6	3 (42.9)	2	2	0 (0)	0	0	4 (57.1)	4	4	3 (30)
062	46	18 (39.1)	6	4 (33.3)	1	1	1 (8.3)	1	1	4 (33.3)	4	4	6 (33.3)
066	52	11 (21.2)	6	3 (33.3)	2	2	0 (0)	0	0	4 (44.4)	4	4	2 (18.2)
075	48	11 (22.9)	6	1 (12.5)	1	1	2 (25)	1	1	4 (50)	4	4	3 (27.3)
092	48	9 (18.8)	6	4 (44.4)	2	2	2 (22.2)	1	1	3 (33.3)	3	3	0 (0)
019	48	8 (16.7)	5	3 (50)	3	3	2 (33.3)	1	2	1 (16.7)	1	1	2 (25)
020	51	10 (19.6)	5	4 (50)	2	2	1 (12.5)	1	1	2 (25)	2	2	2 (20)
035	52	12 (23.1)	5	4 (57.1)	2	2	0 (0)	0	0	3 (42.9)	3	3	5 (41.7)
038	53	6 (11.3)	5	3 (60)	3	3	1 (20)	1	1	1 (20)	1	1	1 (16.7)
041	52	12 (23.1)	5	3 (50)	2	2	1 (16.7)	1	1	2 (33.3)	2	2	6 (50)
043	50	12 (24)	5	8 (80)	3	3	0 (0)	0	0	2 (20)	2	2	2 (16.7)
050	40	6 (15)	5	2 (40)	2	2	0 (0)	0	0	3 (60)	3	3	1 (16.7)
056	52	13 (25)	5	3 (33.3)	2	2	3 (33.3)	1	1	2 (22.2)	2	3	4 (30.8)
064	51	16 (31.4)	5	3 (33.3)	2	2	0 (0)	0	0	3 (33.3)	3	3	7 (43.8)
071	48	10 (20.8)	5	6 (75)	4	4	0 (0)	0	0	1 (12.5)	1	1	2 (20)
080	50	9 (18)	5	2 (28.6)	1	1	0 (0)	0	0	4 (57.1)	4	4	2 (22.2)
085	49	11 (22.4)	5	2 (28.6)	1	1	0 (0)	0	0	4 (57.1)	4	4	4 (36.4)
002	33	8 (24.2)	4	4 (57.1)	2	2	0 (0)	0	0	2 (28.6)	2	2	1 (12.5)

Table 6.4 Continued. Distribution of RV-species and number of types and RV-TDEs in infants

SID	Available swabs	Number of RV-positive samples (%)	Total number of types	RV-A positive samples (%of RV-typeable samples)	Number of species A-types	Number of RVA-TDEs	RV-B positive samples (%of RV-typeable samples)	Number of species B-types	Number of RVB-TDEs	RV-C positive samples (%of RV-typeable samples)	Number of species C-types	Number of RVC-TDEs	UT samples (% of RV-posivies)
004	53	6 (11.3)	4	1 (25)	1	1	0 (0)	0	0	3 (75)	3	3	2 (33.3)
029	50	13 (26)	4	8 (88.9)	3	3	0 (0)	0	0	1 (11.1)	1	1	4 (30.8)
030	44	8 (18.2)	4	4 (66.7)	3	3	0 (0)	0	0	1 (16.7)	1	1	2 (25)
039	42	10 (23.8)	4	5 (50)	2	2	2 (20)	1	1	1 (10)	1	2	0 (0)
045	47	10 (21.3)	4	0 (0)	0	0	4 (50)	2	2	2 (25)	2	2	2 (20)
068	51	7 (13.7)	4	4 (66.7)	2	2	0 (0)	0	0	2 (33.3)	2	2	1 (14.3)
079	53	7 (13.2)	4	1 (20)	1	1	0 (0)	0	0	3 (60)	3	3	2 (28.6)
090	49	10 (20.4)	4	2 (40)	2	2	0 (0)	0	0	2 (40)	2	2	5 (50)
005	51	8 (15.7)	3	1 (33.3)	1	1	0 (0)	0	0	2 (66.7)	2	2	5 (62.5)
012	35	7 (20)	3	2 (66.7)	2	2	0 (0)	0	0	1 (33.3)	1	1	4 (57.1)
013	49	6 (12.2)	3	0 (0)	0	0	1 (25)	1	1	2 (50)	2	2	2 (33.3)
044	38	7 (18.4)	3	1 (33.3)	1	1	1 (33.3)	1	1	1 (33.3)	1	1	4 (57.1)
049	49	8 (16.3)	3	5 (100)	3	3	0 (0)	0	0	0 (0)	0	0	3 (37.5)
061	39	5 (12.8)	3	1 (33.3)	1	1	1 (33.3)	1	1	1 (33.3)	1	1	2 (40)
077	51	6 (11.8)	3	2 (50)	1	1	0 (0)	0	0	2 (50)	2	2	2 (33.3)
081	47	5 (10.6)	3	2 (40)	1	1	0 (0)	0	0	2 (40)	2	2	0 (0)
087	52	6 (11.5)	3	0 (0)	0	0	0 (0)	0	0	3 (100)	3	3	3 (50)
003	43	3 (7)	2	0 (0)	0	0	0 (0)	0	0	2 (100)	2	2	1 (33.3)
006	52	7 (13.5)	2	3 (75)	1	1	0 (0)	0	0	1 (25)	1	1	3 (42.9)
015	53	6 (11.3)	2	4 (80)	1	1	0 (0)	0	0	1 (20)	1	1	1 (16.7)
021	53	10 (18.9)	2	5 (100)	2	2	0 (0)	0	0	0 (0)	0	0	5 (50)
028	52	3 (5.8)	2	0 (0)	0	0	0 (0)	0	0	2 (100)	2	2	1 (33.3)
034	50	8 (16)	2	2 (40)	1	1	3 (60)	1	1	0 (0)	0	0	3 (37.5)
052	43	8 (18.6)	2	0 (0)	0	0	5 (83.3)	1	1	1 (16.7)	1	1	2 (25)
058	49	5 (10.2)	2	1 (50)	1	1	0 (0)	0	0	1 (50)	1	1	3 (60)
063	49	2 (4.1)	2	1 (50)	1	1	1 (50)	1	1	0 (0)	0	0	0 (0)
089	47	6 (12.8)	2	0 (0)	0	0	0 (0)	0	0	2 (100)	2	2	4 (66.7)
091	52	4 (7.7)	2	0 (0)	0	0	0 (0)	0	0	2 (50)	2	3	0 (0)
018	51	3 (5.9)	1	0 (0)	0	0	0 (0)	0	0	1 (100)	1	1	2 (66.7)
024	50	5 (10)	1	0 (0)	0	0	2 (100)	1	1	0 (0)	0	0	3 (60)
031	52	3 (5.8)	1	0 (0)	0	0	0 (0)	0	0	1 (100)	1	1	2 (66.7)
032	52	6 (11.5)	1	1 (100)	1	1	0 (0)	0	0	0 (0)	0	0	5 (83.3)
076	48	4 (8.3)	1	1 (100)	1	1	0 (0)	0	0	0 (0)	0	0	3 (75)
084	44	3 (6.8)	1	0 (0)	0	0	0 (0)	0	0	1 (100)	1	1	2 (66.7)
048	49	0 (0)	0	0 (0)	0	0	0 (0)	0	0	0 (0)	0	0	0 (0)

The lowest proportion of typeable samples was observed in one infant (032; table 6.4) with just one of the six RV-positive samples able to be genotyped. This type was RV-A/18, which was also the only RV-A/18 positive sample identified among all typeable samples (Table 6.3). The distribution of RV-genotypes in infants was highly variable (Table 6.4).

At the species level, and when excluding untypeable samples, only one species was identified in twelve infants (16.9% of total RV-positive infants). RV-C was the only species identified in eight infants (003, 018, 028, 031, 084, 087, 089 and 091) with ten different RV-C genotypes observed (RV-C/02, RV-C/05, RV-C/06, RV-C/10 RV-C/19, RV-C36, RV-C/51, RV-C/seq-4, RV-C/seq-7 and RV-C/seq-8) (Tables 6.3 and 6.4). Typeable RV-A was the only species identified in four infants (049, 021, 076 and 032) (Table 6.4). Among the four infants, five RV-A genotypes were identified (RV-A/18, RV-A/22, RV-A/60, RV-A/63 and RV-A/78). While for RV-B, it was the only species detected in just one infant (024) with only one genotype RV-B/84 found (Table 6.3 for details about genotypes distribution). Other than the above mentioned sole RV-species detections, RV-B was not observed in a further 31 infants (total of 42 RV-B negative infants; 58.3% of total RV-positive infants), RV-A species was not observed in four infants (total of twelve RV-A negative infants; 16.9% of total RV-positive infants), and RV-C was not observed in two further infants (total of nine RV-C negative infants; 12.7% of total RV-positive infants).

As shown in Table 6.5, after adjustment in the multivariate regression model for possible confounding factors, RVA-TDEs were significantly less likely to be observed in the second quarter of the first year of life and during the autumn months. Because of small numbers the model could not be applied to co-detections of RVA-TDEs with other viruses, although with the univariate analysis there was a significant association with DNA-viruses and weak evidence for an association with RNA-viruses too. Unexpectedly, the number of RVA-TDEs was significantly higher in infants whose mothers had received the influenza vaccine sometime prior to delivery.

Table 6.5 Analysis of RVA-TDEs with possible confounding factors; the multivariate regression was adjusted to age, gender, number of children in the house and season

	Variable	Total number of episodes	RV-A		
			No. (%)	Univariate OR (95%); P value	Multivariate OR (95%); P value
Age (months)	0-3	67 (13.1)	18 (26.9)	1	1
	3-6	116 (22.6)	14 (12.1)	**0.37 (0.17-0.81); 0.013**	**0.36 (0.16-0.79); 0.012**
	6-9	174 (33.9)	50 (28.7)	1.09 (0.58-2.05); 0.773	1.13 (0.58-2.17); 0.708
	9-12	156 (30.4)	43 (27.6)	1.035 (0.54-1.97); 0.915	1.01 (0.51-1.96); 0.986
Gender	Male	249 (48.5)	61 (24.5)	1	1
	Female	264 (51.5)	64 (24.2)	0.98 (0.65-1.47); 0.946	0.95 (0.62-1.45); 0.819
Exposure to smoking	Mother	8 (1.6)	3 (37.5)	1.12 (0.58-2.13); 0.735	2.79 (0.61-12.81); 0.185
	Father	54 (11.02)	14 (25.9)	1.91 (0.45-8.16); 0.378	0.99 (0.49-2.0); 0.<982
	No exposure	428 (87.4)	102 (23.8)	1	1
Mother IFV vaccine status	Any IFV vaccine	384 (76.7)	102 (26.6)	**1.75 (1.03-2.98); 0.038**	**1.85 (1.04-3.28); 0.034**
	No IFV vaccine	117 (23.4)	20 (17.1)	**1**	1
Infant order (mother)	First born	324 (63.2)	73 (22.5)	1	1
	Second	120 (23.4)	31 (25.8)	1.19 (0.73-1.94); 0.466	1.26 (0.42-3.82); 0.672
	Third	55 (10.7)	16 (29.1)	1.41 (0.74-2.66); 0.29	1.90 (0.21-16.5); 0.56
	Other	14 (2.7)	5 (35.7)	1.91 (0.62-5.87); 0.259	4.87 (0.39-60.6); 0.218
Number of children in the household	No other children in the household	298 (58.1)	68 (22.8)	1	1
	One child	151 (29.4)	38 (25.2)	1.13 (0.72-1.79); 0.58	1.07 (0.66-1.73); 0.765
	≥ two children	64 (12.5)	19 (26.7)	1.42 (0.78-2.6); 0.245	1.2 (0.64-2.24); 0.555
Household size	3	282 (54.9)	63 (22.3)	1	1
	4	130 (25.3)	33 (25.4)	1.18 (0.72-1.91); 0.497	1.1 (0.67-1.8); 0.688
	5	82 (15.9)	23 (28.1)	1.35 (0.77-2.36); 0.285	1.12 (0.62-2); 0.714
	≥ 6	19 (3.7)	6 (31.6)	1.6 (0.58-4.39); 0.358	2.04 (0.725.8); 0.181
Feeding	Exclusively breastfed	114 (22.2)	20 (17.5)	1	1
	Other milk	387 (75.4)	100 (25.8)	1.67 (0.96-2.79); 0.07	1.63 (0.69-3.83); 0.258
	Solids	12 (2.3)	5 (41.7)	3.35 (0.96-11.7); 0.057	2.39 (0.54-10.59); 0.248
Childcare status	Formal	93 (18.3)	28 (30.1)	1.47 (0.88-2.43); 0.135	1.3 (0.76-2.36); 0.298
	Informal	35 (6.8)	8 (22.3)	1.01 (0.44-2.3); 0.979	0.9 (0.36-2.22); 0.827
	Formal and informal	6 (1.2)	3 (50)	3.41 (0.67-17.21); 0.137	2.2 (0.41-11.71); 0.355
	No childcare	375 (73.7)	85 (22.7)	1	1
Season	Spring	125 (24.4)	41 (32.8)	1	1
	Summer	89 (17.4)	29 (32.6)	0.99 (0.55-1.76); 0.974	1.2 (0.63-2.13); 0.625
	Autumn	147 (28.7)	21 (14.3)	**0.34 (0.18-0.62); 0.001**	**0.35 (0.19-0.65); 0.001**
	Winter	152 (29.6)	34 (22.4)	0.59 (0.34-1.01); 0.053	0.61 (0.34-1.05); 0.077
Co-detection with other viruses	DNA-viruses	54 (10.5)	21 (38.9)	**2.2 (1.22-3.99); 0.008**	Not included[w]
	RNA-viruses	5 (0.9)	3 (60)	5.2 (0.86-31.62); 0.073	Not included[w]
	Both DNA and RNA	2 (0.4)	0	Not included[w]	Not included[w]
	No co-detection	452 (88.1)	101 (22.4)	1	Not included[w]
Clinical outcome	URTIs	264 (51.5)	70 (26.5)	1.5 (0.97-2.34); 0.069	1.89 (0.92-3.87); 0.082
	LRTIs	47 (9.2)	16 (34.0)	**2.2 (1.07-4.33); 0.031**	1.43 (0.89-2.27); 0.136
	No symptoms	202 (39.4)	39 (19.3)	1	1

[w]Small number of observations, so the OR and 95% CI were omitted.

Similarly, as displayed in Table 6.6, in the regression model RVB-TDEs were significantly less likely to be encountered in infants older than six months of age and in female infants. They were more

commonly observed in summer compared to other seasons, but less likely to be associated with symptoms, significantly so for URTIs.

Table 6.6 Analysis of RVB-TDEs with possible confounding factors; the multivariate regression was adjusted to age, gender, number of children in the house and season

	Variable	Total number of episodes	RV-B		
			No. (%)	Univariate OR (95%); P value	Multivariate OR (95%); P value
Age (months)	0-3	67 (13.1)	8 (11.9)	1	1
	3-6	116 (22.6)	15.12.9)	1.09 (0.43-2.7); 846	0.95 (0.36-2.46); 0.92
	6-9	174 (33.9)	8 (4.6)	**0.35 (0.12-0.98): 0.048**	**0.33 (0.12-0.95); 0.04**
	9-12	156 (30.4)	5 (3.2)	**0.24 (0.07-0.77); 0.017**	**0.24 (0.75-0.81); 0.021**
Gender	Male	249 (48.5)	24 (9.6)	1	1
	Female	264 (51.5)	12 (4.55)	**0.44 (0.21-0.91); 0.027**	**0.38 (0.18-0.81); 0.013**
Exposure to smoking	Mother	8 (1.6)	1 (12.5)	1.76 (0.21-14.8); 0.599	1.01 (0.11-9.46); 0.992
	Father	54 (11.02)	3 (5.6)	0.72 (0.21-2.46); 0.610	0.70 (0.19-2.55); 0.592
	No exposure	428 (87.4)	32 (7.5)	1	1
Mother IFV vaccine status	Any IFV vaccine	384 (76.7)	27 (7.1)	0.92 (0.41-1.98); 0.809	0.88 (0.37-2.06); 0.772
	No IFV vaccine	117 (23.4)	9 (7.7)	1	1
Infant order (mother)	First born	324 (63.2)	24 (7.4)	1	Not included[w]
	Second	120 (23.4)	7 (5.8)	0.77 (0.32-1.84): 0.970	Not included[w]
	Third	55 (10.7)	4 (7.3)	0.08 (0.32-2.94); 0.72	Not included[w]
	Other	14 (2.7)	1 (7.1)	0.96 (0.12-7.66); 0.97	Not included[w]
Number of children in the household	No other children in the household	298 (58.1)	20 (6.7)	1	1
	One child	151 (29.4)	11 (7.3)	1.09 (0.5-2.34); 0.821	1.03 (0.46-2.28); 0.934
	≥ two children	64 (12.5)	5 (7.8)	1.17 (0.42-3.26); 0.753	1.82 (0.62-5.38); 0.273
Household size	3	282 (54.9)	19 (6.7)	1	1
	4	130 (25.3)	7 (5.4)	0.78 (0.32-1.92); 0.6	0.77 (0.31-1.94); 0.586
	5	82 (15.9)	8 (9.7)	1.46 (0.62-3.55); 0.361	1.39 (0.55-3.448);0.475
	≥ 6	19 (3.7)	2 (10.5)	1.62 (0.35-7.57); 0.534	2.72 (0.54-13.52); 0.22
Feeding	Exclusively breastfed	114 (22.2)	16 (14.0)	1	1
	Other milk	387 (75.4)	20 (5.2)	**0.33 (0.16-0.66); 0.002**	0.5 (0.19-1.34); 0.173
	Solids	12 (2.3)	0	Not included[w]	Not included[w]
Childcare status	Formal	93 (18.3)	4 (4.3)	1.01 (0.3-3.41); 0.981	Not included[w]
	Informal	35 (6.8)	3 (8.8)	1.17 (0.3-4.56); 0.818	Not included[w]
	Formal and informal	6 (1.2)	0	Not included[w]	Not included[w]
	No childcare	375 (73.7)	28 (7.5)	1	1
Season	Spring	125 (24.4)	3 (2.4)	1	1
	Summer	89 (17.4)	10 (11.2)	**5.16 (1.36-19.52); 0.015**	**4.62 (1.19-17.9); 0.027**
	Autumn	147 (28.7)	11 (7.5)	3.3 (0.89-12.29); 0.074	3.07 (0.81-11.5); 0.097
	Winter	152 (29.6)	12 (7.9)	3.49 (0.96-12.69); 0.58	3.24 (0.87-12.04); 0.078
Co-detection with other viruses	DNA-viruses	54 (10.5)	3 (5.6)	Not included[w]	Not included[w]
	RNA-viruses	5 (0.9)	0	Not included[w]	Not included[w]
	Both DNA and RNA	2 (0.4)	0	Not included[w]	Not included[w]
	No co-detection	452 (88.1)	33 (7.3)	Not included[w]	Not included[w]
Clinical outcome	URTIs	264 (51.5)	11 (4.2)	**0.32 (0.15-0.67); 0.003**	**0.40 (0.18-0.88); 0.023**
	LRTIs	47 (9.2)	1 (2.1)	0.16 (0.02-1.22); 0.078	0.24 (0.03-1.94); 0.184
	No symptoms	202 (39.4)	24 (11.8)	1	1

[w]Small number of observations, so the OR and 95% CI were omitted.

Finally, Table 6.7 demonstrates that in contrast, the regression model showed that RVC-TDEs were significantly more common in infants in the second half of their first year of life, occurred more often during autumn and were more likely to be associated with symptoms of URTIs and LRTIs.

Table 6.7 Analysis of RVC-TDEs with possible confounding factors; the multivariate regression was adjusted to age, gender, number of children in the house and season

	Variable	Total number of episodes	RV-C		
			No. (%)	Univariate OR (95%); P value	Multivariate OR (95%); P value
Age (months)	0-3	67 (13.1)	12 (17.9)	1	1
	3-6	116 (22.6)	32 (27.6)	1.74 (0.82-3.67); 0.143	1.73 (0.81-3.68); 0.152
	6-9	174 (33.9)	64 (36.8)	**2.66 (1.32-5.35); 0.006**	**2.6 (1.32-5.4); 0.006**
	9-12	156 (30.4)	56 (35.9)	**2.56 (1.26-5.19); 0.009**	**2.77 (1.35-5.69): 0.005**
Gender	Male	249 (48.5)	79 (31.7)	1	1
	Female	264 (51.5)	85 (32.2)	1.02 (0.7-1.48); 0.9	1.04 (0.71-1.53); 0.805
Exposure to smoking	Mother	8 (1.6)	3 (37.5)	1.3 (0.3-5.52); 0.72	1.18 (0.26-5.28); 0.827
	Father	54 (11.02)	17 (31.5)	0.99 (0.54-1.83); 0.993	0.96 (0.5-1.85); 0.924
	No exposure	428 (87.4)	135 (31.5)	1	1
Mother IFV vaccine status	Any IFV vaccine	384 (76.7)	118 (30.7)	0.85 (0.55-1.32); 0.481	0.93 (0.58-1.5); 0.796
	No IFV vaccine	117 (23.4)	40 (34.2)	1	1
Infant order (mother)	First born	324 (63.2)	104 (32.1)	1	1
	Second	120 (23.4)	41 (34.2)	1.09 (0.7-1.71); 0.68	1.26 (0.49-3.24); 0.621
	Third	55 (10.7)	14 (25.5)	0.72 (0.37-1.38); 0.327	1.77 (0.23-13.2); 0.576
	Other	14 (2.7)	5 (35.7)	1.17 (0.38-3.59); 0.777	2.88 (0.26-31.95); 0.388
Number of children in the household	No children in the household	298 (58.1)	96 (32.2)	1	1
	One child	151 (29.4)	51 (33.7)	1.07 (0.71-1.62); 0.739	1.14 (0.74-1.75); 0.537
	≥ two children	64 (12.5)	17 (26.6)	0.7 (0.41-1.39); 0.377	0.76 (0.41-1.43); 0.411
Household size	3	282 (54.9)	88 (31.2)	1	1
	4	130 (25.3)	46 (35.4)	1.2 (0.78-1.87); 0.4	1.28 (0.81-2.02); 0.278
	5	82 (15.9)	24 (29.3)	0.91 (0.53-1.56); 0.738	0.97 (0.56-1.71); 0.941
	≥ 6	19 (3.7)	6 (31.6)	1.01 (0.37-2.78), 0.973	0.86 (0.30-2.42); 0.778
Feeding	Exclusively breastfed	114 (22.2)	21 (18.4)	1	1
	Other milk	387 (75.4)	141 (36.4)	**2.5 (1.51-4.25); <0.001**	1.98 (0.97-4.04); 0.06
	Solids	12 (2.3)	2 (16.7)	0.88 (0.18-4.34); 0.881	0.72 (0.13-4.08); 0.720
Childcare status	Formal	93 (18.3)	30 (32.3)	0.98 (0.61-1.61); 0.965	0.74 (0.43-1.26); 0.277
	Informal	35 (6.8)	7 (20)	0.51 (0.21-1.23); 0.138	0.45 (0.18-1.1); 0.082
	Formal and informal	6 (1.2)	3 (50)	2.07 (0.41-10.5); 0.377	1.84 (0.34-9.77); 0.472
	No childcare	375 (73.7)	122 (32.5)	1	1
Season	Spring	125 (24.4)	32 (25.6)	1	1
	Summer	89 (17.4)	23 (25.8)	1.02 (0.54-1.88); 0.968	1.14 (0.6-2.18); 0.672
	Autumn	147 (28.7)	55 (37.4)	**1.73 (1.03-2.92); 0.038**	**1.86 (1.09-3.19); 0.023**
	Winter	152 (29.6)	54 (35.5)	1.6 (0.95-2.69); 0.077	1.69 (0.98-2.89); 0.055
Co-detection with other viruses	DNA-viruses	54 (10.5)	19 (35.2)	0.46 (0.05-4.23); 0.494	Not included[w]
	RNA-viruses	5 (0.9)	1 (20)	1 (0.54-1.84); 0.992	Not included[w]
	Both DNA and RNA	2 (0.4)	0	Not included[w]	Not included[w]
	No co-detection	452 (88.1)	144 (31.9)	1	Not included[w]
Clinical outcome	URTIs	264 (51.5)	96 (36.4)	**1.88 (1.21-2.92); 0.004**	**1.72 (1.09-2.67); 0.018**
	LRTIs	47 (9.2)	20 (42.5)	**2.43 (1.23-4.82); 0.011**	**2.09 (1.04-4.18); 0.036**
	No symptoms	202 (39.4)	48 (23.7)	1	1

[w]Small number of observations, so the OR and 95% CI were omitted.

Table 6.8 shows that there were no statistically significant independent associations for RV-DEs caused by untypeable samples with any of the variables included in the model.

Table 6.8 Analysis of RV-DEs caused by untypeable samples with possible confounding factors; the multivariate regression was adjusted to age, gender, number of children in the house and season

Variable		Total number of episodes	Episodes with untypeable RV-samples		
			No of samples (%)	Univariate OR (95%); P value	*Multivariate OR (95%); P value
Age (months)	0-3	67 (13.1)	29 (43.3)	1	1
	3-6	116 (22.6)	55 (47.4)	1.23 (0.65-2.32); 0.512	1.29 (0.68-2.44); 0.425
	6-9	174 (33.9)	51 (29.3)	0.55 (0.3—1); 0.051	0.55 (0.3-1); 0.053
	9-12	156 (30.4)	52 (33.3)	0.65 (0.35-1.19); 0.169	0.61 (0.33-1.14); 0.123
Gender	Male	249 (48.5)	84 (33.7)	1	1
	Female	264 (51.5)	103 (390	1.24 (0.84-1.84); 0.274	1.3 (0.87-1.95); 0.192
Exposure to smoking	Mother	8 (1.6)	20 (37)	**0.23 (0.02-2.18); 0.02**	0.24 (0.02-2.31); 0.219
	Father	54 (11)	1 (12.5)	1.01 (0.52-1.92); 0.976	1.14 (0.57-2.28); 0.69
	No exposure	480 (87.4)	158 (36.9)	1	1
Mother IFV vaccine status	Any IFV vaccine	384 (76.7)	136 (35.4)	0.79 (0.49-1.26); 0.327	0.68 (0.42-1.09); 0.116
	No IFV vaccine	117 (23.4)	48 (41)	1	1
Infant order (mother)	First born	324 (63.2)	122 (37.7)	1	1
	Second	120 (23.4)	41 (34.2)	0.86 (0.53-1.39); 0.545	0.89 (0.34-2.3); 0.819
	Third	55 (10.7)	21 (38.2)	1.02 (0.53-1.94); 0.941	0.45 (0.04-5.07); 0.52
	Other	14 (2.7)	3 (21.4)	0.45 (0.11-1.79); 0.262	0.12 (0.01-2.14); 0.152
Number of children in the household	No children in the household	298 (58.1)	113 (37.9)	1	1
	One child	151 (29.4)	51 (33.8)	0.83 (0.53-1.31); 0.438	0.8 (0.51-1.27); 0.351
	≥ two children	64 (12.5)	23 (35.9)	0.923 (0.49-1.72) ?	0.94 (0.50-1.74): 0.425
Household size	3	282 (54.9)	111 (39.4)	1	1
	4	130 (25.3)	44 (33.9)	0.78 (0.49-1.3); 0.324	0.75 (0.47-1.21); 0.245
	5	82 (15.9)	27 (32.9)	0.74 (0.42-1.32); 0.313	0.81 (0.46-1.44); 0.494
	≥ 6	19 (3.7)	5 (26.3)	0.56 (0.18-1.69); 0.302	0.51 (0.16-1.57); 0.241
Feeding	Exclusively breastfed	114 (22.2)	57 (50)	1	1
	Other milk	387 (75.4)	125 (30.3)	**0.46 (0.22-0.73); 0.001**	0.59 (0.31-1.14); 0.118
	Solids	12 (2.3)	5 (41.7)	0.73 (0.20-2.6); 0.622	0.91 (0.21-3.75); 0.895
Childcare status	Formal	93 (18.3)	31 (33.3)	0.83 (0.51-1.38); 0.488	1.04 (0.6-1.79); 0.882
	Informal	35 (6.9)	17 (48.6)	1.61 (0.77-3.3; 0.2	2.04 (0.93-4.46); 0.073
	Formal and informal	6 (1.2)	0	Not included[w]	Not included[w]
	No childcare	375 (73.7)	139 (37.1)	1	1
Season	Spring	125 (24.4)	49 (39.2)	1	1
	Summer	89 (17.4)	27 (30.3)	0.66 (0.63-1.2); 0.177	0.54 (0.29-1); 0.05
	Autumn	147 (28.7)	60 (40.8)	1.04 (0.63-1.7); 0.854	0.95 (0.57-1.59); 0.859
	Winter	152 (29.6)	51 (33.5)	0.77 (0.46-1.27); 0.316	0.72 (0.42-1.21); 0.217

[w]Small number of observations, so the OR and 95% CI were omitted.

6.3.3.1 Prolonged RV-DEs and type replacement

As reported already, prolonged detection of RV was observed in this infant cohort (chapter-3; section 3.3.3.4 and table 3.3). Overall, there were 39 RV-DEs (11.9% of 327 RV-DEs) that persisted for more than three consecutive weeks with a median duration of five weeks and a maximum of nine weeks (IQR 4-6 weeks). The VP4/VP2 genotyping revealed only eight RV-TDEs (1.5% of total RV-TDEs) that could be defined as prolonged RV-DEes with continued shedding of the same RV-type for more than three weeks. These episodes were caused by five distinct RV-A types (two RV-A/seq-2, one RV-A/38, one RV-A/78, one RV-A/59 and one RV-A/63) and two RV-B types (one RV-B/06 and one B/84). One of the RV-A/seq-2 episodes was accompanied by PIV-1 and was also the only episode, of the eight prolonged RV-TDEs, that was associated with ARI symptoms. Untypeable RV-detections were observed in 31 of the 39 prolonged RV-DEs (79.5%), sixteen of which (41%) contained one or more interval of untypeable detections and in all cases, a new RV-type was detected in the following nasal swab. For one RV-DE that continued for four consecutive weeks, all four swabs contained untypeable RV detections.

Figure 6.4 illustrates the prolonged detection of RV-DEs in infant 027 and the results of RV-typing demonstrating the diverse and dynamic nature of the different types and species.

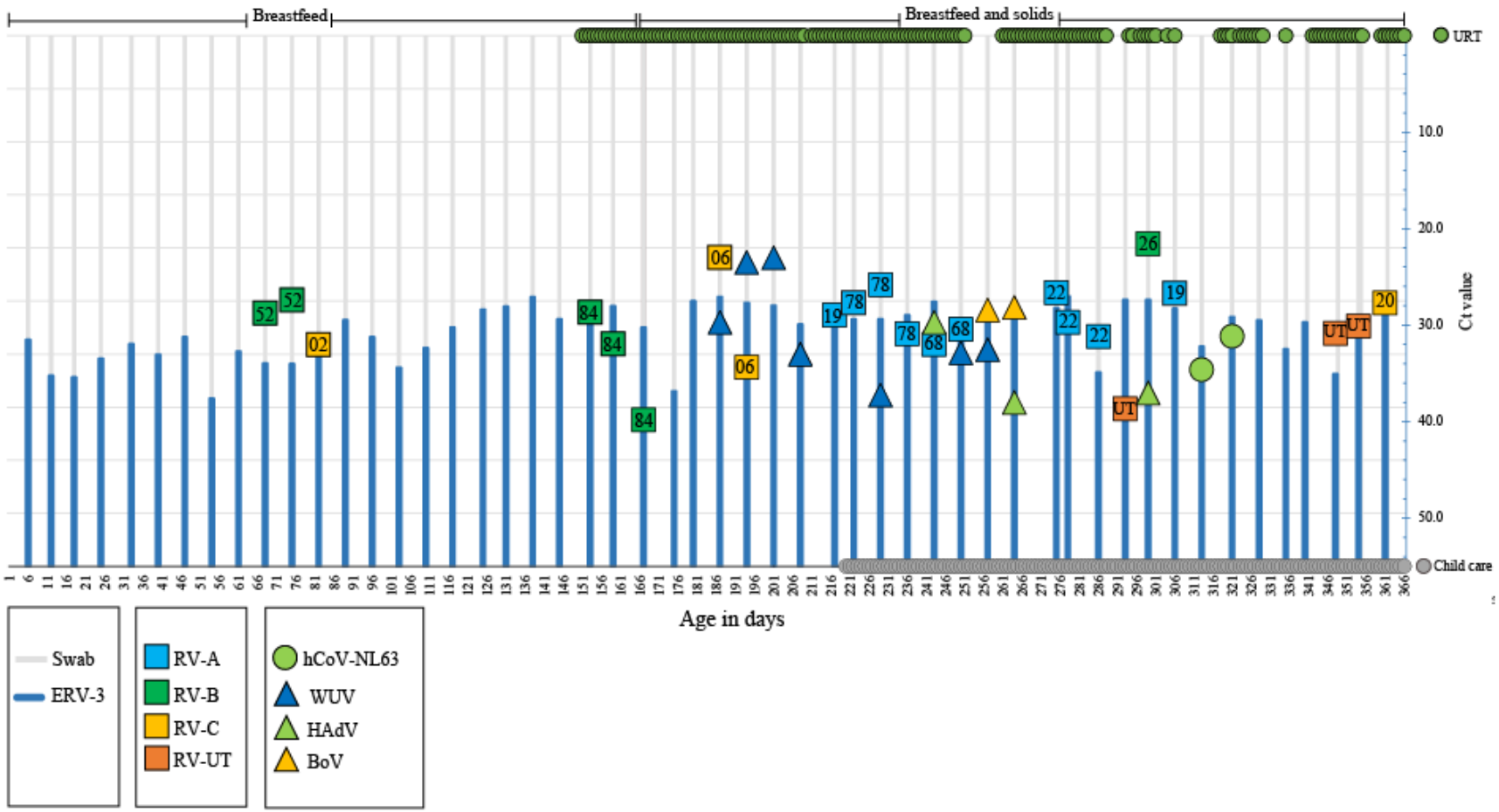

Figure 6.4 Data obtained from one infant participating in the study (infant 027). Vertical bars indicate sampling events (nasal swabs-grey bars) and ERV-3 Ct values (blue bars,Y axis). RV-detection is identified by shape in the legend and different colors refer to RV-species (A-blue, B-green, C-yellow and untypeable samples-orange). The numbers inside each box refers to the genotype. Different shapes indicate respiratory virus detections and their associated Ct values. The presence of acute respiratory illness symptoms over time is displayed at the top of the graph (green dots) and two major confounding factors, breastfeeding and childcare are also shown at the top (solid lines) and bottom (grey dots) of the figure respectively.

6.3.3.2 The first RV-TDEs

By the age of six months, 65 infants (90.2% of total infants) had experienced at least one RV-TDE. Six infants (8.3% of total infants) did not have their first RV until the second half of the first year and one infant did not experience any RV-DEs. Overall, the first RV-DEs included 34 different RV-types: 17 RV-A types caused 19 episodes, 12 RV-C types 17, and five RV-B types caused 11 episodes. The remaining 24 first RV-TDEs were caused by untypeable RV-detections. In total, 34 (47.8%) of the first RV-TDEs were associated with symptoms and 14 of these symptomatic RV-TDEs were RVC-TDEs (Figure 6.5).

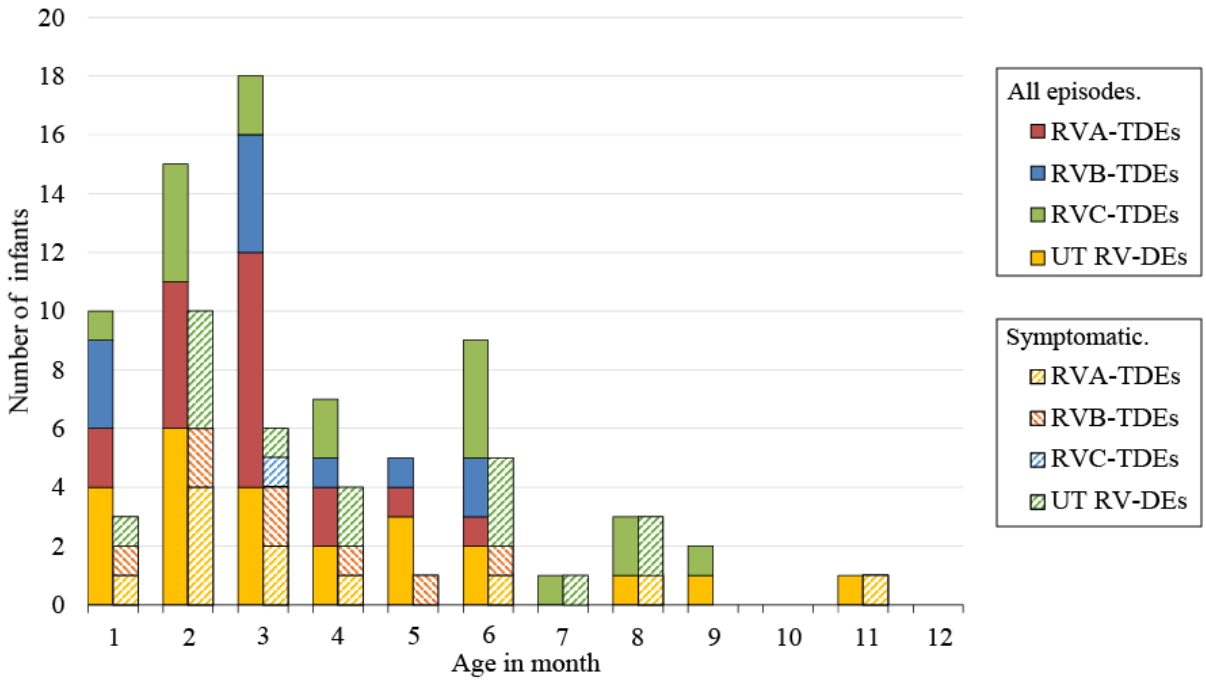

Figure 6.5 The age and number of infants for which the first RV-TDE was detected. The contribution of RV-A (red-filled bars) surpassed that of RV-C (green filled bars). The diagonally lined bars illustrate the number of first RV-TDEs that were associated with symptoms.

6.3.4 RV-TDEs and ARIs

Overall, 424 ARI episodes were reported for members of the infant cohort (described in chapter-3; section 3.3.4). Of these episodes, 234 were associated with 79 RV-types with a median of one type per episode and a maximum of five types in one continuous ARI episode only (Figure 6.4).

RV-C was more likely to be associated with symptoms. Of 164 RVC-TDEs, 116 (70.7%) were associated with both URTIs and LRTIs. LRTIs were also more likely to be associated with RVC-TDEs (Table 6.7). RV-A was the next most common species associated with symptoms where 86 of 127 (67.7%) RVA-TDEs were symptomatic (Table 6.5). Compared to other RV-species, RV-B was significantly less likely to be associated with symptoms (12/37; 32.4%; Table 6.6) and this was statistically significant for URTIs. For RV-A types, all of the five RV-A/80 detection episodes were associated with symptoms, none of which were accompanied by another respiratory virus (Table 6.3), while for RV-C types, association with symptoms by type was not as obvious, with the exception of types RV-C/51 and RV-C/seq-6 for which all of their eight detection episodes were associated with symptoms (Table 6.3).

6.3.5 RV-types in initial swabs from parents

Of the eight positive RV nasal swabs provided by parents during the initial visit, four were successfully typed (50%). Three of these were collected from mothers and all contained RV-A types (RV-A/19, RV-A/63 and RV-A/78), while the only positive swab collected from one father contained the unclassified RV-C/seq-1. None of these types identified in the parental swabs were detected in their infants during the first three months of life. The same types, however, were detected in two infants in the last three months of their first year. Figure 6.6 illustrates the dynamic nature of RV types during the first year of life in infants whose parents were positive for RV at the time of their birth.

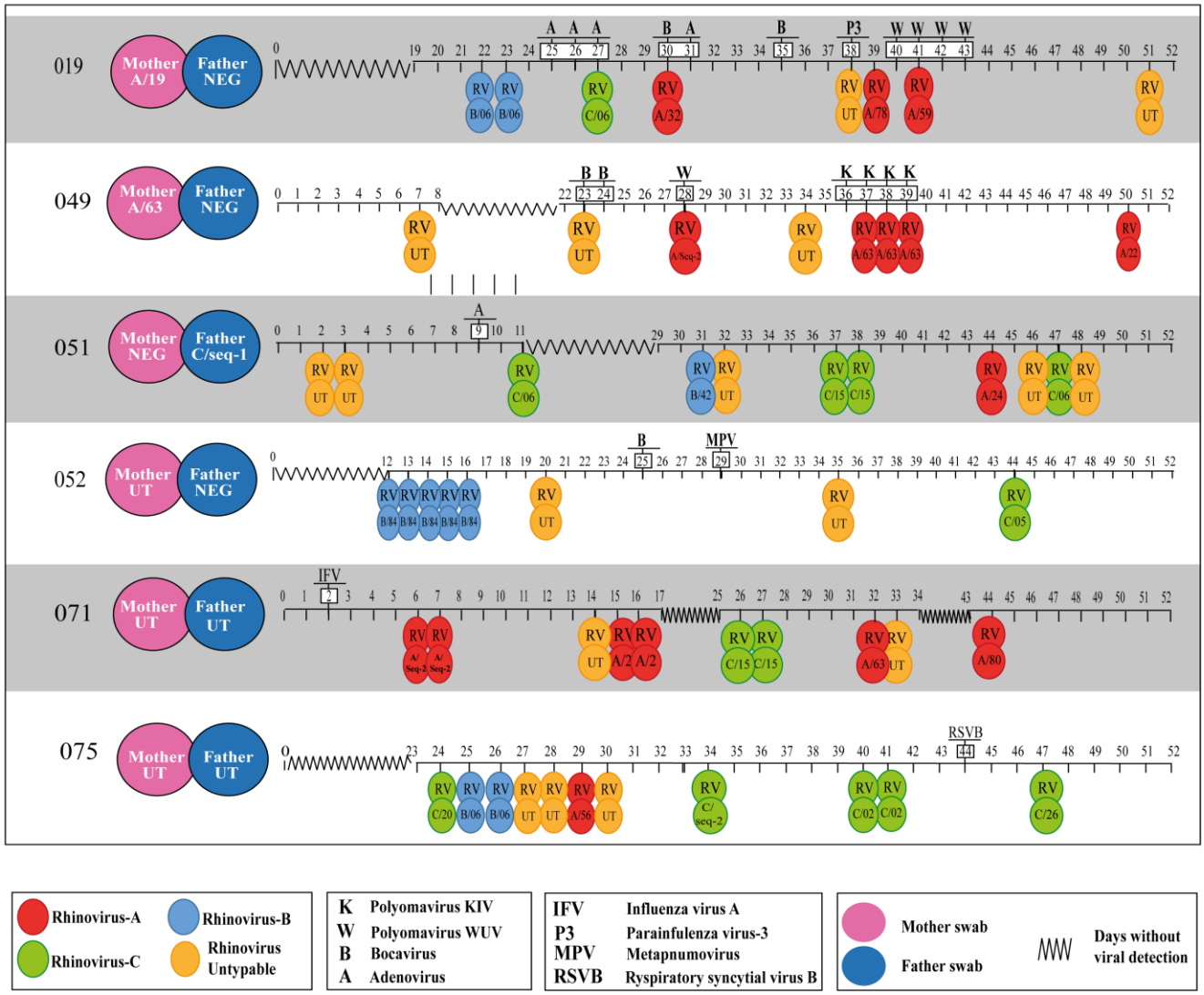

Figure 6.6 Respiratory virus detection during the first year of life in six infants whose parents were RV-positive at the time of initial visit and sampling by research staff within one-to-two days of birth

6.3.6 Investigation of untypeable samples

Overall, there were 189 samples (28.3% of RV-positive samples) that provided negative results for the VP4/VP2 typing protocol and these were investigated further. Both the quality of RV-amplification curves obtained from the initial real-time PCR screening and the Ct values for RV-positives in both typeable and untypeable samples were compared. The nonparametric Mann-Whitney U test was performed to identify any associations with Ct values from the screening RV RT-PCR assay.

The average RV-Ct value for the 470 typeable samples (30.2; 95%CI 29.7-30.5) was significantly lower, indicating greater RV-template load, than the average RV-Ct values for the 189 untypeable

ones (36.5; 95%CI 35.7-37.2; P<0.0001), Accordingly, samples that provided Ct values numerically higher than 35.7 cycles (n=121) were excluded from any further investigation and were not subjected to the 5`UTR typing because they were unlikely to provide a typing result due to low template load (Figure 6.7).

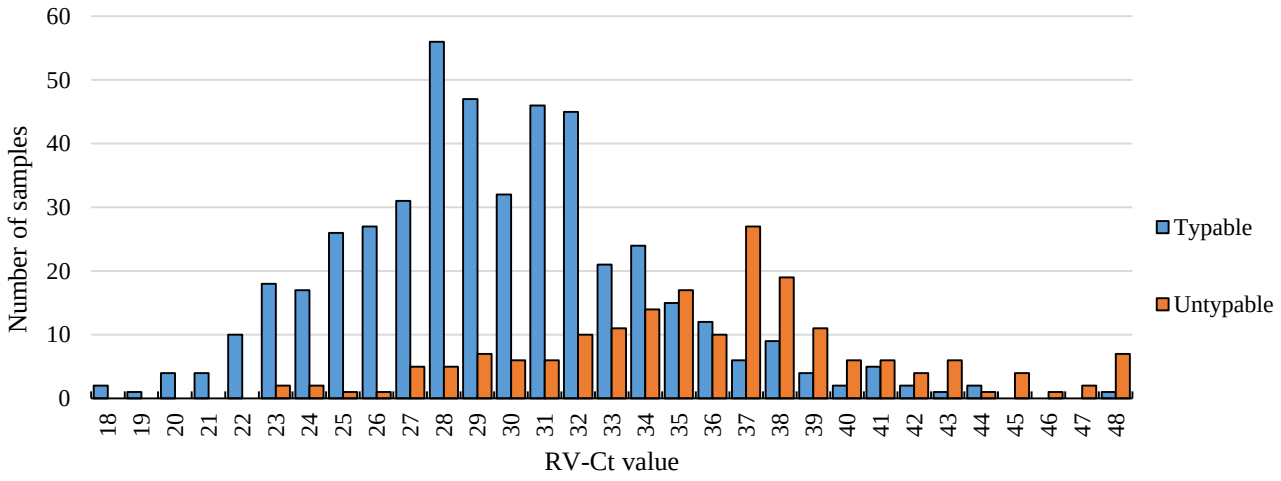

Figure 6.7 Comparison between RV cycle threshold (Ct) values in typeable (blue bars) versus untypeable (orange bars) samples. In typeable samples, the average RV-Ct values (30.2) was significantly lower (higher RNA load) than the average RV-Ct values (36.5) from untypeable samples (crude difference=6.3).

The analysis of the amplification curves obtained from the initial RV-screening revealed three categories of amplification curve shape that were defined as: optimal (sigmoidal), semi-linear (some curving) and linear (Figure 6.8). For the optimal curves, the phases of the amplification could be clearly distinguished as described in chapter-2 (section 2.1.4).

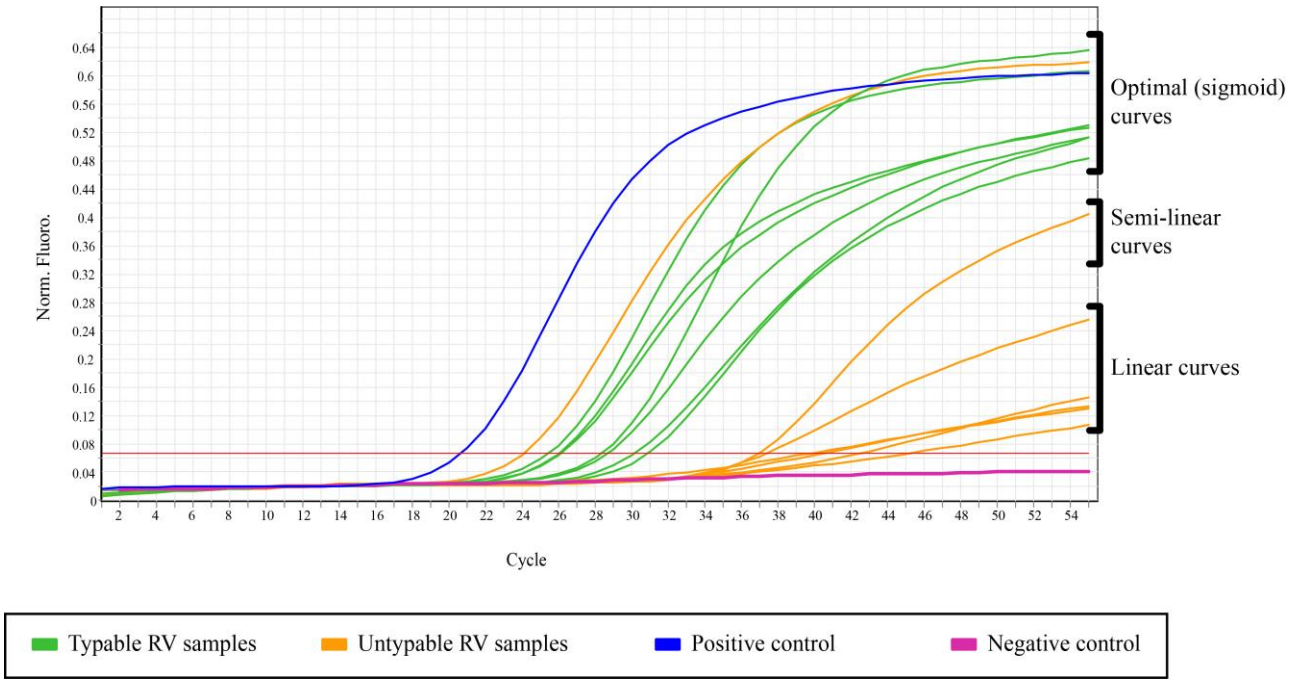

Figure 6.8 Comparison between the types of amplification curves showing the final VP4/VP2 results

Overall, there were 343 samples producing optimal amplification curves, of which 303 (88.3%) were typeable; 194 samples provided semi-linear amplification curves, of which 127 (65.5%) were typeable, and 130 samples provided linear signal, of which only 39 (30.0%) were typeable. For the latter, the average Ct value of these samples was relatively high (average= 37.3, 95%CI 35.9-38.7). Therefore, all samples that provided linear amplification curves were excluded from further investigations.

6.3.7 5`UTR typing

Overall, 107 RV-positive samples were examined using the 5`UTR typing assay because they could not be typed using the VP4/VP2 assay (Table 6.1) of which, 28 samples had provided optimal amplification curves with Ct values >35.7 cycles, while another 15 samples provided semi-linear curves and Ct values >35.7 cycles. However, only two samples (4.7%) were successfully amplified; they provided uninterpretable sequences.

6.4 Discussion

This chapter described RV-infections during the first year of life in a subset of 72 infants participating in the ORChID study. This included the incidence and prevalence of different RV-species and their association with ARIs (URTIs and LRTIs), the RV-types acquired during the first year of life, multiple detection of RV-species with other respiratory viruses, the seasonality of RV-species and prolonged RV shedding that in some infants continued for as long as nine consecutive weeks. All RV-positive samples underwent VP4/VP2 typing and samples that failed typing were investigated further by targeting the more conserved 5`UTR in the RV-genome without additional success.

6.4.1 Incidence and prevalence

Overall, when compared with the other respiratory viruses, we observed an overall high incidence and prevalence of RV infections during the first year of life, and this was accompanied by a high degree of diversity in the RV-genotypes. During the course of this study, other community-based cohort studies were published that investigated RV genotypes in children. Van der Zalm et al (2011) described RV genotypes in a healthy, yet older child cohort, while Lee et al (2012) described RV types in otherwise healthy infants with one or both atopic parents. The former study collected samples regardless of ARI symptoms being present (van der Zalm et al., 2011), while the latter obtained samples only during periods of illness (Lee et al., 2012). Both studies showed a high diversity amongst the RV genotypes detected. Our nested cohort RV detection prevalence of 19.1% was approximately half that found in these two aforementioned studies (37% and 46.4% respectively). Possible explanations for this difference include the reduced potential for exposure in our infant cohort compared to an older aged one (van der Zalm et al., 2011), the screening of many samples that were collected independently of ARI symptoms as well as other possible technical aspects of this study. These factors are discussed further in chapter 7.

The prevalence of RVA-TDEs increased in infants of mothers who had received the IFV vaccine recently. Maternal vaccination with inactivated IFV vaccine can reduce IFV illness by as much as 63% in infants up to six months of age due to the passive immunity from transplacentally acquired maternal antibodies (Zaman et al., 2008). However, in agreement with our findings, another study it was found that there was an associated increased risk of infection with other (non-IFV) respiratory viruses (Cowling et al., 2012). The reasons for this increased susceptibility to other virus infections is unknown, although the authors suggested that vaccination could somehow interfere with innate or cell mediated immune responses. Alternatively, one might also speculate that parents gained a false

sense of security following maternal vaccination and allowed their infants to be exposed to infection risks that they may have ordinarily avoided had vaccination not taken place. Interestingly, this association between RV-species and IFV vaccination was only observed in RVA-TDEs, which if based upon biological principles rather than changes in behaviour raises further questions over the role of IFV vaccine either in somehow protecting against RV-species other than RV-A or in increasing the risk of RV-A infections.

6.4.2 RV-types, including association with ARI episodes

During the 26 months of investigation, 97 different RV-genotypes were identified in 71 infants. RV-C/02 and RV-A/78 were among the most frequently observed types in otherwise healthy infants. The same findings were reported from infants and children hospitalised with community-acquired pneumonia (Daleno et al., 2013). In our cohort, 17% of detection episodes caused by the two types were associated with wet cough, however none were associated with pneumonia. Despite the small difference in the proportions of RV-A and RV-C, RV-A species provided the greatest diversity, which agrees with the findings of other community-based studies (Lee et al., 2012); Peltola et al., 2008; van der Zalm et al., 2011). The low prevalence of RV-B observed in our study is also consistent with other community and hospital cohort studies (Lee et al., 2012; Miller et al., 2011; van der Zalm et al., 2011). RV-C types were significantly more often associated with ARIs, including LRTIs, followed by RV-A, while RV-B had the least clinical impact. This agrees with a previous study in children under five years of age from Perth that reported a strong association between RV-C and wheezing illnesses from a prospective cohort of 197 children who presented to hospital with acute wheezing episodes, while no such association was observed in children with either RV-A or RV-B infections (Cox et al., 2013). Furthermore, those with RV-C presentations were also more likely to have had an increased risk of prior and subsequent hospital admissions for respiratory illnesses, especially if they were also atopic. In contrast, another hospital-based, but retrospective, study from Hong Kong, reported both RV-A or RV-C were associated with wheezing ARIs in children without a history of asthma (Mak et al., 2011). These disparities in results could be due to differences in geographic locations, study population or design. Nevertheless, both studies indicated the significant association between RV-C and wheezing illnesses. Recent studies have found that RV-C species have higher G + C content than either RV-A or RV-B and it has been speculated that this could signify its adaptability to replicating at higher temperatures in the lower airways, providing a mechanistic explanation for its apparent higher pathogenicity in ARIs, including inducing wheeze, than the other two RV species (Ashraf et al., 2013; Linsuwanon et al., 2011).

In this study, 60 samples contained ten unclassified RV sequences. All of these matched sequences reported on the GenBank database from other study groups since 2011. This suggests that these unclassified types were not newly emerged RVs, nor were they restricted to a specific geographic area. The patterns of shedding, co-detection with other viruses and association with symptoms for these RV-types were not different to the patterns of other assigned RV-types. Except for one type, RV-A/seq-2, the prevalence of the unclassified types was comparable to the assigned types as well. Nevertheless, further investigation is needed to fully understand the evolutionary position of these particular RV types.

The high number of untypeable samples in this study was not unique. In one of the abovementioned community-based studies, 30% of 101 RV positive samples collected from children were also untypeable (van der Zalm et al., 2011). Similar to ours, this study investigated RV-genotypes in samples collected at regular intervals, independent of respiratory symptoms being present. In contrast, in a recent hospital-based cohort, only 10.8% of 204 RV-positive samples were untypeable (Lauinger et al., 2013). Host biology, geography and technical factors that result in low template loads could be major reasons for genotyping failures as well as the possibility that further RV, or other *Enterovirus*, variants may exist. Untypeable samples were observed throughout the study without evidence of clustering and pose some limitations upon our analyses. In particular, every untypeable RV detection was considered a distinct RV-TDE, while for other RV-species consecutive detections of the same type were considered to be part of the same RV-TDEs. This may lead to overestimating RV-TDEs. However, until better RV typing methods are established we see this as an unavoidable limitation.

As an interesting aside, I identified EV-D68 on a single occasion from an untypeable RV sample from infant 062 in January-2012, who remained asymptomatic during and after this viral detection episode. This virus was first detected in California in 1962 and until recently had been sought and reported rarely. However, it has been implicated recently in two outbreaks of severe ARIs in the US Midwest where young children with prior wheezing illnesses seem to be particularly susceptible (Stephenson, 2014). The full spectrum of illness caused by EV-D68 is unknown, but my results suggest it can also cause subclinical infection.

6.4.3 Co-detection with other respiratory viruses

For RV-A there was a significantly higher proportion of co-detection with DNA-viruses. However, because of limited numbers of co-detections involving RNA-viruses a full regression model could not be completed. Caution must therefore be exercised when interpreting these data as the results may be from random fluctuations involving small numbers or simply because of the higher proportions and shedding durations of DNA-viruses compared to RNA-viruses and the high proportion of RV-A species.

6.4.4 Seasonality

While RV-A was least likely to be detected during autumn, this was the season where RV-C was the most common RV species found in the infant cohort. Previous studies suggested that differences in geographic locations appear to influence seasonal patterns of RV-species (Lau et al., 2010). There are some reports of a higher activity of RV-C in late autumn, early winter or wet seasons in most temperate or subtropical regions (Lau et al., 2009; Linsuwanon et al., 2009; McErlean et al., 2007; Xiang et al., 2008). RV-A is reported to be more common in Aboriginal children in Kalgoorlie, Western Australia during the hot, dry summer months (Annamalay AA et al., 2012), while in Hong Kong RV-A has also shown autumn peaks on occasions (Lau et al., 2009). Recent observers have proposed a theory of alternating activity between RV-A and RV-C species (Lau et al., 2009) however, this was not confirmed in my study, although it only covered two autumn seasons and thus could miss such patterns if they emerge over more time. In this cohort, RVB-DEs were more likely to occur in the summer months, unlike previous reports of autumn peaks (Lau et al., 2009). This result may simply reflect the small numbers of RV-B identified in this study and it is worth noting that autumn and winter seasons were also well represented by RV-B detections. Finally, it should be noted that with the exception of the Kalgoorlie study, the available data for seasonality of RV-species were mainly derived from hospital-based studies investigating samples from clinically attended ARIs. Thus, their conclusions may not be completely representative of young infants in the community.

6.4.5 Prolonged RV-detection

The prolonged detection of RV for more than three weeks during RV-screening in the ORChID study was due to RV-type replacements and not because of continuous shedding of the same RV-type. In fact, prolonged shedding of the same RV-type was very uncommon in this otherwise healthy birth-cohort. Even with prolonged detection of the same RV-species, intraspecies type replacement was frequently observed and was more frequent than replacement of genotypes by

those from a different species. Shedding of the same RV type for more than four weeks was more likely to be observed with RV-B types. This is possibly due to the slower replication of this RV species compared to RV-A and RV-C but may relate to these types eliciting more mild immune responses. Although not very common, there are some factors that may generate the appearance of interruption to continuous RV-TDE. These include poor sample collection that may lead to negative RV results and failure in typing some RV-positive samples that were sometimes observed in the middle of continuous RV-TDEs.

6.5 Conclusion

The relatively long duration of this investigation, large number of participating families, high frequency of sampling and comprehensive testing compared to other studies created a rich data set to support the hypotheses of this investigation. The predominant RV-species during the first year of life were RV-A followed closely by RV-C, while RV-B was observed in smaller numbers. The majority of RV-C episodes were associated with symptoms and with a relatively high likelihood of an LRTI episode. The highest variability of genotypes was observed however in RV-A species. Finally, the prolonged detection of RV in our longitudinal study was due to RV-type replacement. Persistent shedding of the same RV-type in otherwise healthy infants was uncommon.

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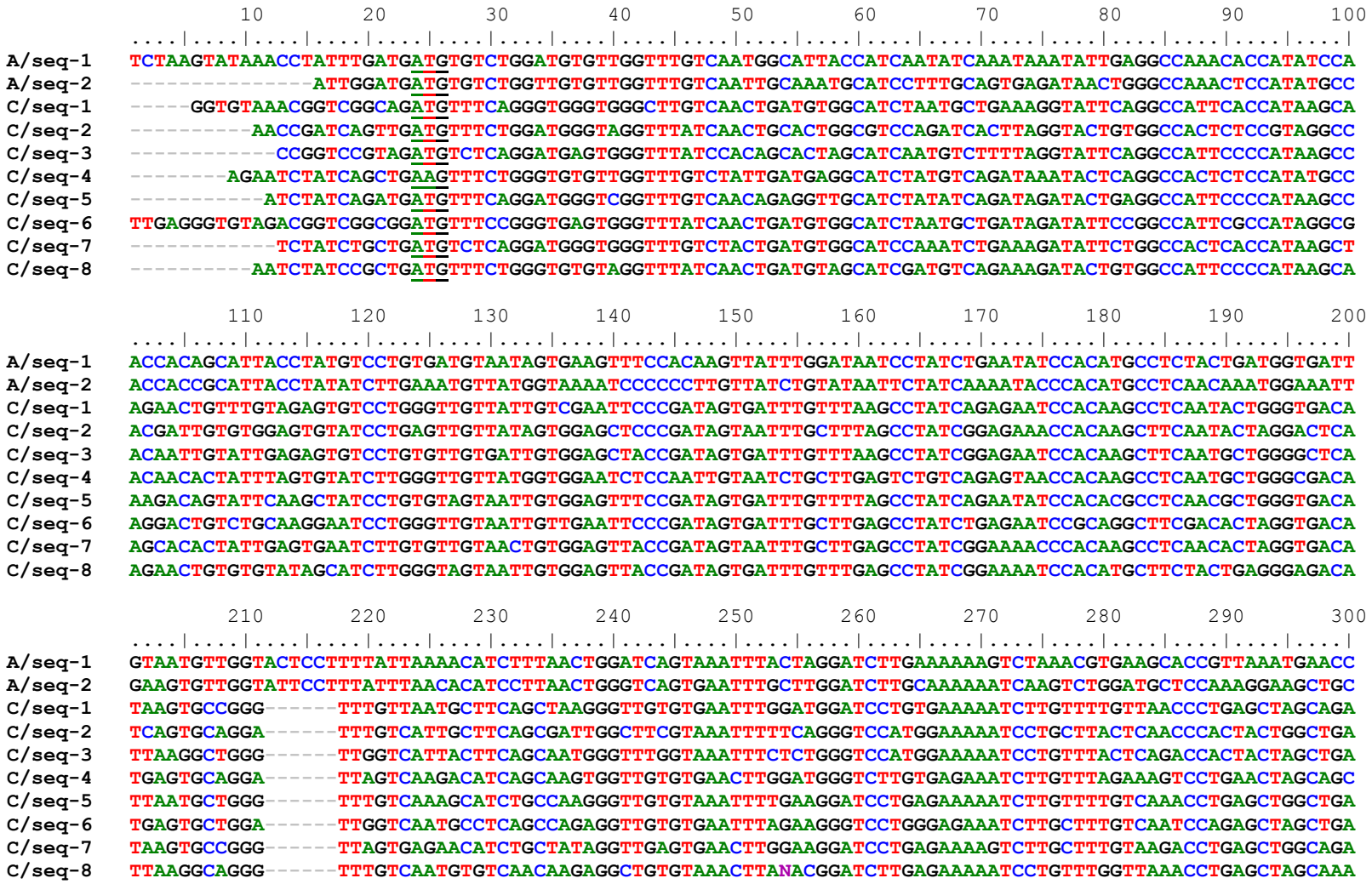
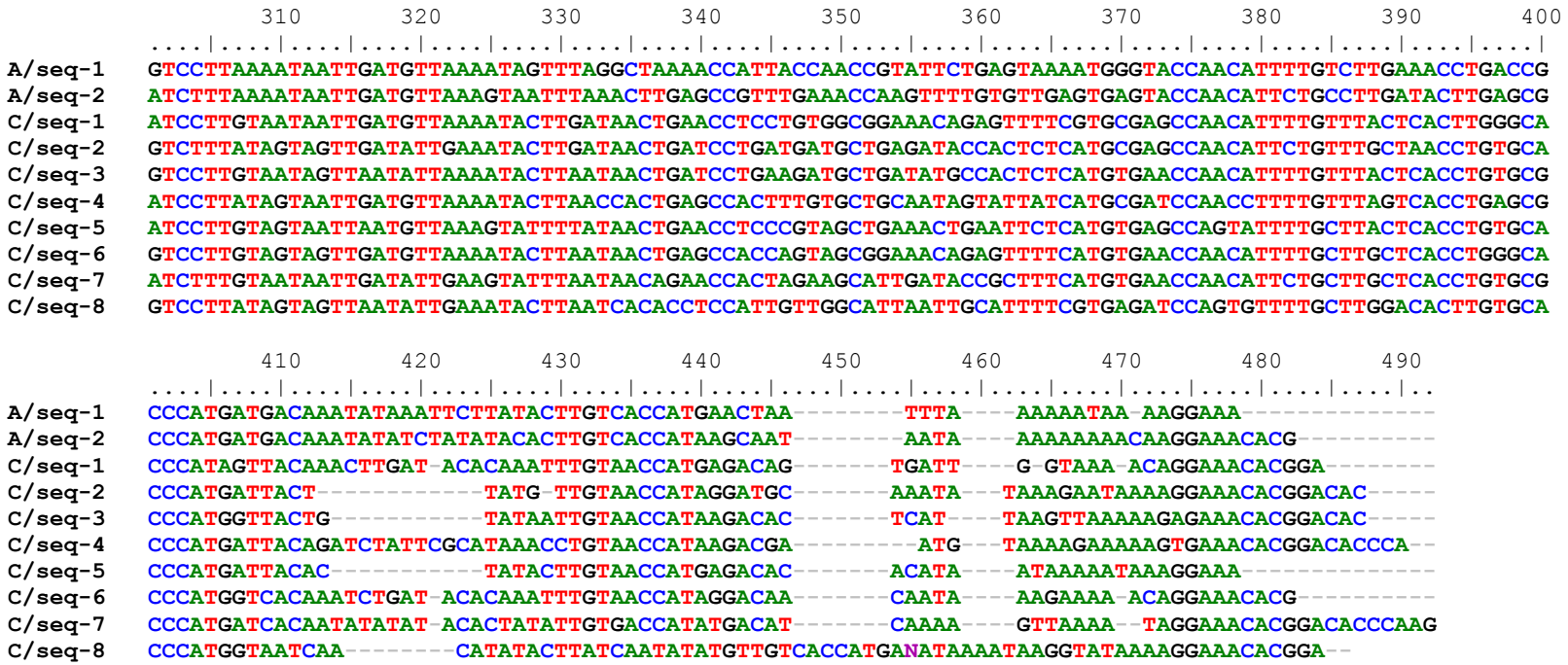
Chapter-6 Appendices

Appendix 6-1 Ten unclassified VP4/VP2 sequences identified in 60 samples collected from 31 infants




310 320 330 340 350 360 370 380 390 400
A/seq-1 GTCCTTAAAATAATTGATGTTAAAATAGTTTAGGCTAAAACCATTACCAACCGTATTCTGAGTAAAATGGGTACCAACATTTTGTCTTGAAACCTGACCG
A/seq-2 ATCTTTAAAATAATTGATGTTAAAGTAATTTAAACTTGAGCCGTTTGAAACCAAGTTTTGTGTTGAGTGAGTACCAACATTCTGCCTTGATACTTGAGCG
C/seq-1 ATCCTTGTAATAATTGATGTTAAAATACTTGATAACTGAACCTCCTGTGGCGGAAACAGAGTTTTCGTGCGAGCCAACATTTTGTTTACTCACTTGGGCA
C/seq-2 GTCTTTATAGTAGTTGATATTGAAATACTTGATAACTGATCCTGATGATGCTGAGATACCACTCTCATGCGAGCCAACATTCTGTTTGCTAACCTGTGCA
C/seq-3 GTCCTTGTAATAGTTAATATTAAAATACTTAATAACTGATCCTGAAGATGCTGATATGCCACTCTCATGTGAACCAACATTTTGTTTACTCACCTGTGCG
C/seq-4 ATCCTTATAGTAATTGATGTTAAAATACTTAACCACTGAGCCACTTTGTGCTGCAATAGTATTATCATGCGATCCAACCTTTTGTTTAGTCACCTGAGCG
C/seq-5 ATCCTTGTAGTAATTAATGTTAAAGTATTTTATAACTGAACCTCCCGTAGCTGAAACTGAATTCTCATGTGAGCCAGTATTTTGCTTACTCACCTGTGCA
C/seq-6 GTCCTTGTAGTAGTTGATGTTAAAATACTTAATAACTGAGCCACCAGTAGCGGAAACAGAGTTTTCATGTGAACCAACATTTTGCTTGCTCACCTGGGCA
C/seq-7 ATCTTTGTAATAATTGATATTGAAGTATTTAATAACAGAACCACTAGAAGCATTGATACCGCTTTCATGTGAACCAACATTCTGCTTGCTCACCTGTGCG
C/seq-8 GTCCTTATAGTAGTTAATATTGAAATACTTAATCACACCTCCATTGTTGGCATTAATTGCATTTTCGTGAGATCCAGTGTTTTGCTTGGACACTTGTGCA
410 420 430 440 450 460 470 480 490
A/seq-1 CCCATGATGACAAATATAAATTCTTATACTTGTCACCATGAACTAA--------TTTA----AAAAATAA-AAGGAAA----------
A/seq-2 CCCATGATGACAAATATATCTATATACACTTGTCACCATAAGCAAT--------AATA----AAAAAAAACAAGGAAACACG------
C/seq-1 CCCATAGTTACAAACTTGAT-ACACAAATTTGTAACCATGAGACAG--------TGATT----G-GTAAA-ACAGGAAACACGGA---
C/seq-2 CCCATGATTACT----------TATG-TTGTAACCATAGGATGC--------AAATA---TAAAGAATAAAAGGAAACACGGACAC--
C/seq-3 CCCATGGTTACTG----------TATAATTGTAACCATAAGACAC--------TCAT----TAAGTTAAAAAGAGAAACACGGACAC--
C/seq-4 CCCATGATTACAGATCTATTCGCATAAACCTGTAACCATAAGACGA---------ATG---TAAAAGAAAAAGTGAAACACGGACACCCA--
C/seq-5 CCCATGATTACAC----------TATACTTGTAACCATGAGACAC--------ACATA----ATAAAAATAAAGGAAA----------
C/seq-6 CCCATGGTCACAAATCTGAT-ACACAAATTTGTAACCATAGGACAA--------CAATA----AAGAAAA-ACAGGAAACACG------
C/seq-7 CCCATGATCACAATATATAT-ACACTATATTGTGACCATATGACAT--------CAAAA----GTTAAAA--TAGGAAACACGGACACCCAAG
C/seq-8 CCCATGGTAATCAA--------CATATACTTATCAATATATGTTGTCACCATGANATAAAATAAGGTATAAAAGGAAACACGGA--

Chapter 7

General discussion

7.1 Overview

ARIs caused by viruses are the most common illnesses experienced by all age groups. They are particularly important in children younger than two years of age who experience the highest rate of infections and complications. Most of the available data are derived from hospital-based studies that represent less than 2% of children with viral ARIs (Lambert et al., 2008). The relatively few previous community-based studies have suffered from one or more important methodological limitations, including using less sensitive detection methods, targeting of particular (often high-risk) groups that are not necessarily representative of the general population, screening for only a limited number of respiratory viruses, and only sampling from those with more severe symptoms (e.g. fever with wet cough or wheeze, suggesting lower respiratory involvement) or from a relatively short period of time (Budge et al., 2013; Kusel et al., 2006; Legg et al., 2005; Regamey et al., 2008; van der Zalm et al., 2009).

Molecular-based methods, used for both the diagnosis and characterisation of respiratory viruses, have led to further understanding of the role and characteristics of the established respiratory viruses, including RSV, IFV, PIV, HAdV, hCoV-OC43, hCoV-229E and RV. They have also led to the discovery of several other novel respiratory viruses. Between 2000 and 2010, seven newly identified respiratory viruses have been described. These include hBoV, hMPV, two PyVs: KIV and WUV, and three coronaviruses, SARS-CoV, hCoV-NL63 and hCoV-HKU1, as well as a third RV species where in 2009 RV-C joined the previously described species RV-A and RV-B. Since 2010, other novel viruses have also been identified, including MERS-CoV, and influenza A (H7N9) virus (which, in addition to SARS-CoV were not sought as part of this study). These last three mentioned respiratory viruses have been associated with outbreaks in specific parts of the world, although none have occurred in Australia.

The increased employment of highly sensitive molecular-based methods for respiratory virus diagnosis had also led to other important observations being made, such as the frequent detection of more than one respiratory virus in a single sample, the persistent shedding of some viruses, most notably RV, HAdV, hBoV and the two PyVs: WUV and KIV, and detecting respiratory viruses in samples collected from healthy, asymptomatic individuals. Nevertheless, until this study, we had limited knowledge of the nature and epidemiology of respiratory viruses in otherwise-healthy infants in the community.

The ORChID study is a prospective community-based, dynamic, longitudinal cohort study that aims to describe the respiratory viruses, including their shedding patterns and association with ARIs, encountered in infants and young children during their first two years of life. It was designed to

overcome some of the major limitations of previous studies (Lambert et al., 2012) and required parents to collect weekly nasal swab specimens, which were sent to the laboratory by regular mail. Progressive recruitment over a two year period allowed testing for respiratory viruses during multiple seasons.

The overall aims of my thesis were to *(a)* describe respiratory virus detections and their association with ARI symptoms during the first year of life in a subset (nested cohort) of 72 healthy infants from the ORChID study, *(b)* examine the impact of some technical issues upon respiratory virus detection during this longitudinal study (including the quality of nasal swab sample collection and transportation as well as sequence variation in DNA viruses) and *(c)* describe the nature of consecutive RV detections observed in the nested cohort.

My key findings were:

1. In otherwise healthy infants, RVs were the most frequently detected viruses and they were also the virus most commonly associated with ARIs during infancy.
2. After RVs, the most commonly detected respiratory viruses were the DNA viruses, HAdV, hBoV and the two PyVs: KIV and WUV, followed by RSV-A.
3. Established viruses (e.g. IFA and RSV) may be detected in asymptomatic infants.
4. ERV-3 was used successfully to measure the quality of nasal swab collection. By establishing this association we were able to monitor the quality of nasal swab collection in real-time.
5. Mould in nasal swabs appeared after prolonged periods between the site of collection and the laboratory, especially during spring and summer months and adversely affected sample quality. As more than 20% of our samples were at risk of mould contamination, this highlights the importance of examining samples as soon as they reach the laboratory and encouraging participating families to mail samples promptly.
6. Sequence variation is one factor that may impact upon the performance of real-time PCR assays in a longitudinal study. This can be avoided by reviewing the performance of established real-time PCR assays by comparison to alternative updated assays.
7. Detection of RV for more than three weeks in consecutive longitudinal samples was from type replacement rather than persistent shedding of a single RV-type.
8. At any one time, large numbers of different RV-types circulate in a single geographic region.

9. RV-C appears to be more pathogenic than either RV-A and RV-B. Of the three RV species, RV-B was the least likely to be associated with symptoms of an ARI.

In this chapter, I discuss further issues raised during the course of these studies that relate to clinical factors and the general performance of longitudinal studies. These include limitations, possible solutions and discussion of further implications of my findings.

7.2 Respiratory viruses and disease

In chapters 3 and 6, I have discussed key findings relating to viral detection and disease. Here I explore three factors that I believe warrant further consideration.

7.2.1 Potential virus-virus interactions

Understanding the distribution of respiratory viruses in the community helps to improve our knowledge of virus-virus interactions. During the course of this study the national IFV surveillance program recorded an increase in the activity of IFVs in QLD during August 2012 (Australian Government Department of Health and Ageing, 2012). Several theories have been proposed to explain the seasonal variation and the prevalence of certain respiratory viruses. These include viral interference at a population level whereby the peak activity of a specific virus may at the same time inhibit the spread of other viruses in that location (Mackay et al., 2013). The aforementioned peak of IFVs in August-2012 was the only reported change in respiratory virus activity throughout the study but was not mirrored by extra detections in our cohort. While the data generated from this nested infant cohort cannot be assumed to represent the wider general community, it was of interest there was also a notable decline in RV proportions in August 2012 too (described in chapter 3; section 3.3.3.4). This decrease was also associated with a slight increase in hCoV activity within the cohort (Chapter 3; section 3.3.3.4, Table 3.3) and in RV activity in the local hospital-based samples (personal communication, Dr. Ian M. Mackay). Although this observation could be simply explained by the small number of infants remaining in the study at that time-point, preliminary data from the larger ORChID study showed similar respiratory virus activity patterns at the aforementioned time-point (data not shown) and others have also reported an inverse relationship between RV and hCoV activities (van den Bergh et al., 2012).

The rates of co-detections in nasal swabs were higher for DNA-viruses compared to RVs (Chapter 3; Section 3.3.3.5; Table 3.4). This could also be explained by the relatively high prevalence of these viruses in the cohort. Nevertheless, previous studies showed that in preschool-aged children, RVs were less likely to be co-detected with another respiratory virus than DNA-viruses and co-detections involving RVs were less than expected by chance alone (Greer et al., 2009; Mackay et al., 2013). Both aspects were notable in the cohort (Chapter 3; Section 3.3.3.5, Figure 3.5). However, the small number of observations, especially for RNA-viruses, prevents an accurate assessment of these interactions between respiratory viruses. Also, the number of VDEs for each respiratory virus, other than RV, was relatively small compared to the large number of confounding factors present and this prevented a robust regression model being developed for use in this study.

Nevertheless, direct comparisons suggest that the rate of co-detection in VDEs caused by DNA-viruses was relatively higher than in VDEs caused by RV.

7.2.2 Further questions on detecting viruses in symptomatic and asymptomatic infants

Detecting viruses in respiratory secretions from asymptomatic infants can result from: (i) symptoms being present, but going unrecognised, (ii) a nascent or incubating infection (iii) a recent infection that has resolved or (iv) genuine subclinical infection. The longitudinal design of this study allowed nascent and resolved infections to be addressed and helped define the association between VDEs and their accompanying symptoms. I found that approximately one-third of RVDEs were asymptomatic and were recorded at least once in almost three-quarters of RV infected infants, while more than 90% of infants in my cohort had at least one symptomatic RVDE.

For RV-C, 164 RVC-TDEs were observed and 12.5% of these were associated with LRTIs, underlining the significant burden of RV-infections, particularly RV-C, in the community and their relatively important clinical role. Nevertheless, almost 30% of RV-C TDEs were asymptomatic. Our data and the literature (Daleno et al., 2013) also suggest there may be more virulent RV-C types than others (e.g. RV-C/02, RV-C/15 and RV-C/06). However, studies involving thousands, rather than hundreds of positive RV-C samples, are required to substantiate these observations.

In this cohort, the proportion of IFVs detected was very low. The five IFV-DEs observed represented only 0.8% of the overall 618 VDEs (post RV-typing), two of which were asymptomatic, two were associated with URTIs and one with symptoms of a LRTI. Of the two asymptomatic IFV infections, one was in an infant aged less than three months whose mother was vaccinated during pregnancy and maternal antibodies may have provided this infant with some degree of protection. Further studies, such as the Australian FluMum national prospective cohort study of more than 10,000 mother-infant pairs will help determine the effectiveness of maternal IFV vaccination during pregnancy at preventing IFV illness in infants before six months of age (O'Grady et al., 2014). We also observed asymptomatic RSV cases. It is well established that maternal antibodies play a protective role against RSV infections in young term infants and the timing of birth with respect to the onset of the RSV season is a risk factor for severe disease. Infants born in the months prior to the RSV season have a greater risk of severe illness than those born once the RSV season is established (Birkhaug et al., 2013; Englund, 1994; Glezen, 2003; Grimwood et al., 2008). Asymptomatic RSV cases may otherwise have arisen from repeated infections as observed in infant 065 (Appendix-1; Page XVII). These subclinical infections may

however be important as by shedding viruses they may act as a reservoir for transmitting RSV to others within the community.

7.2.3 RV during infancy

Despite the large number of RV-positive samples, including RVs able to be typed from this cohort, the high level of variation in RV-types (ie. the sheer diversity of individual types) prevented further analysis of whether specific types were more likely to be associated with ARI symptoms. Nonetheless, the data from my nested cohort suggest that some types (e.g. RV-A/78 and RV-C/02; Chapter 6, Table 6.3) were of interest given that they were the most frequently detected types in our infants, were often associated with symptoms, and have been reported previously to be associated with community-acquired pneumonia in older children (Daleno et al., 2013). More data related to RV-types, their distribution over a four year sampling period, and their association with ARIs will be available at the completion of the ORChID study. Identifying an accurate association between specific RV-types and ARIs will help with developing a candidate prophylactic vaccine or possibly novel therapeutics, which currently have had limited impact on preventing infection or reducing viral replication and duration of symptoms respectively. For vaccine development, a similar example is with IFV vaccines, which are updated every year according to the major IFV-serotype responsible for epidemics (Papi and Contoli, 2011). However, due to the high variability of types observed and the short duration of my study that only covered eight seasons (Chapter 6; Section 6.4.4), it was impossible to determine whether the predominant RV types circulating in the community could be predicted accurately in a year-on-year basis.

For the untypable samples, the data show that there are more types yet to be detected and may include new RV-divergent species or types, especially from untypable samples with relatively early Ct values (Chapter 6; Section 6.3.7). There may also be further RV-infections that are yet to be identified, e.g. RV/RV co-detections have been reported in previous studies (e.g. Lee et al., 2007; Renwick et al., 2007) however, this was not examined in our cohort. As we observed so many diverse RV genotypes, it is reasonable to speculate that RV/RV co-detections occurred, but went undetected by our methods. The VP4/VP2 typing assay was developed to comprehensively type all known RV types, as well as some *Enterovirus* strains (Wisdom et al., 2009). The variation in amplicon sizes allows both to be discriminated from one another. However, as RV types have PCR-products of similar sizes, it is not possible to differentiate accurately between them. Moreover, there is a possibility that the assay only amplifies the predominant type in the sample. Support for this speculation comes from the high failure rate when attempting to type RV-samples with low viral loads (Chapter 6; Section 6.3.6). Therefore, selecting or developing more specific RV-typing

protocols may be beneficial for future investigations of RV molecular epidemiology in the community.

This, to my knowledge, was the most comprehensive study investigating the first episode of RV-DEs in individual infants in the first year of life. Interestingly, only half of the first RV-DEs that were observed in the cohort infants were associated with ARI symptoms. Additionally, RV-A types were more often seen in the first RV-DEs compared to other RV species, especially in infants who experienced their initial infection during the first three months of life. Further questions regarding the role of early RV-infections, and how they might influence subsequent respiratory virus infections will be answered by the completion of the ORChID study.

7.3 Issues related to conducting community-based longitudinal studies

7.3.1 Sensitivity of ARI definitions

Establishing a definition of ARIs when investigating infants was particularly challenging. The symptom diary card used in the ORChID study was designed to maximise the number of the recorded ARI episodes. It included all possible categories of ARI symptoms and signs, some of which were intended to capture influenza-like symptoms in older children (Lambert et al., 2007; Lambert et al., 2012). The ARI definition used in this study, however, considered only respiratory-related symptoms. Other symptoms, such as headaches, sore throats and muscle pain were excluded as non-verbal infants cannot complain of these symptoms and irritability is too non-specific to be reliable in this context. From our data, almost one-third of the VDEs were not associated with symptoms. This outcome did not change significantly when we included into the ARI definition symptoms such as headaches and muscle pain recorded by parents in the symptom diary. Overall, given the longitudinal design of the study, this suggests that asymptomatic detections were either associated with very mild and unrecognised symptoms of ARIs or were genuine subclinical infections. Moreover, this indicates that our ARI definition is not likely to have grossly underestimated the total number of ARIs in our community cohort, and therefore is an important finding for planning similar studies in the future.

7.3.2 Symptom data collection and transformation – the potential benefits of new technology?

One problem that became increasingly evident during the study was that of data management. Basically, it was very time consuming, especially when transferring data from the symptom diary cards to soft copies. In future studies, replacing regular symptom diary cards with electronic cards (E-cards) may help solve this problem. Such E-cards can be developed as smart-phone applications for ease of access. Also, simple functions can be added, such as reminder notifications for data recording, missing data monitoring or sample collection and transportation. This may be especially relevant when considering the sample quality issues raised in chapter 4, namely the failure to mail samples promptly. All data can also be synchronised in defined databases to larger on-line databases. Similar E-card systems, if supported by data illustration tools, could provide invaluable resources for families by educating them about study tasks and helping them to recognise specific symptoms and signs of ARI in their infants. Taken together, these measures may also help with cohort maintenance and retention. Finally, taking a much broader 'over the horizon' perspective, E-cards could also be used in the future to help health practitioners monitor ARIs in their patients as part of an integrated health care package and for researchers to survey ARIs in a given population and to link them to subsequent chronic pulmonary disorders, such as asthma or COPD.

7.3.3 Communicating with participating families

In this study, no results were communicated to parents until the completion of the two years of investigation (as in the ORChID study). We did however, on occasion, contact them in response to issues with the ERV3 PCR results, to remind them of the optimal sample collection methods. This lack of communication of results was done deliberately to avoid any possible information bias as knowledge of a viral infection may alter the way parents report symptoms (Lambert et al., 2007). However, during the course of this study, three significant international outbreaks were reported, all of which were caused by newly identified viruses; the novel MERS-CoV and the two IFVs: H9N7 and H10N8. In my opinion, it is likely the influence of media reports and heightened concerns may change participants' behavior in terms of either reporting symptoms or collection of samples. With the volume of gathered data on quality of sample collection (ERV-3; Chapter 4) and by designing surveys to measure their awareness, the influence of media reports on participant's behavior could be examined in subsequent studies.

7.4 Further laboratory aspects; low viral load and real-time PCR sensitivity

When using real-time PCR for respiratory sample screening, the Ct-values are often used as a semi-quantitative measure for viral load that can be used to determine whether the latter correlates with clinical severity. For more severe illnesses, as seen in patients presenting to hospital or emergency clinics, respiratory virus screening often provides early Ct values (indicating high-viral loads) (Christensen et al., 2010; Harvala et al., 2012; Ngaosuwankul et al., 2010). This may however, be related to several factors, including the method obtained for respiratory sample collection, the expertise of those collecting particular specimens (e.g. NPAs), time between collection and screening, and the conditions under which samples were transported to the laboratory. Other patient-related factors, such as severity of symptoms, timing of sample collection (early vs late in the ARI episode) and any underlying chronic health disorders should also be considered.

Unlike the above clinical situations, for this community-based study, we adopted sampling strategies that would be most acceptable to parents and families in order to optimise cohort retention while still conducting intensive viral surveillance. For example, collecting weekly nasal swabs and sending them to the laboratory by regular mail at ambient temperatures was accompanied by nasal swab specimen return rates exceeding 90% throughout the study. Yet, some technical factors should be taken into consideration when using similar strategies in community-based studies. Respiratory virus screening data provided in this study indicated that Ct values may not always accurately reflect the true viral load and thus cannot be relied upon to measure associations between respiratory viruses and illness severity.

Late (or high) Ct values obtained from respiratory virus screening in community-cohorts risk under-estimating the true burden of some respiratory viruses by either misreading associations between Ct values and disease severity or, more importantly, reporting false-negative results. To overcome the latter, the routine weekly sampling strategy obtained in this longitudinal study, was designed to help maximise chances of detecting respiratory viruses, even though sampling may not always have occurred at the time of peak virus shedding in first days of the infection episode. Therefore, when studying or diagnosing infants and children respiratory viruses in the community, taking samples within one week of an ARI should still lead to a respiratory virus detection on most occasions.

One of the aims of this longitudinal study was to determine the kinetics of respiratory virus shedding from the respiratory tract. However, this can also be under-estimated when there are false-negative results due to low template loads and providing a false impression of intermittent virus shedding. In addition, low template loads in nasal swab samples collected from community studies

may compromise investigations of certain viruses, when less sensitive methods are being employed on these samples. An example of this occurred with the RV-typing project where 64% of the untypeable samples provided late Ct values (>35.7 cycles). While in these instances the viral load may have been genuinely low, some of the preventable technical factors may have contributed to produce such late Ct values. These are now discussed further below.

Nasal swab samples and potential variability Samples such as respiratory swabs have the potential for sampling error from poor technique and to introduce considerable variability, even when collected by well-trained health professionals. In this study we demonstrated, by using ERV3 as a marker for collection quality, that considerable variability in specimen quality existed between nasal swab specimens collected by parents and that some parents were not as good as others at obtaining adequate specimens. An example of this is provided by one infant (Infant 048; Appendix-1; page XVII) whereby the swabs collected from this infant were consistently negative for all respiratory viruses until the last month of the study when KIV was detected in two samples over a two week period. By applying the ERV-3 method, we found that ERV-3 load in this infant's nasal swabs were significantly lower than the ERV-3 load in swabs from other infants indicating that the swabs obtained from infant 048 were, in general, suboptimal and likely to have been poorly collected. The quality of serial nasal swab samples collected from the same subject could vary from sample-to-sample and over time. As an example, some parents reported difficulties in collecting samples as the study progressed and their infants became older, or when infants experienced ARIs some resisted having swabs taken. Establishing a simple system to quantitatively measure the quality of sample collections was important for understanding the reasons for any decline in viral detection rate for some infants and to address one possible confounding factor that may impact upon Ct values.

Sequence variation: another factor influencing respiratory virus screening To a lesser extent, this study also demonstrated that sequence variation in our community samples could introduce delayed Ct values for HAdV, and possibly other viruses. For a given established real-time PCR assay and when sequence variation could explain delayed Ct values obtained from community samples, one suggested strategy is to use an alternative assay that targets different genome areas to ensure accurate Ct values are being obtained. This will provide data confirming assay performance is not being compromised in a given study population (i.e. the ORChID cohort) prior to making any decision upon whether an established assay needs replacing. When choosing the pan-HAdV assay for my study I was aware of considerable differences between HAdV types, of new HAdV types and variants being identified, and that this assay had been in use for almost a decade. Consequently, I employed a modified version of the established assay comprising identical oligonucleotides as an

initial quality assurance exercise and I was able to confirm that the established pan-HAdV PCR method that was initially selected for the ORChID study was appropriate for viral detection, but not for quantification purposes. The modified assay however replaced the established pan-HAdV to avoid false-negative results due to true low HAdV load in the nasal swab samples.

Degenerate bases are commonly adopted to overcome sequence variation when designing respiratory virus real-time PCR assays (Arthur et al., 2009; Lu et al., 2008; Orozovic et al., 2010). However, the accumulation of degenerate bases in real-time PCR primers or probes are not recommended as they increase the background signals due to non-specific binding (Rychlik, 1995). This is particularly important when performing large numbers of samples as it may complicate interpretation of the results, especially in samples with true low viral loads. Furthermore, balancing melting temperatures can be very challenging when designing a degenerate real-time PCR assay that is comprehensive for all strains of a specific virus. Therefore, when designing the modified assays, I aimed at distributing degenerate bases over several oligonucleotide versions that were, otherwise, identical to the targeted sequence. The method proved successful at overcoming the problem of sequence variation and avoiding further delays in Ct-values.

The strategy of using an alternative assay targeting a completely different target was used in the RV-typing project that relied upon conventional PCR assays in a carefully designed protocol (Chapter 6; sections; 6.2.3, 6.2.4. Figure 6.1). The aim of this protocol was to eliminate most of the possible factors that may cause failure in VP4/VP2 typing. The VP4/VP2 typing assay is a nested assay that targets a region of the RV-genome comprising high levels of heterogeneity between RV-types. Consequently, late amplification due to sequence variation was predicted to be the most likely cause when the VP4/VP2 typing assay failed in the initial step of the typing strategy. Of the 221 samples that failed to be typed in the first step, 32 (14.5%) RV-positive samples were typed when the VP4/VP2 typing step was repeated. In other words, using this protocol increased the chance of producing a detectable amount of VP4/VP2 PCR-product by less than 5%. The remaining 189 RV-positive samples were subjected to further screening and analysis to confirm positivity and Ct values. Once completed, samples with early Ct values were typed using the more conserved 5`UTR to eliminate the sequence variation factor (Chapter 6; sections 6.3.5, 6.3.6). When this step also failed, other possible reasons (see next section) were examined to explain both delayed Ct values and failure of typing.

Quality of real-time PCR templates This involves genome templates resulting from the nucleic acid extraction step, which itself may be affected by other factors, including the efficiency of the automated system and the performance of the extraction reagents. The high number of nasal swabs expected to be submitted over a relatively short period of time required a time-efficient

system for nucleic acid extraction. The QIAxtractor robotic system, which is a vacuum-based nucleic acid extraction system, was chosen for this purpose. The genomic DNA extraction kit (DX) (Qiagen, Australia) was selected on the basis of the QPID laboratory experience and other previous studies suggesting its suitability for respiratory virus detection and quantification (Wishaupt et al., 2011). However, alternative extraction kits that are more specific for viral nucleic acid extraction purposes may have provided better results. The viral nucleic acid extraction kit (VX) (Qiagen, Australia), which was introduced after the ORChID study commenced is such an example. In a pilot study, I compared the performances of the two kits, using a single batch of ORChID samples. The two viral nucleic acid templates used for this comparison were EHV-1 and RV. EHV-1 was selected as a representative for viral DNA, while RV was selected to represent the viral RNA component as it was the most frequently detected respiratory virus. For the DNA template (EHV-1), the two kits performed similarly for both detection rate and Ct values. In contrast, PCR assays using the viral RNA extraction kit had slightly increased detection rates and lower Ct values for RV when compared with the QIAxtractor system (data not shown), especially in samples with original low RV loads. This suggested that while the DX kit is suitable for broad-based extraction of both viruses and bacteria, it may not be necessarily optimal for RV and possibly other RNA viruses. While we do not expect this to have any significant effect on the main outcomes of the ORChID study, considering its intensive sampling, it may have compromised RV genotyping (i.e. insufficient load for the less sensitive typing methods).

These aforementioned factors highlight that a late Ct-value observed for any given positive sample may not necessarily reflect low viral loads for several technical reasons. In addition, my results caution against using parent-collected nasal swabs being used to accurately assess viral load in samples from community-based studies. While probably not important for understanding the nature of respiratory viruses in young children, high load is often used as a marker of active viral replication and thus alternative strategies such as frequent sampling and monitoring sample quality as employed in ORChID will still need to be used to address this limitation.

7.5 Other potential limitations

The sampling protocol used in the ORChID study, including the swabs used, was established and selected according to work published previously involving community-based sampling in Australian children, including mailing nasal swab specimens (Lambert et al., 2007; O'Grady et al., 2010; Yin et al., 2011). While the available studies report a slightly better performance of flocked swabs compared to rayon swabs, those studies compared flocked swabs with liquid universal transport media to rayon swabs incorporating a sponge reservoir containing VTM (Esposito et al., 2010; Hernes et al., 2011). The design of the ORChID study required the parents to mail samples to the research laboratory using the regular postal service. Importantly, the Australia Post regulations prohibit sending liquid biological samples. Although dry flocked swabs were an option, the absence of comparative data was problematic and by using a sponge soaked with VTM to help maintain respiratory virus particles at ambient temperatures this allowed rayon budded swabs to be chosen for this study.

While the virological data described in this study are important, they may serve as a minimal estimate for at least some viruses. The data for some viruses were generated from small numbers of infections and therefore they may not be representative of the general community. The ORChID study will provide further data to examine the role of these viruses and to understand the significance of asymptomatic VDEs in the older cohort.

Additionally, due to the characteristics of this nested cohort, it may not be representative of other Australian infants or the global community. The infants lived in small, socioeconomically advantaged family units where breast feeding rates were high and daycare attendance and passive exposure to tobacco was low. The data were also derived from an urban subtropical environment, which may influence the epidemiology, including transmission dynamics, of some respiratory viruses as seen with RSV and RV (Chapter 6; Section 6.4.4).

My study included six newly identified viruses. However, the role of other novel respiratory viruses during infancy has attracted more attention recently, such as IFV-C and PIV-4. Recent data suggest that these viruses may have a role in ARIs during infancy comparable to other more established respiratory viruses (Calvo et al., 2013; Fox and Christenson, 2014).

7.6 Future directions

My PhD studies have provided important insights into conducting intensive community-based studies of respiratory viruses in young children. In so doing several research questions have also been raised by my findings. In regard to determining the severity of symptoms, changes in types of respiratory viruses during an ARI were noticed in this cohort and in some cases these were associated with increased symptom severity. This observation needs further study with higher number of both ARIs and proportions of respiratory viruses to help determine whether mixed or sequential infections result in more severe symptoms.

Almost half of the first RV-detections observed in this cohort were asymptomatic. The first RV-DEs in these infants were caused by different RV-types. This observation can lead to several future studies to investigate: *(a)* the association between RV-types that cause the first RV-detection after birth and presence and severity of symptoms, *(b)* the relationship between the age at which the first infection occurs and the number of subsequent RV-detections and *(c)* the impact of early and/or repeated RV exposure upon respiratory health, lung growth and lung function in later childhood.

Longitudinally collected samples and data provide an opportunity to investigate the influence of respiratory virus infections and early life events (breast or milk formula feeding, childcare attendance, antibiotics, tobacco smoke exposure and other environmental factors) upon establishing and maintaining the nasal microbiome during the first two years of life. In addition, these types of data may aid in determining the nature of viral-bacterial interactions within the infant's nasal space, including their relationship with ARIs in early childhood and future respiratory health and lung function. Previous cross-sectional studies reported positive associations between HAdV and RV and both *M. catarrhalis* and *H .influenzae*. However, the direction of such associations was not determined due to lack of intensive follow up in these studies (Moore et al., 2010; Pitkäranta et al., 2006).

By providing an insight into the performance of longitudinal nasal swab collection, alternative approaches may help in improving the quality of parental sample collection technique. These include; the use of a nasal saline spray prior to taking a nasal swab and comparing this with nasal swabs alone in this young age group. Previous studies in older children have indicated this method provided similar results to those obtained by standard nasal washes and deep nasal or nasopharyngeal swabs (Campbell et al., 2013; Emerson et al., 2013). An increased detection sensitivity of 7% was found when comparing nasal swab samples collected from children with CF

aged six to 18 years before and after instillation of nasal sprays (Emerson et al., 2013). However, for young children and when parental collection is required, the level of acceptance of this method is unknown.

Finally, virus discovery can be undertaken using shotgun cloning and next generation sequencing, especially in samples that were collected during well-documented ARIs, but which were negative for all respiratory viruses. Similar techniques could be beneficial in investigating untypable RV-positive, especially those with early Ct values (<35 cycles), to determine the nature of these samples and whether they contained novel RV-divergent types.

7.7 References

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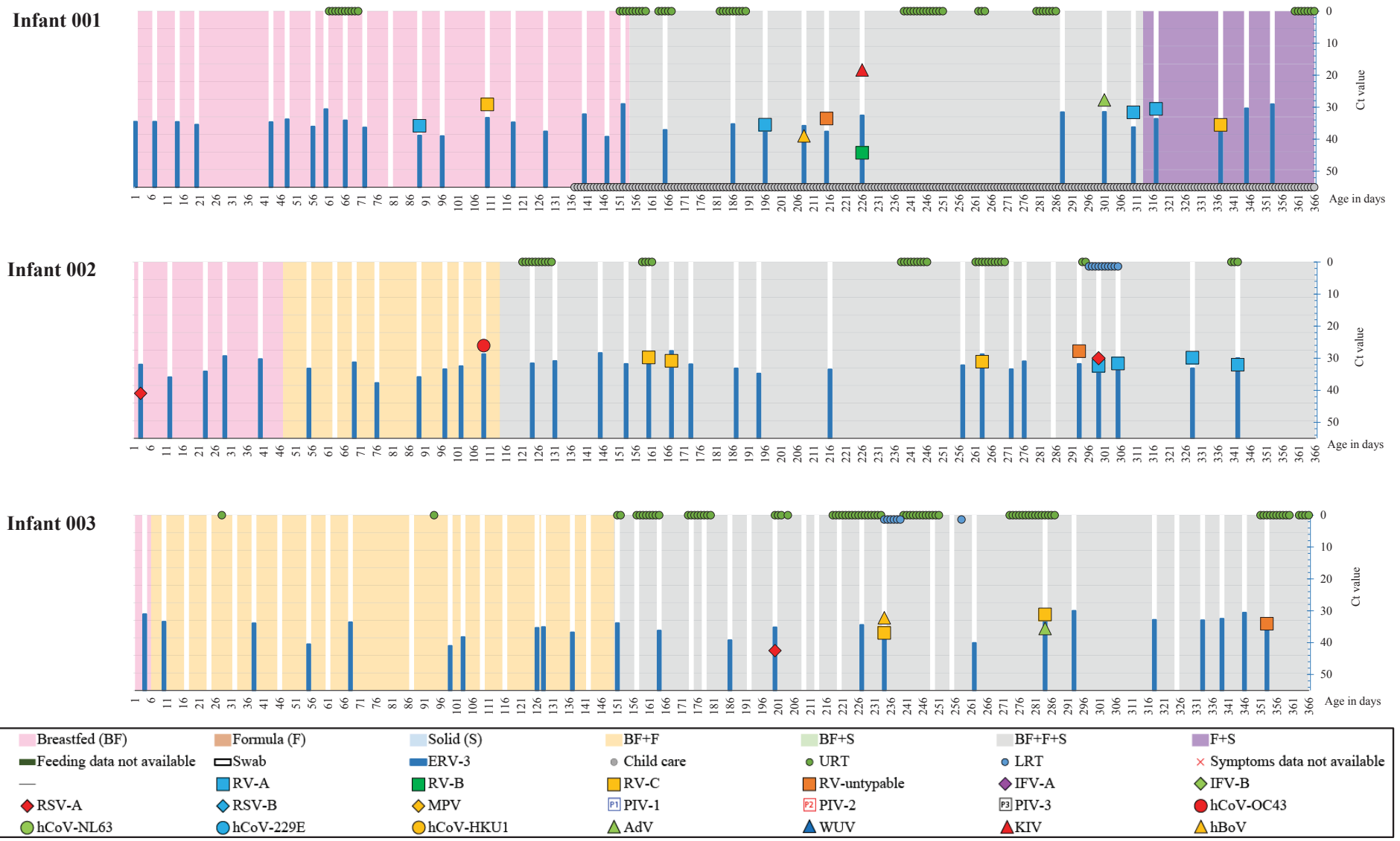
Appendix-1

Illustration of data obtained from infants during the first year of life.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Formula (F)
Solid (S)
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BF+F+S
F+S
Feeding data not available
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ERV-3
Child care
URT
LRT
Symptoms data not available
RV-A
RV-B
RV-C
RV-untypable
IFV-A
IFV-B
RSV-A
RSV-B
MPV
PIV-1
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AdV
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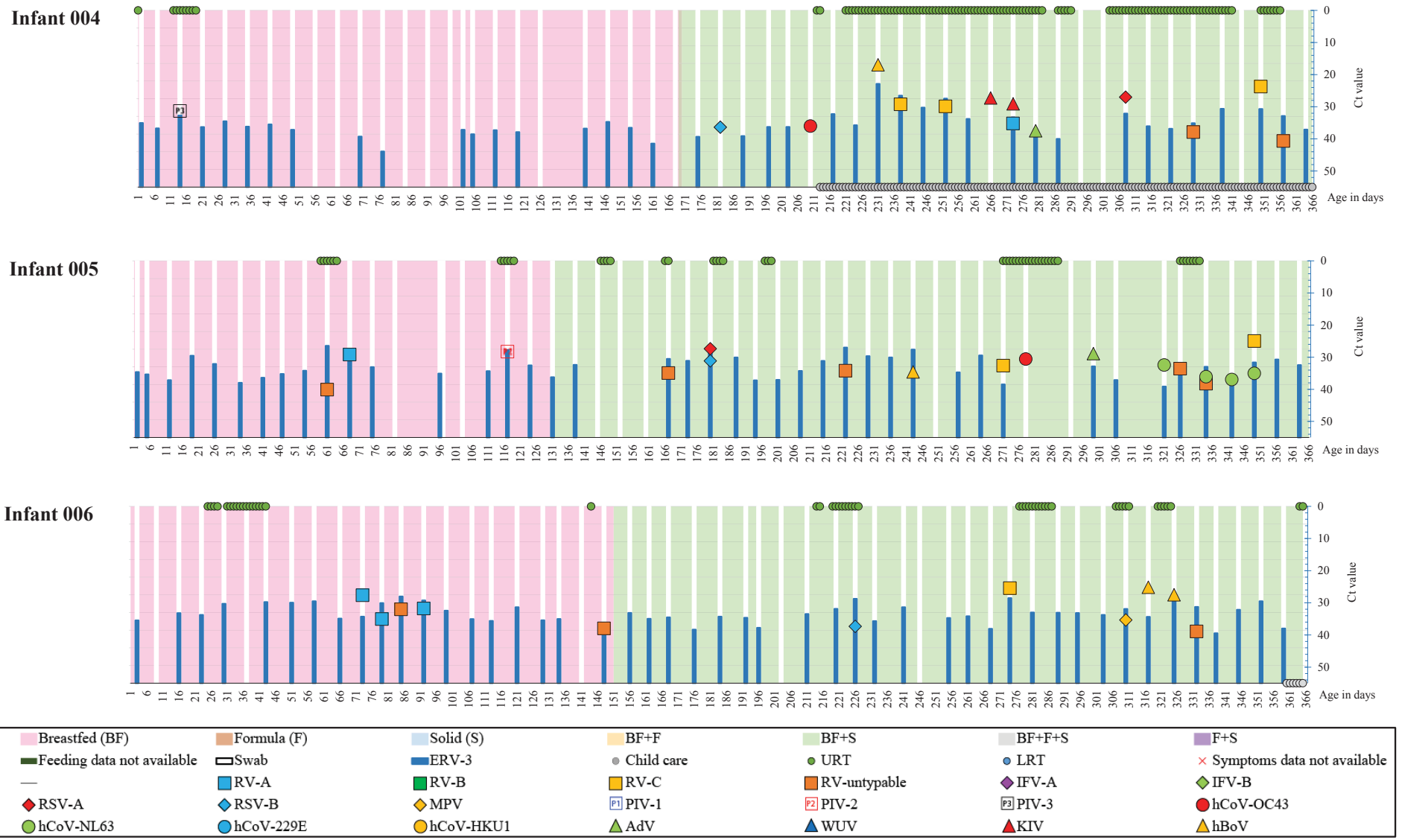
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Child care
URT
LRT
Symptoms data not available
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RV-C
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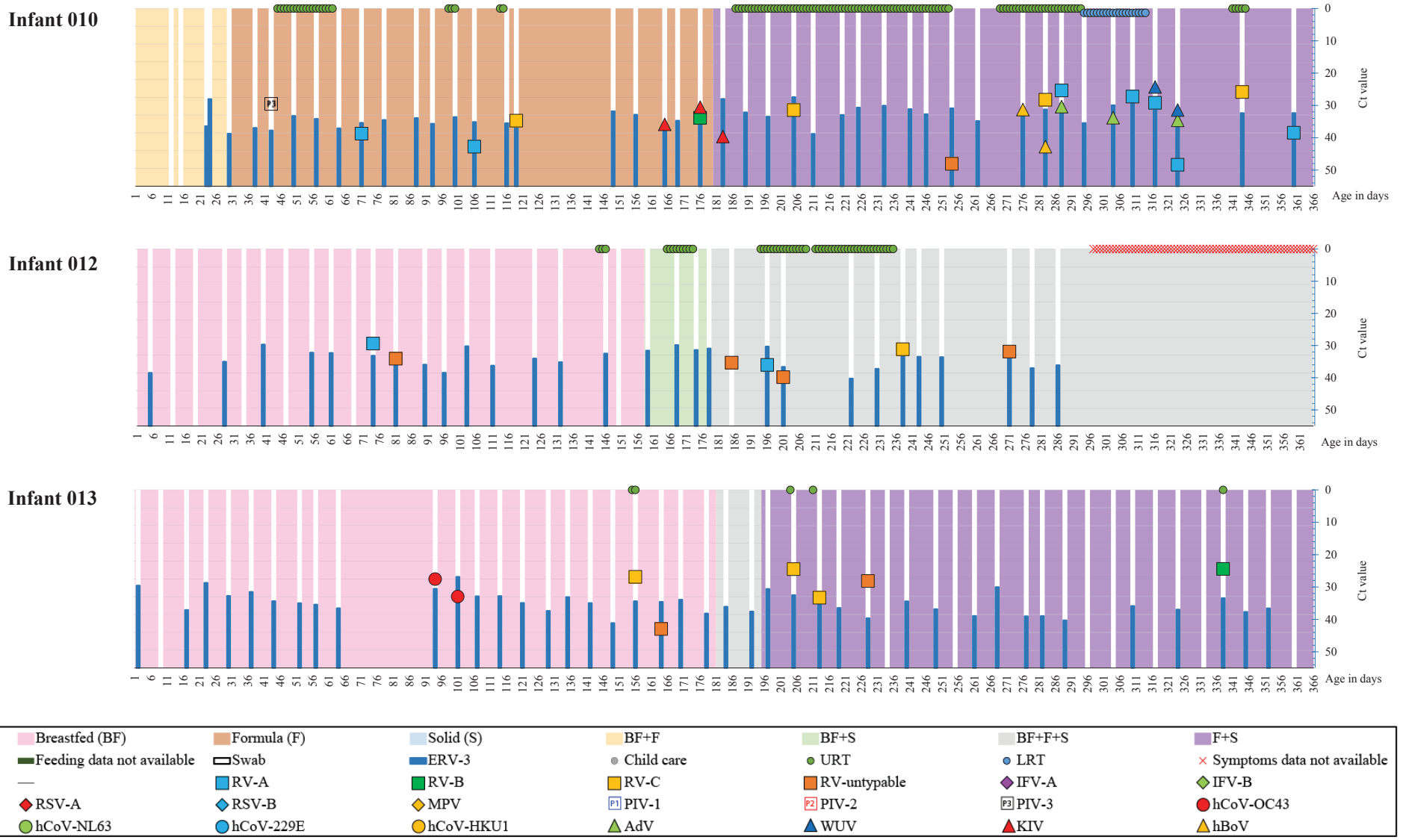
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Child care
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LRT
Symptoms data not available
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RV-B
RV-C
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RSV-A
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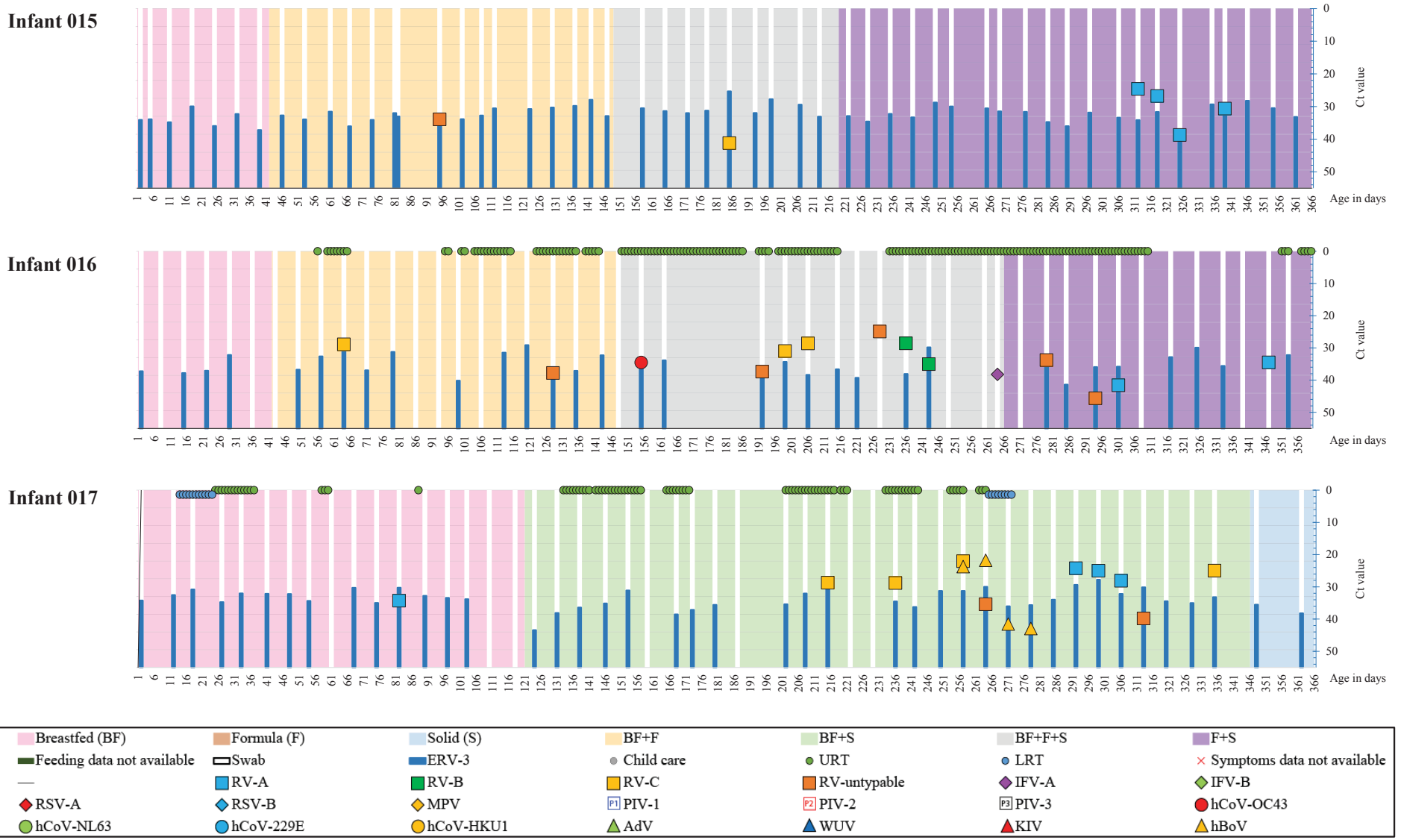
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Child care
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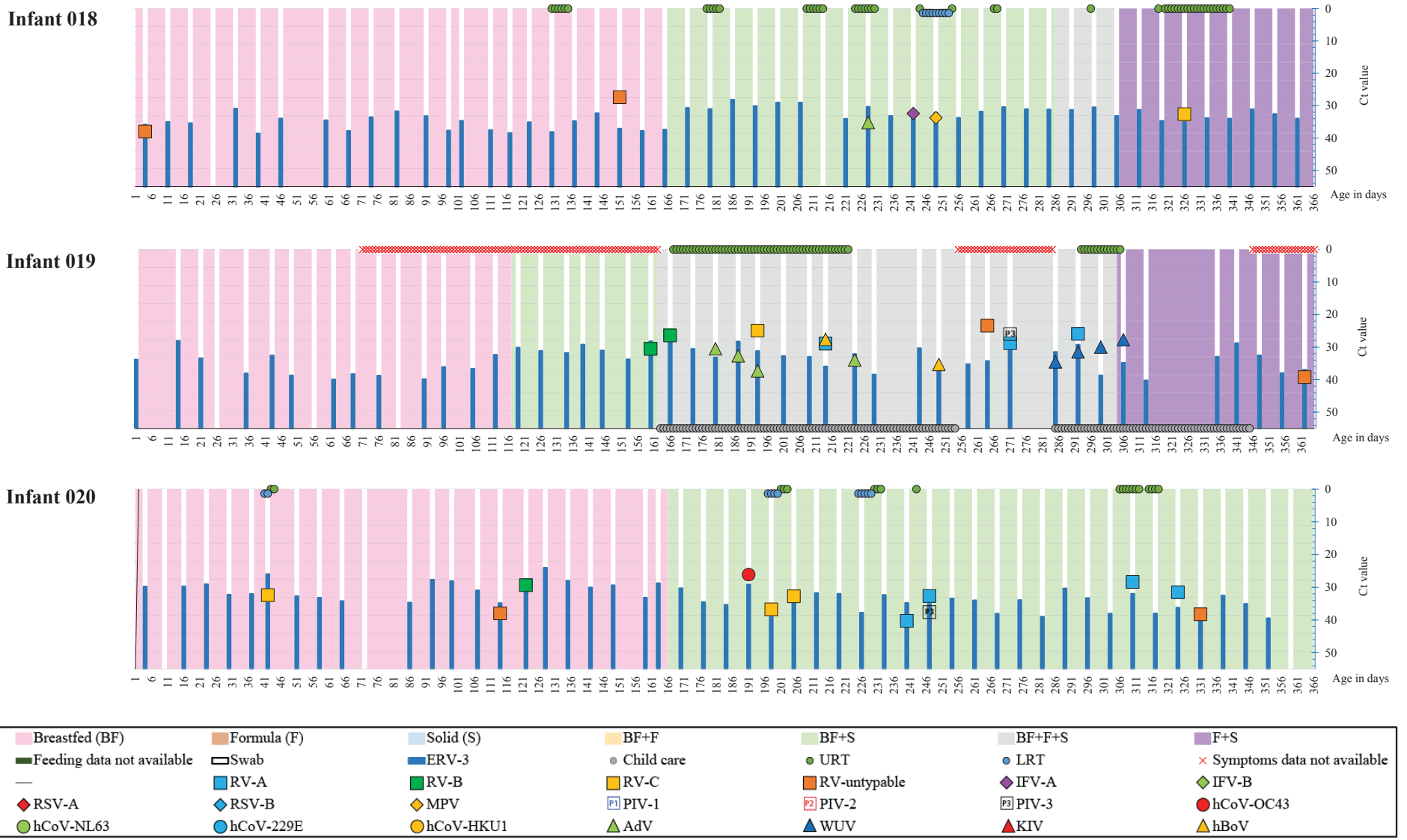
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Feeding data not available
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Child care
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Symptoms data not available
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IFV-A
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RSV-A
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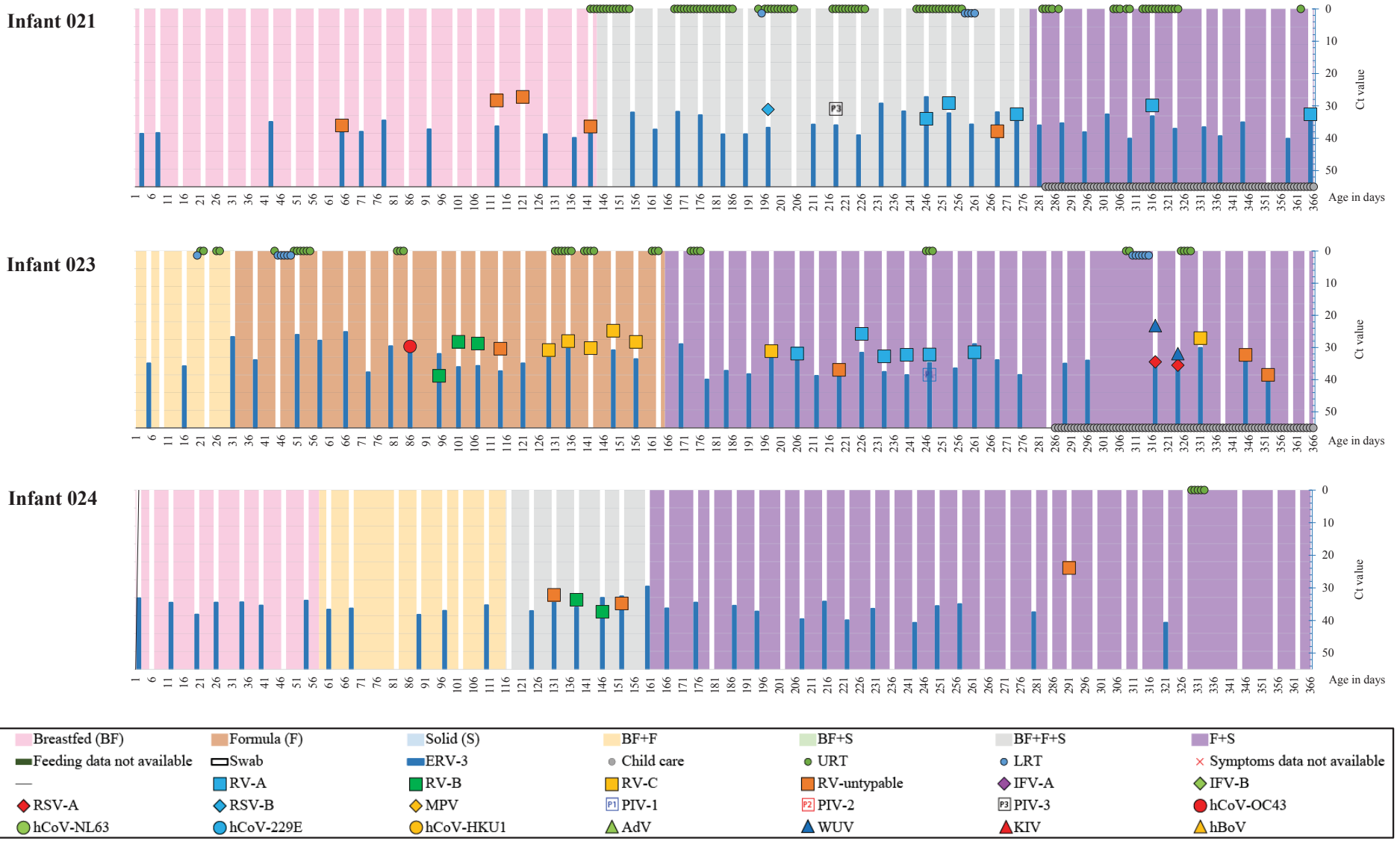
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Child care
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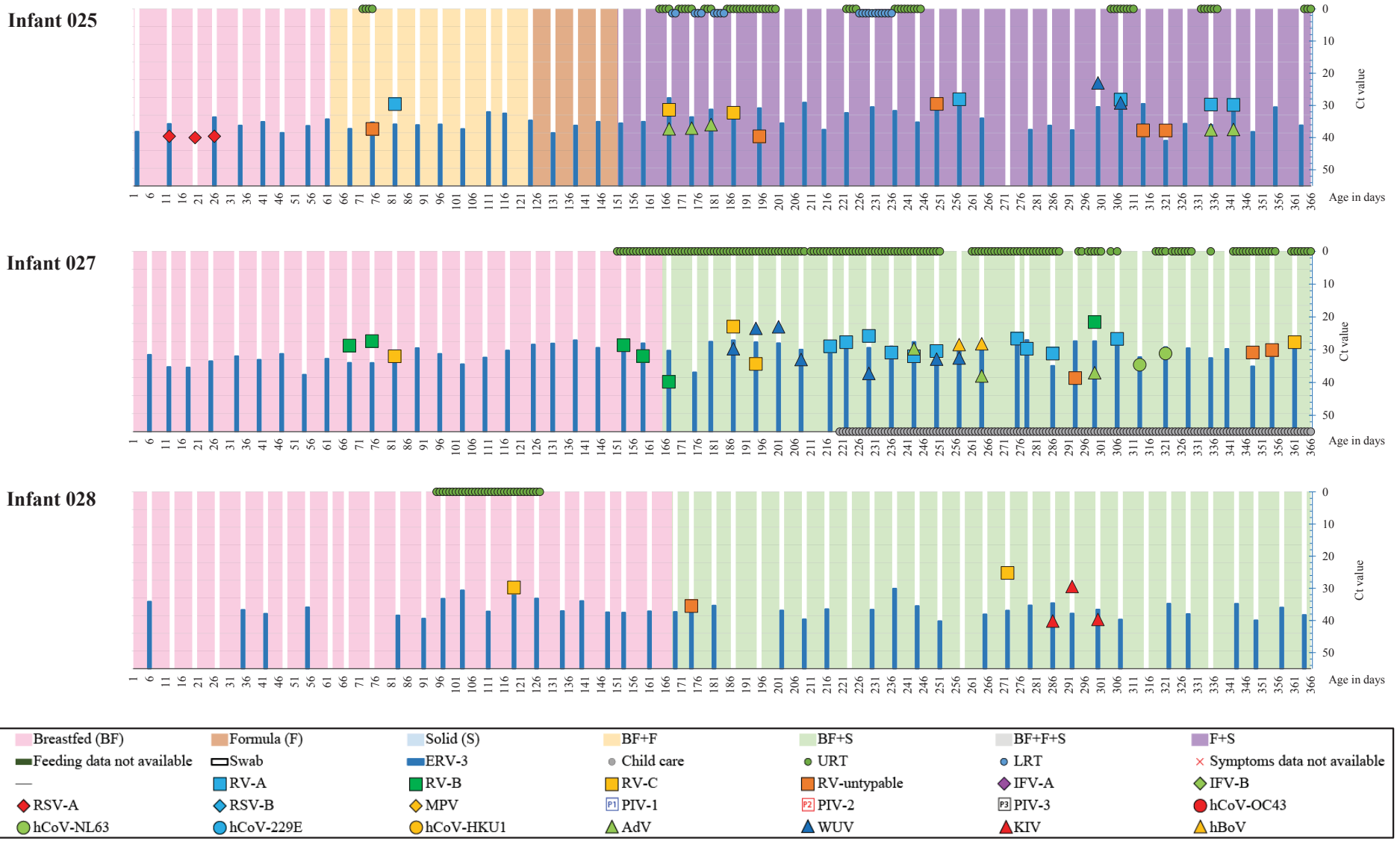
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RSV-A
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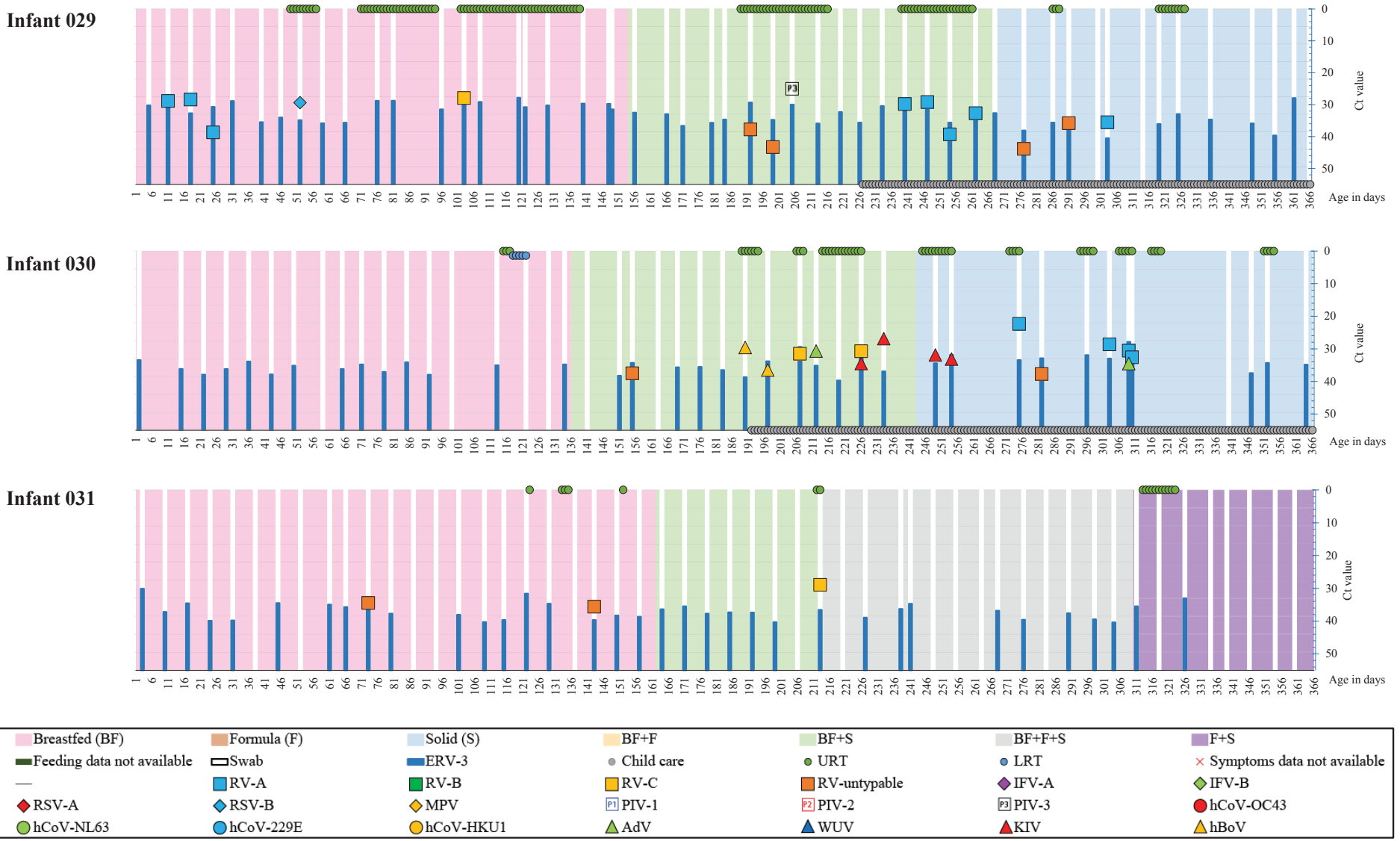
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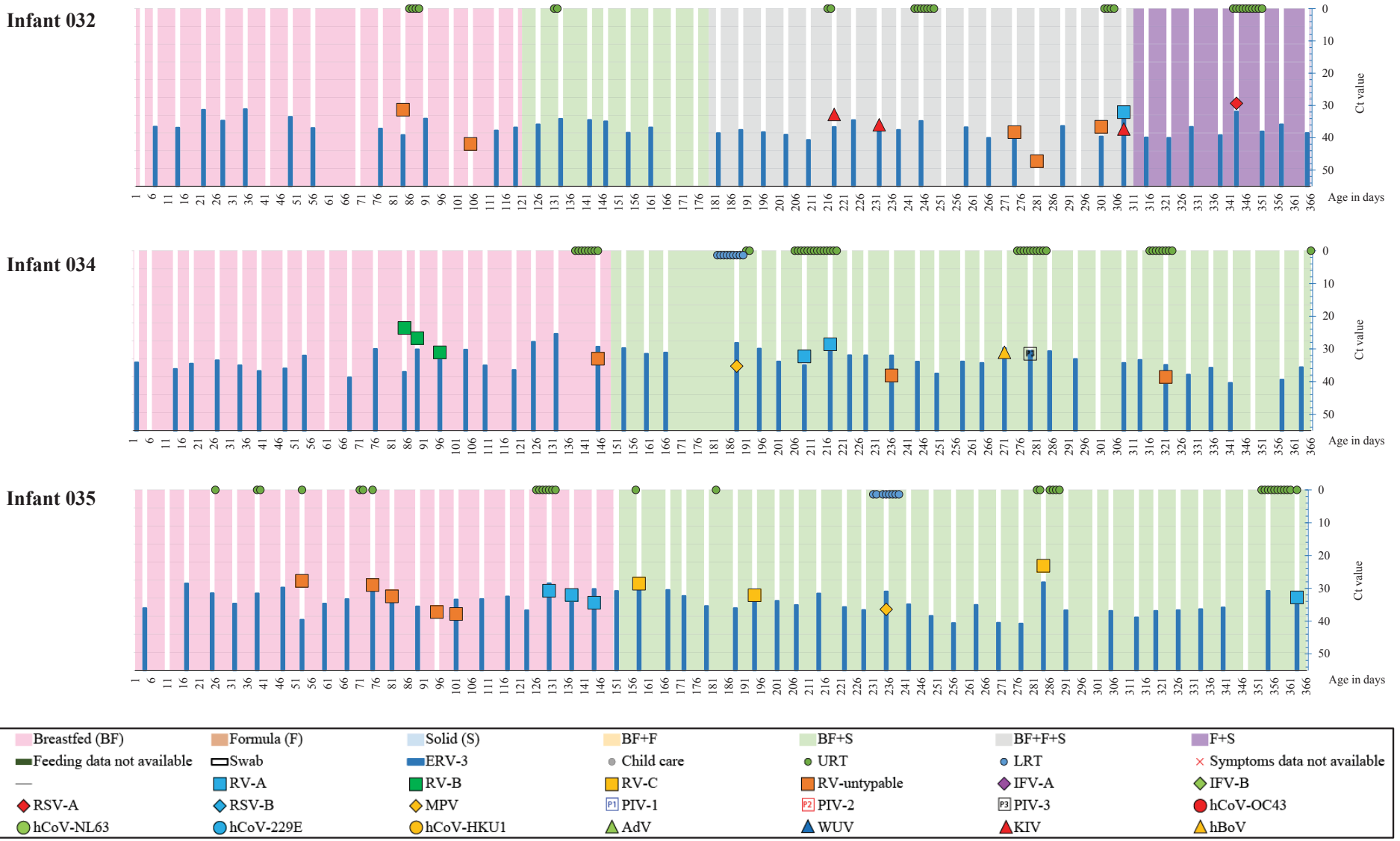
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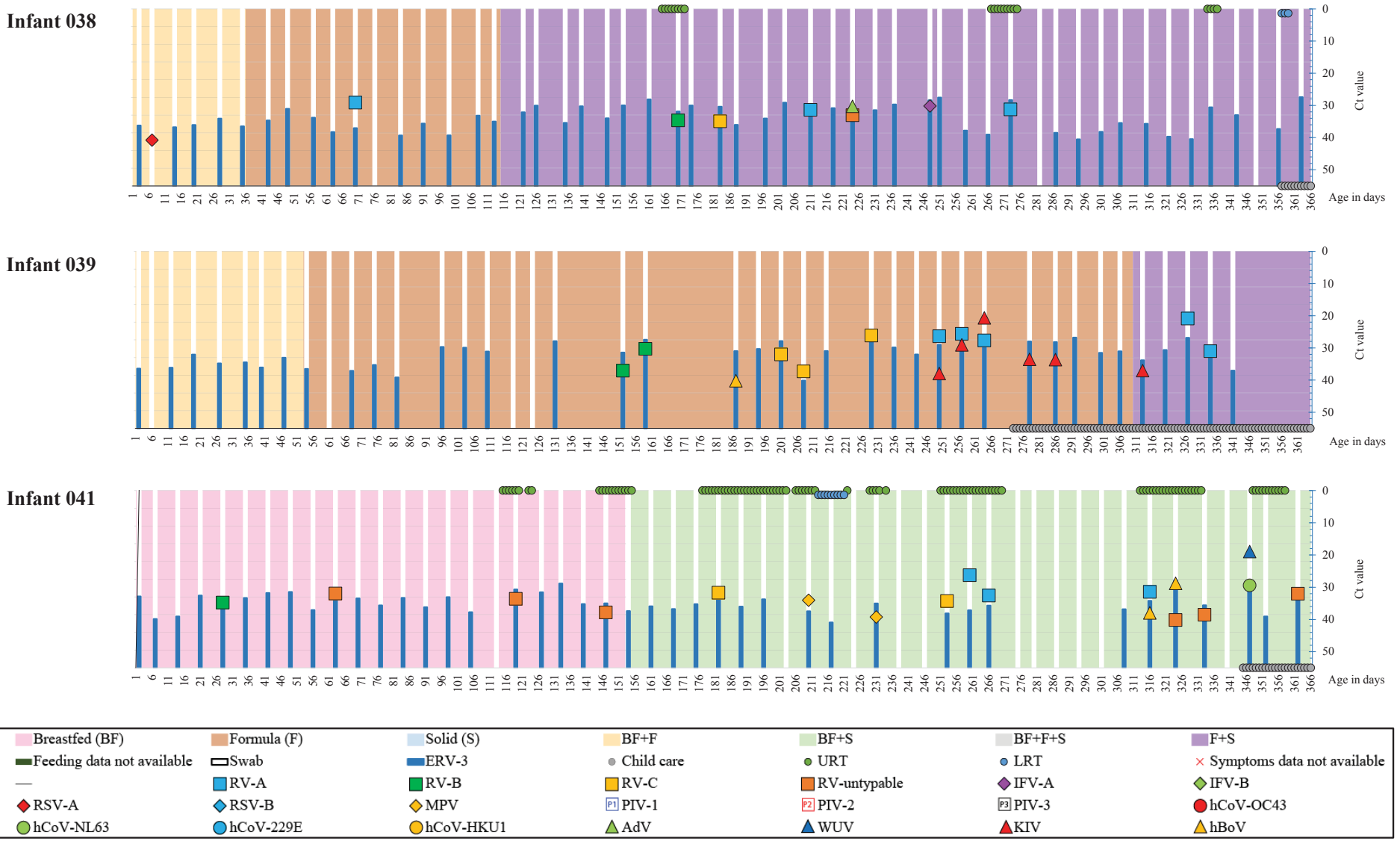
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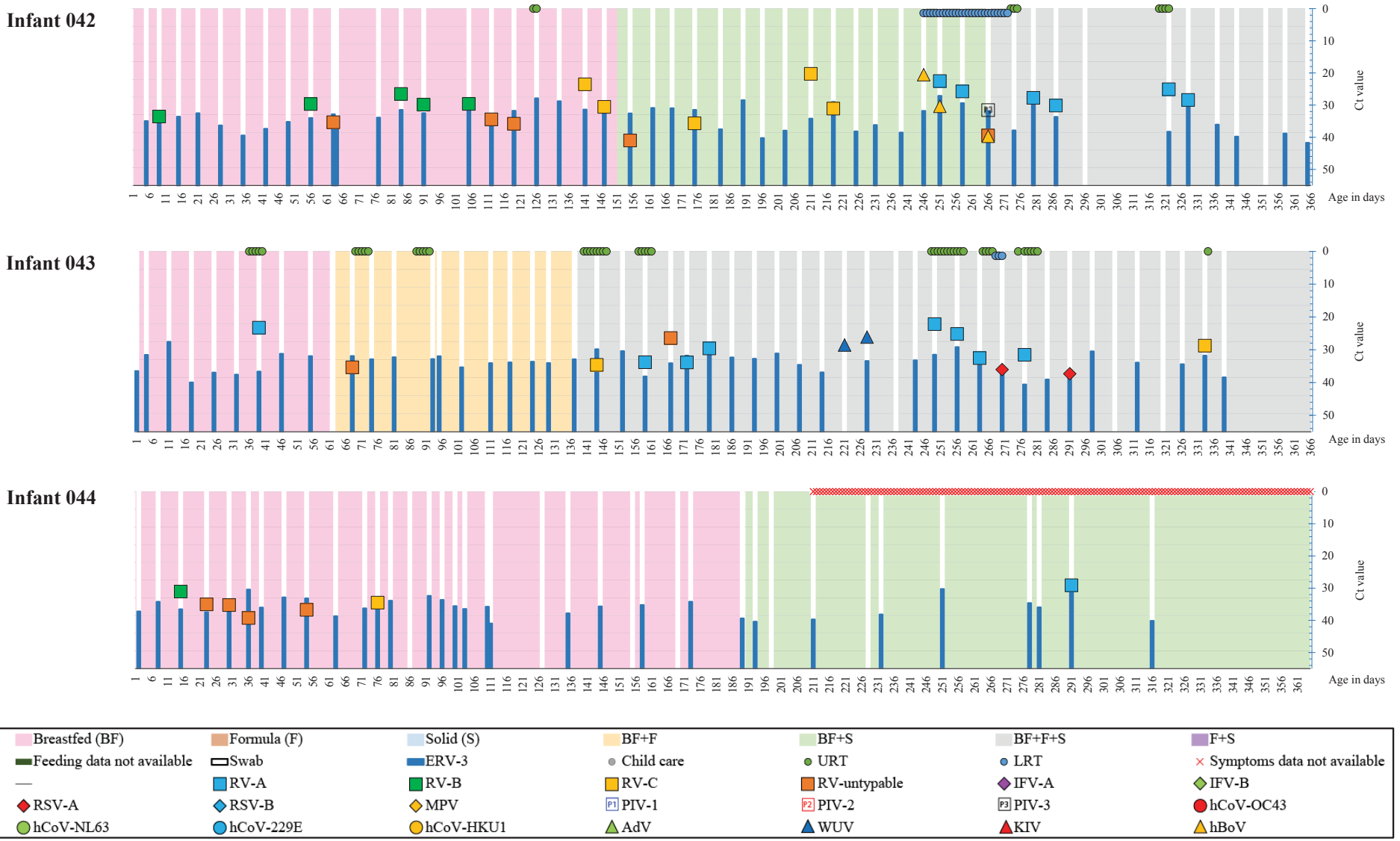
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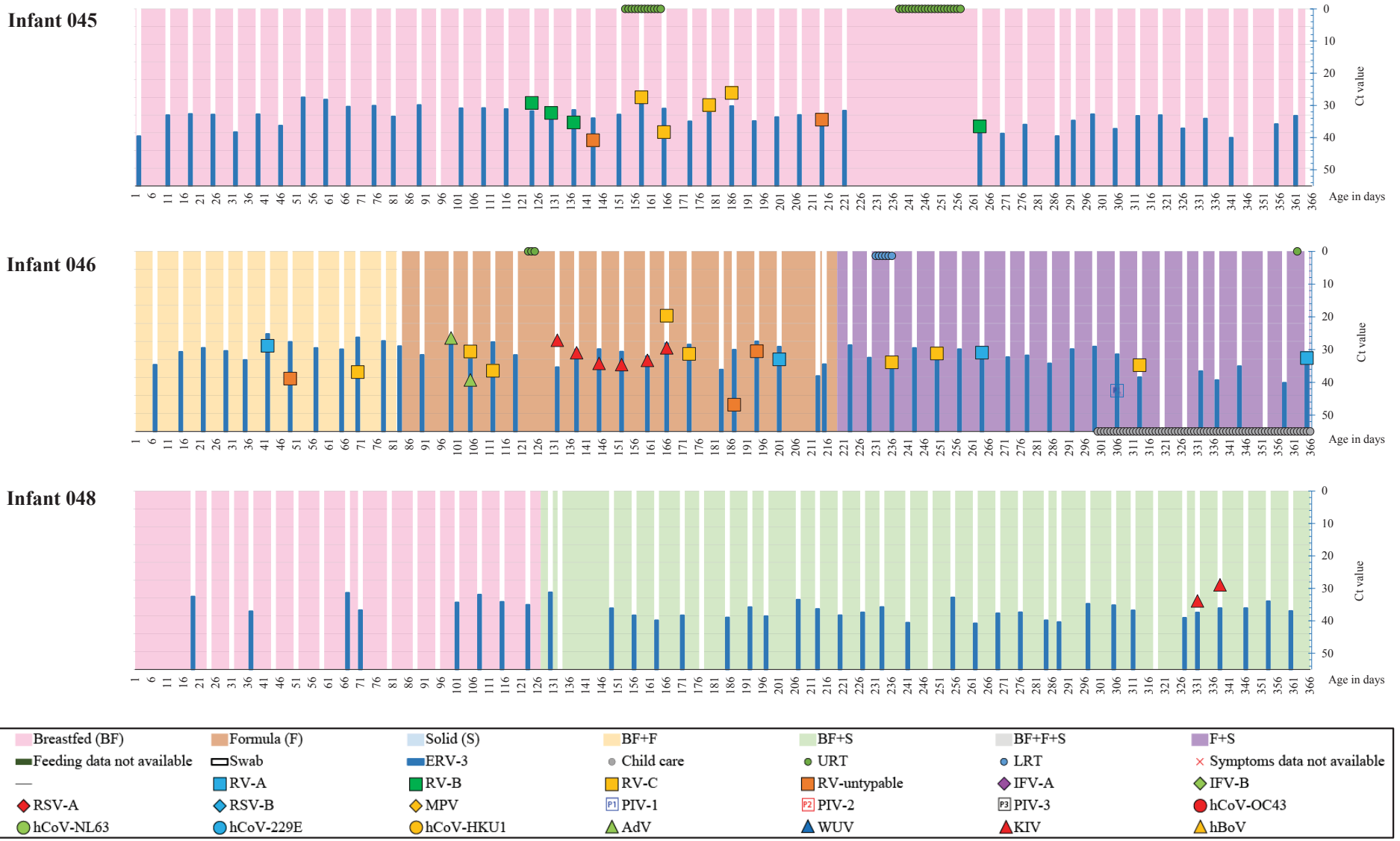
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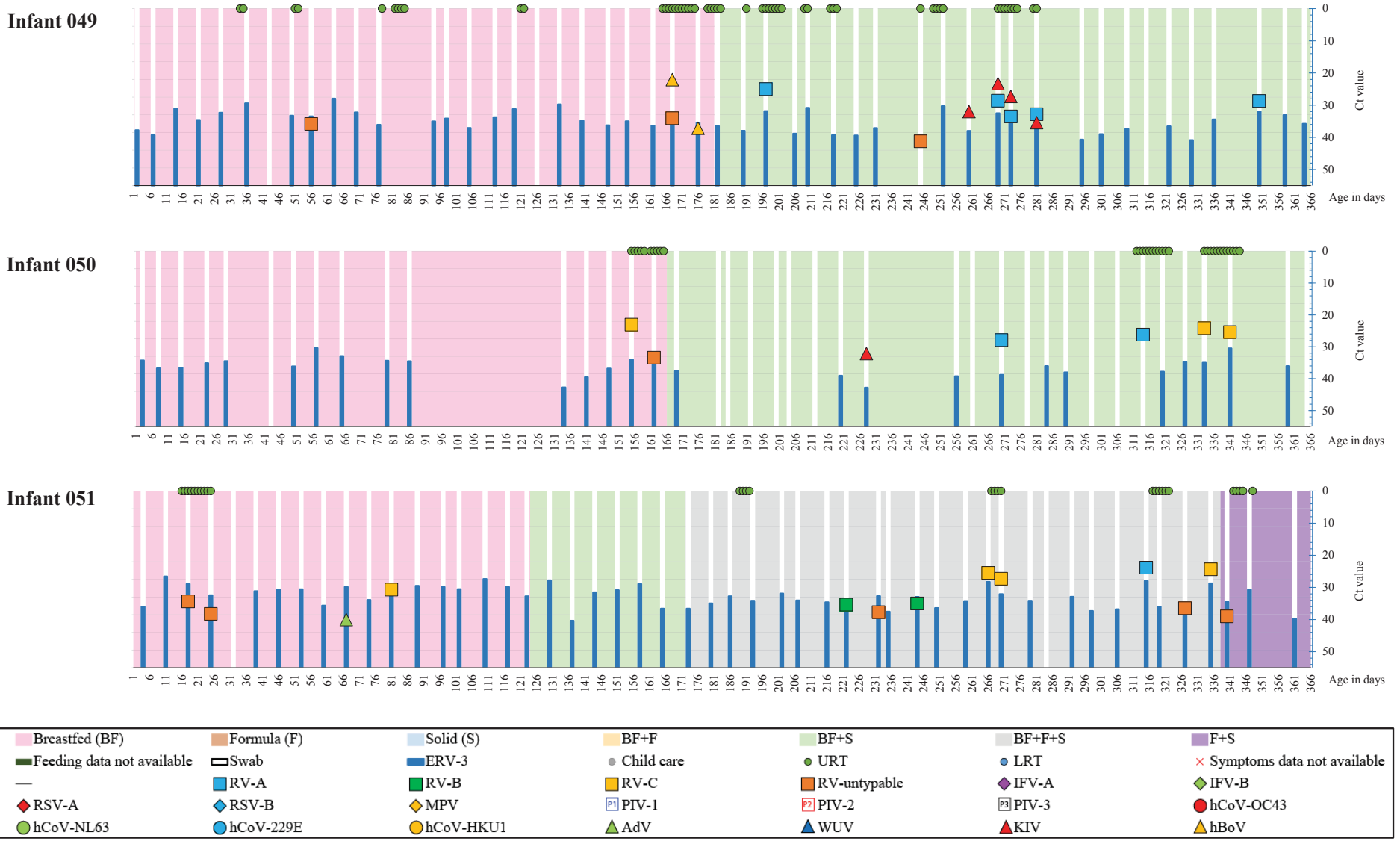
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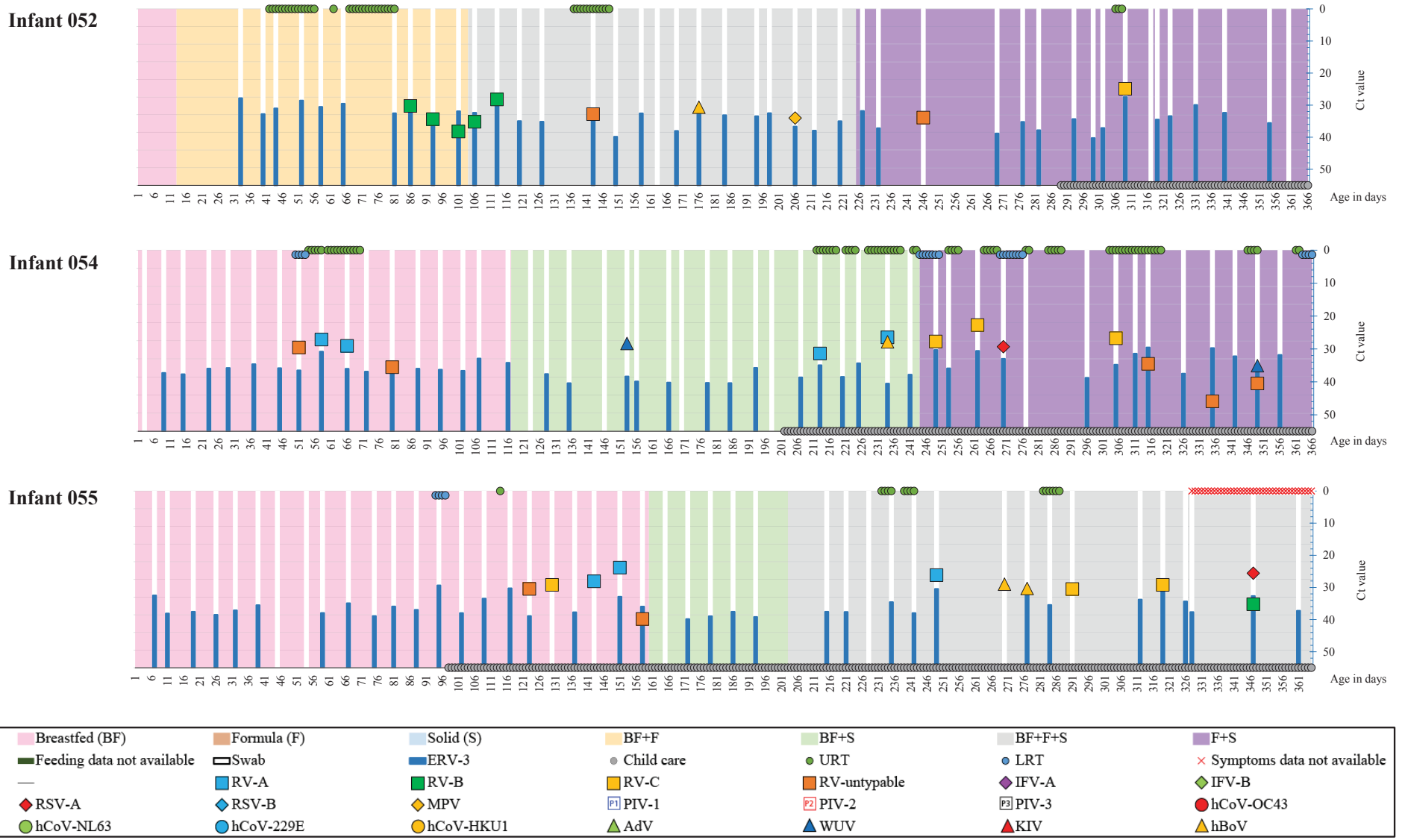
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Feeding data not available
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Child care
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LRT
Symptoms data not available
RV-A
RV-B
RV-C
RV-untypable
IFV-A
IFV-B
RSV-A
RSV-B
MPV
PIV-1
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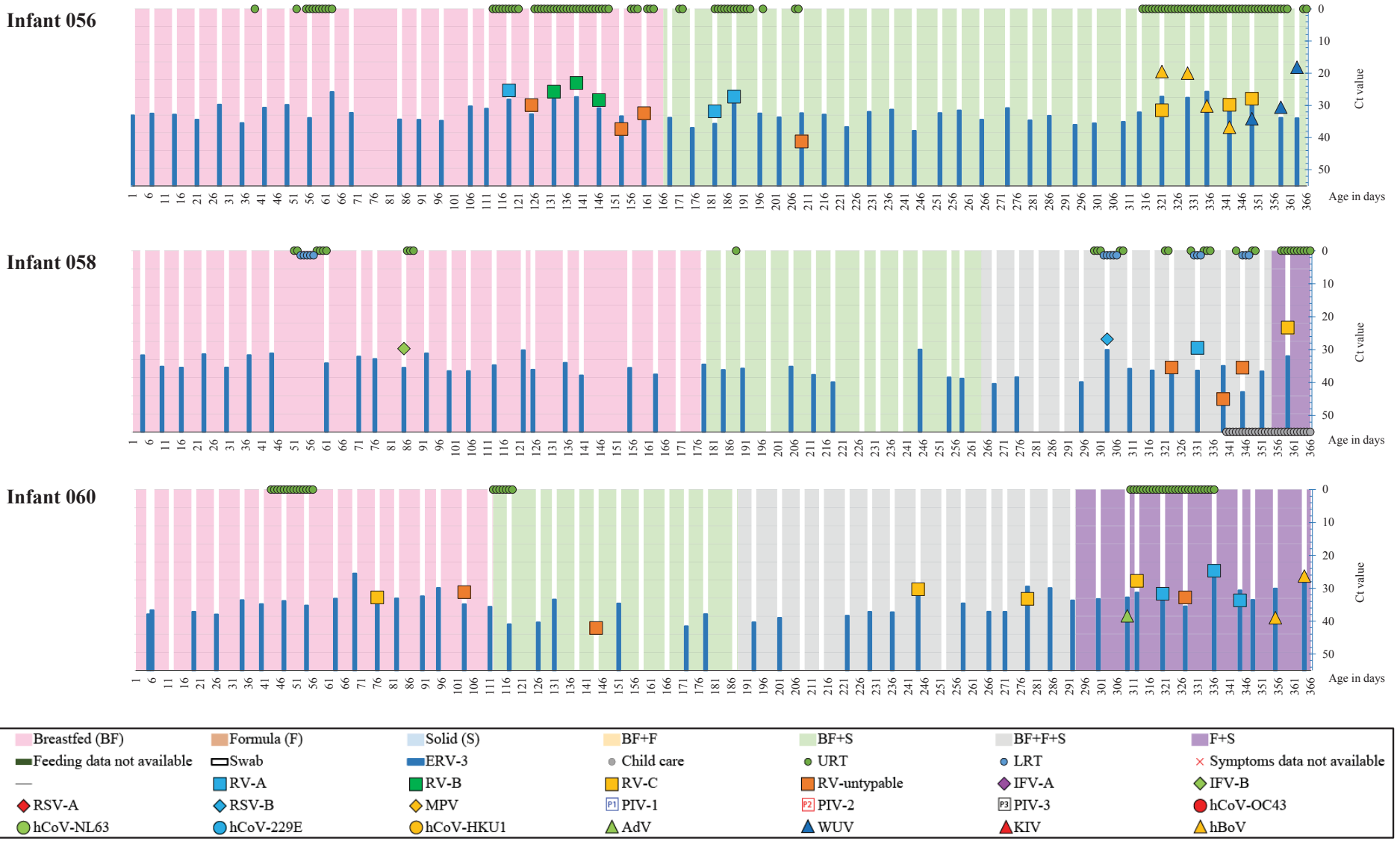
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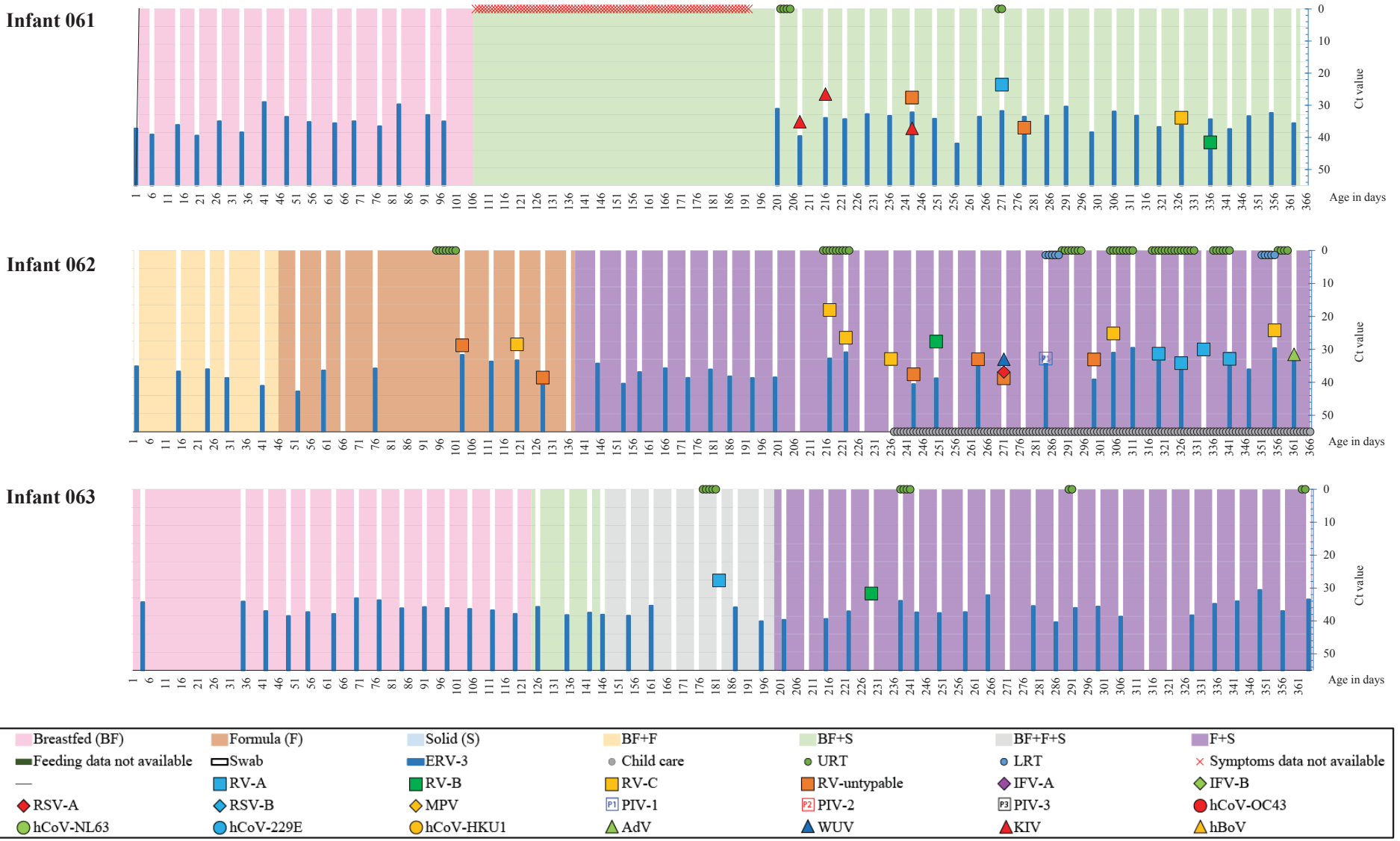
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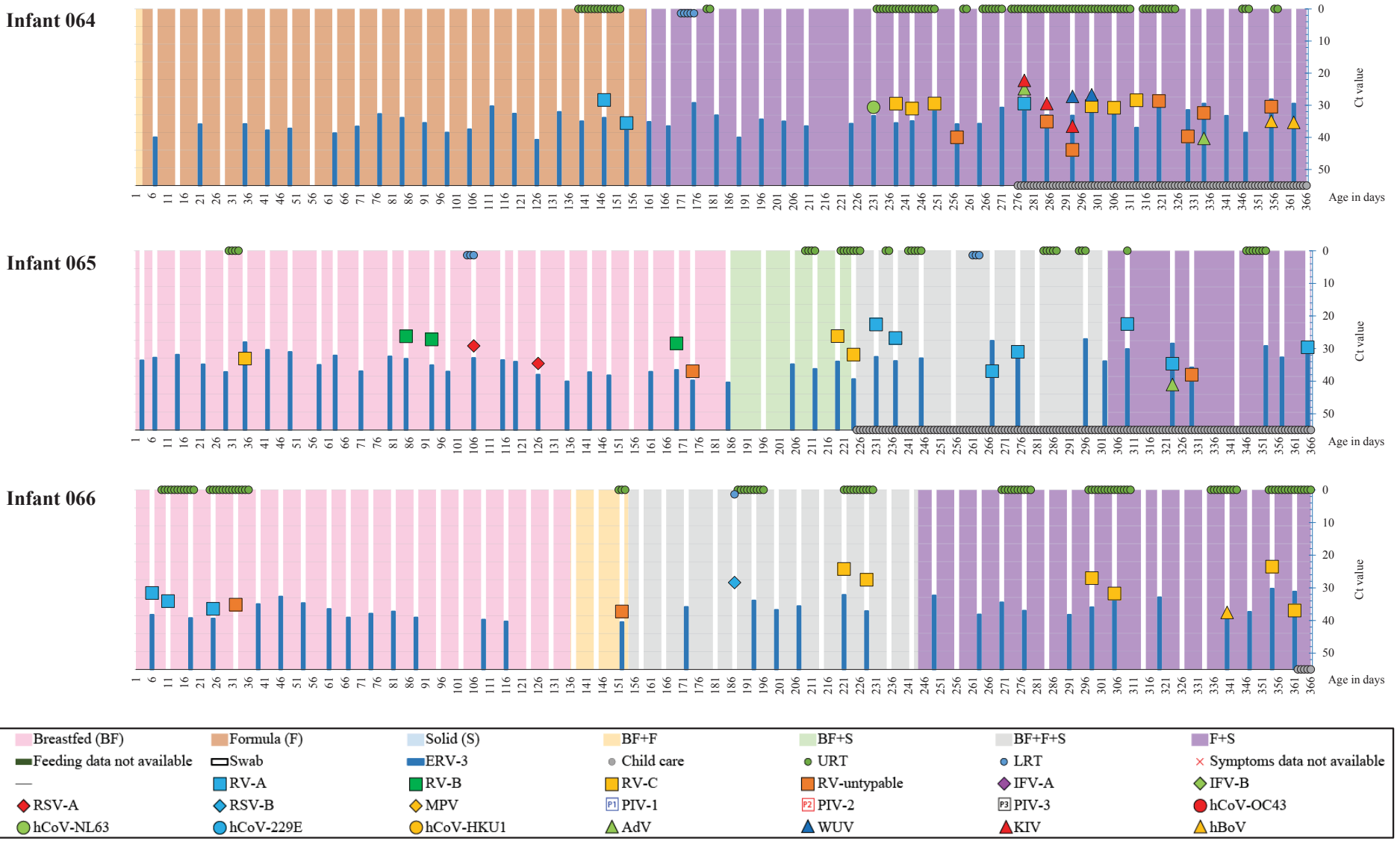
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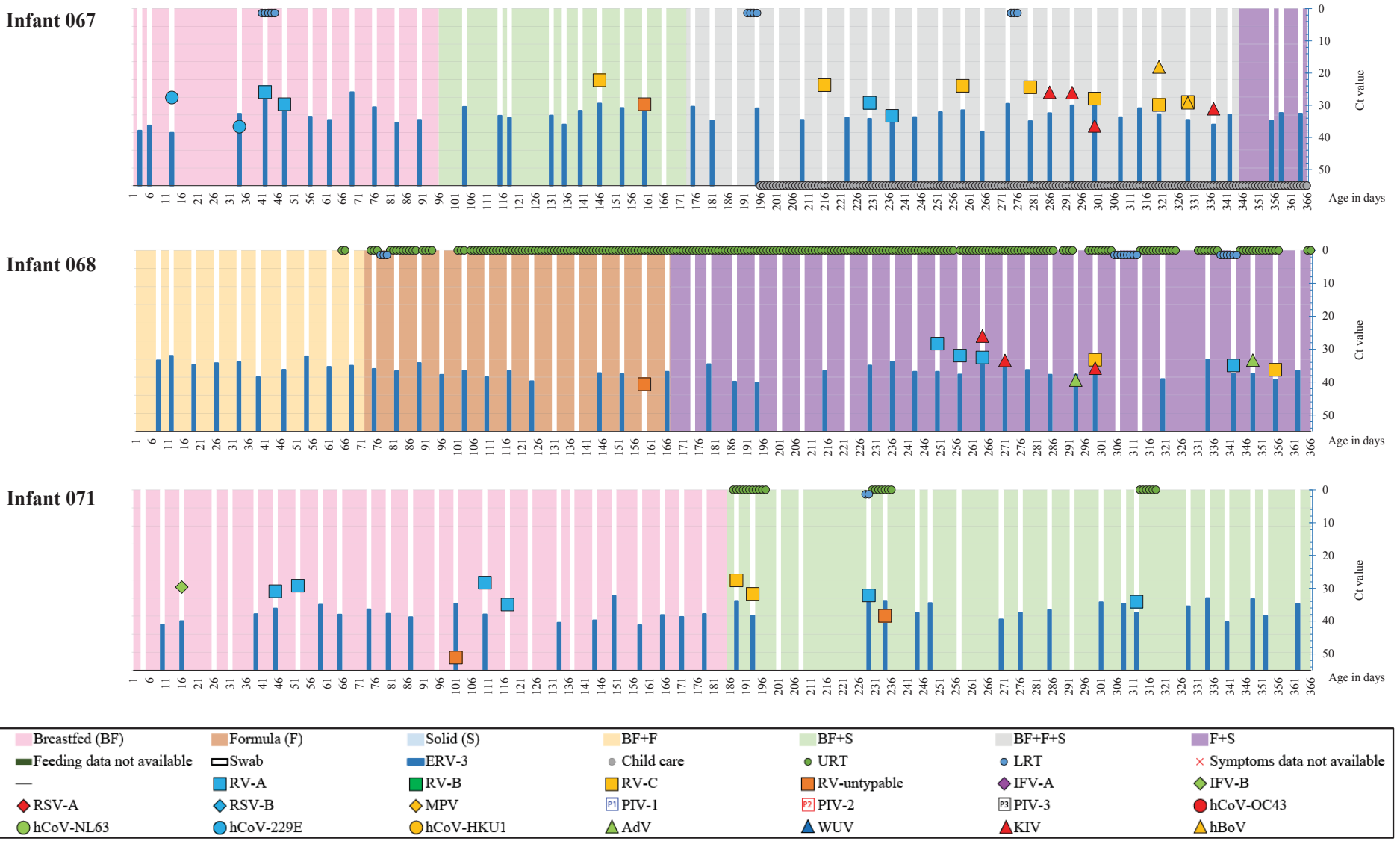
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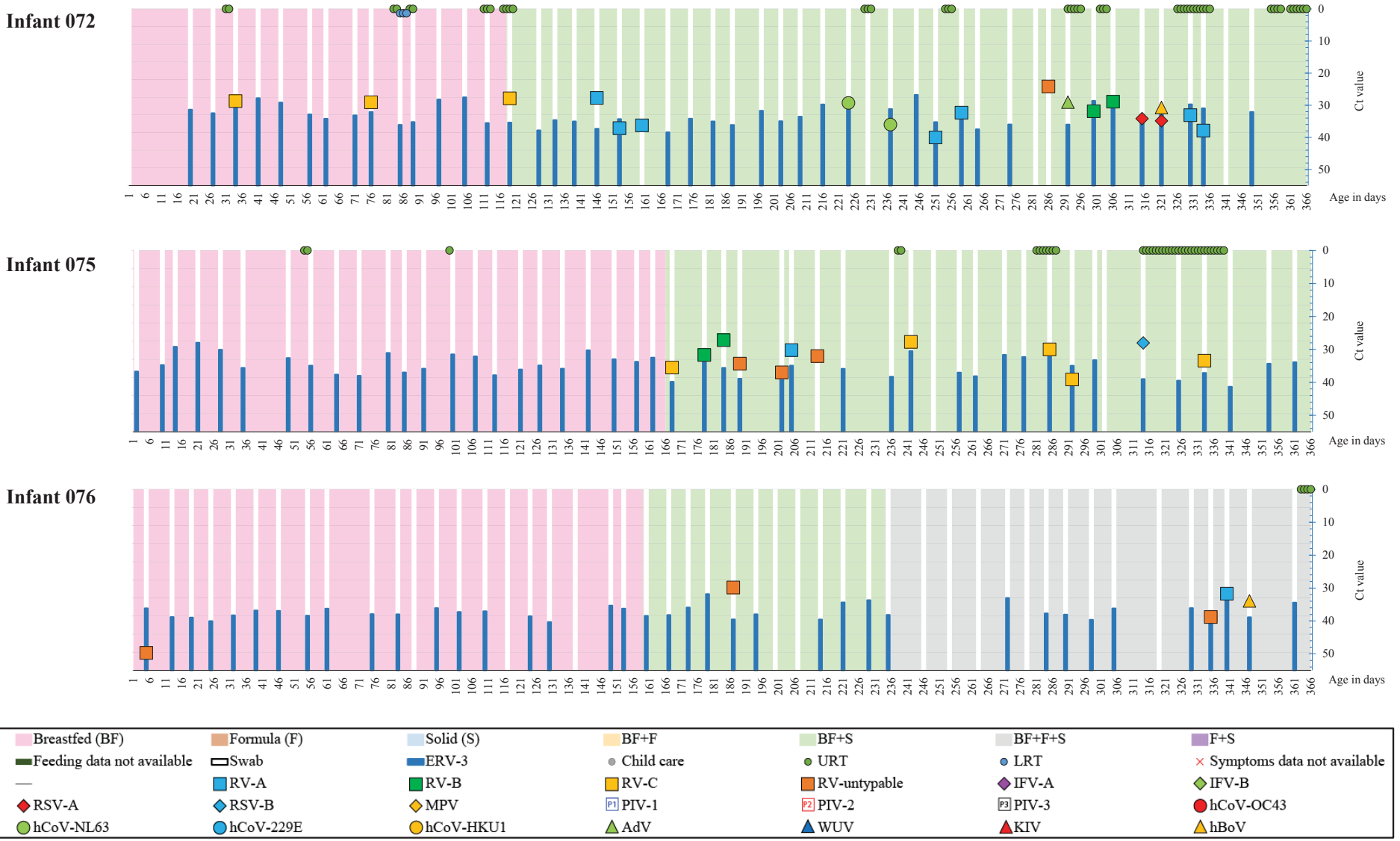
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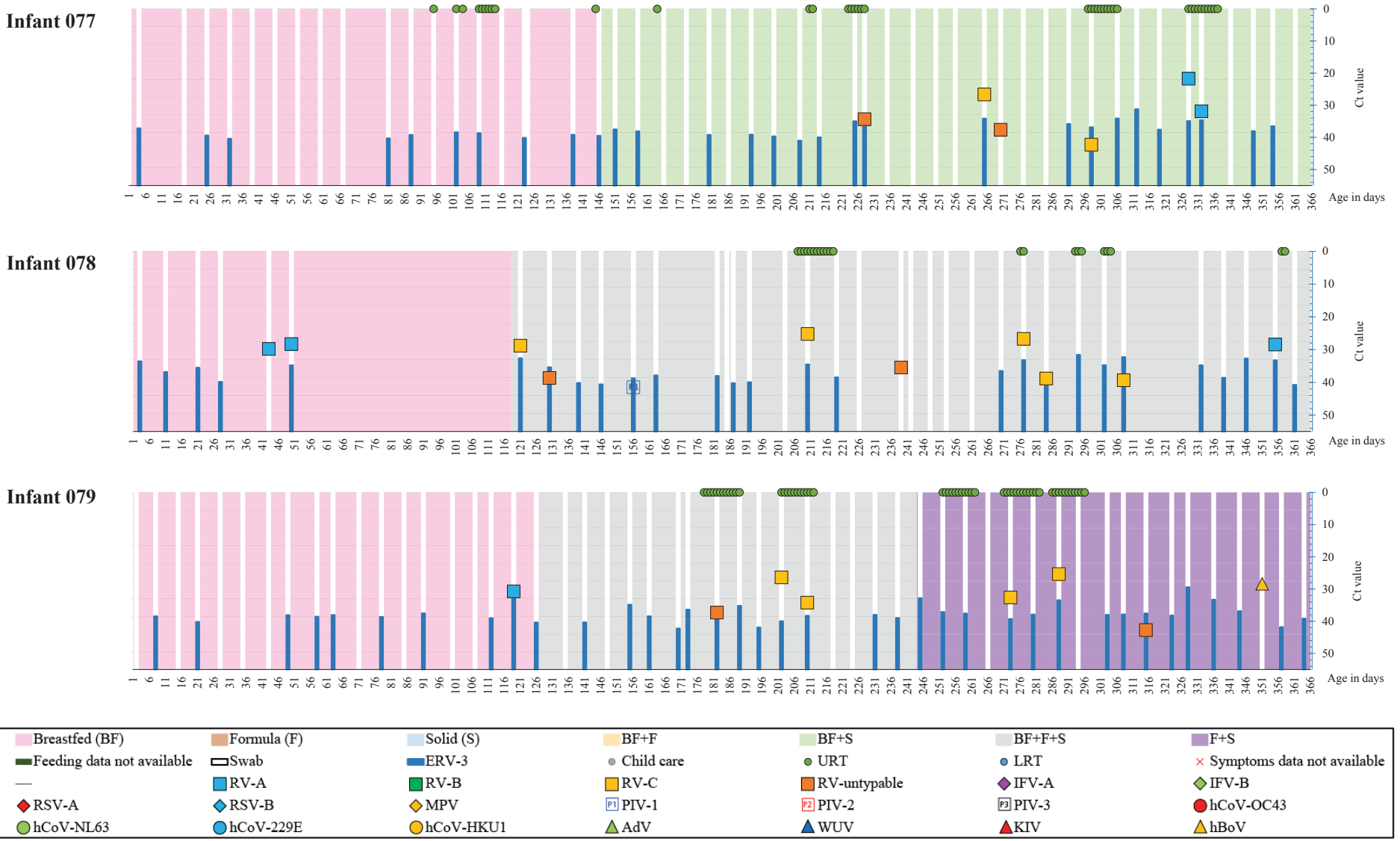
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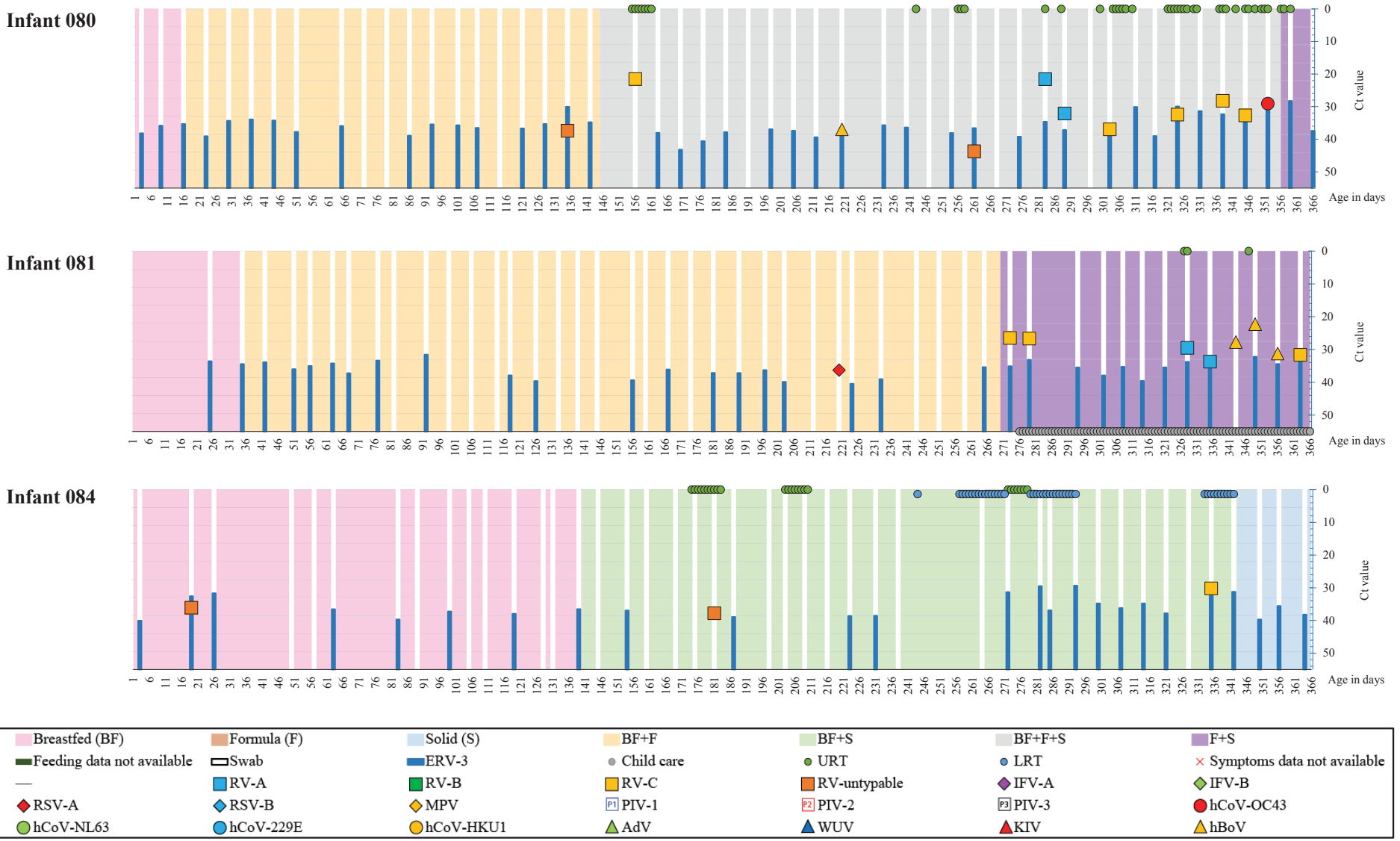
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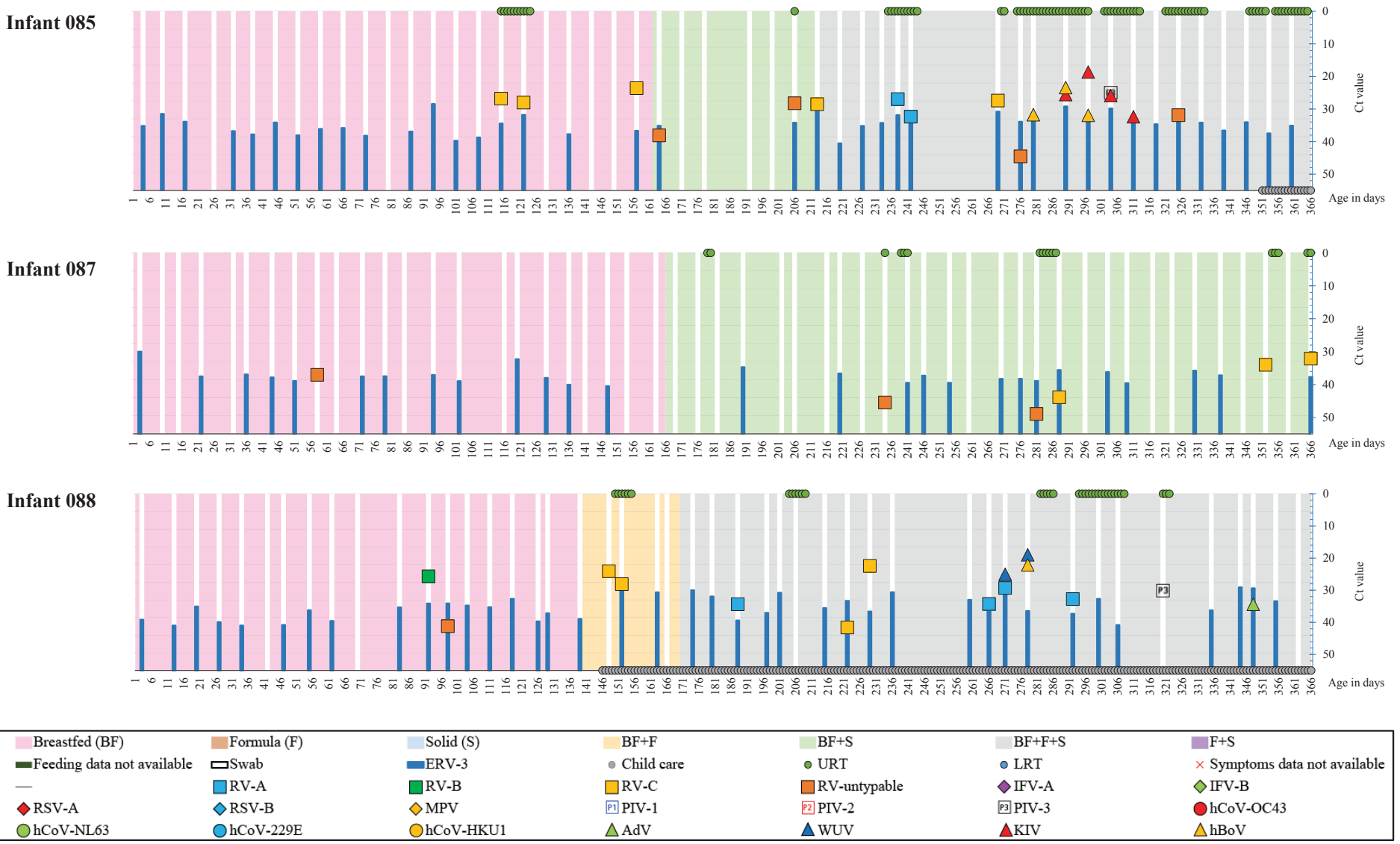
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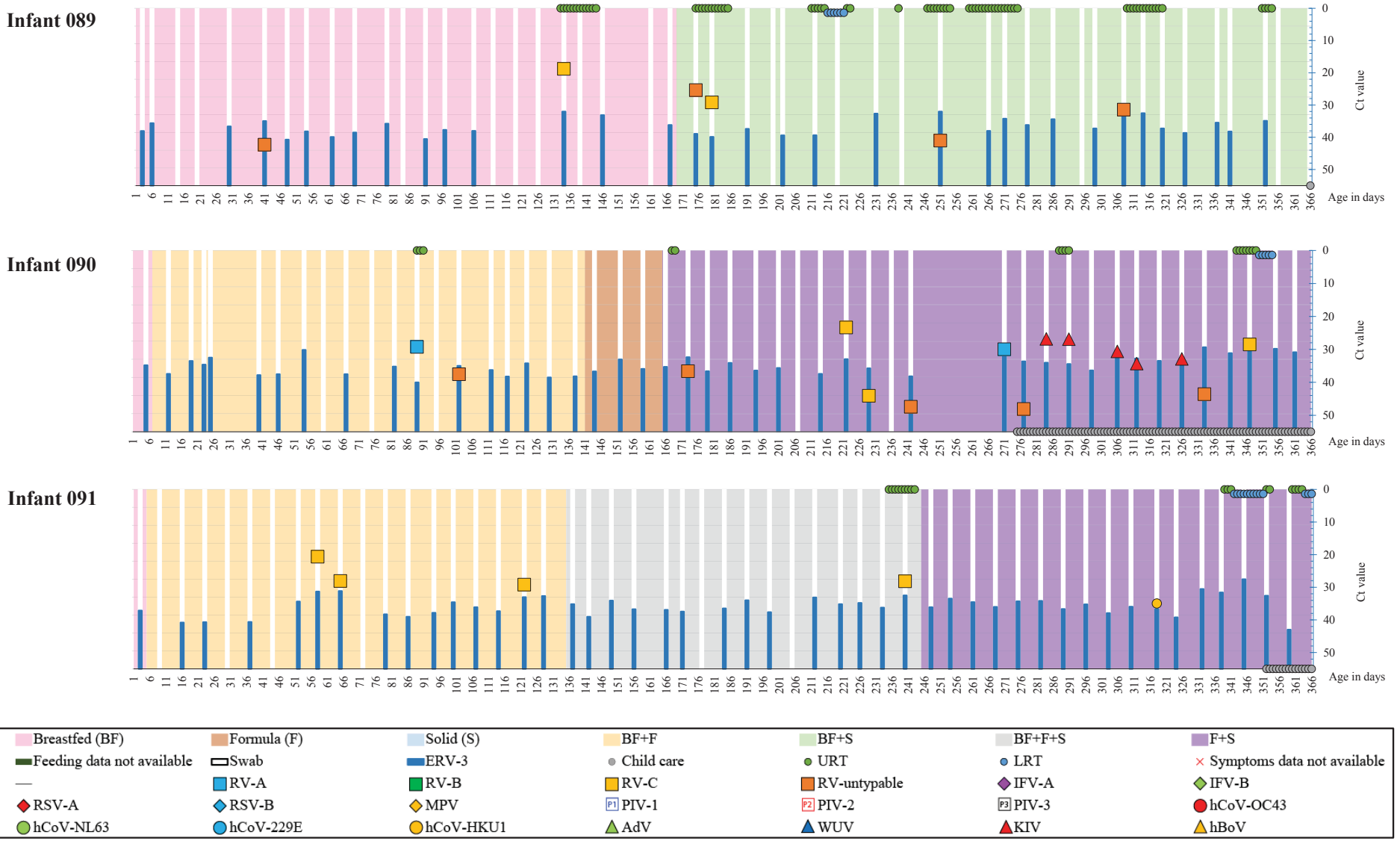
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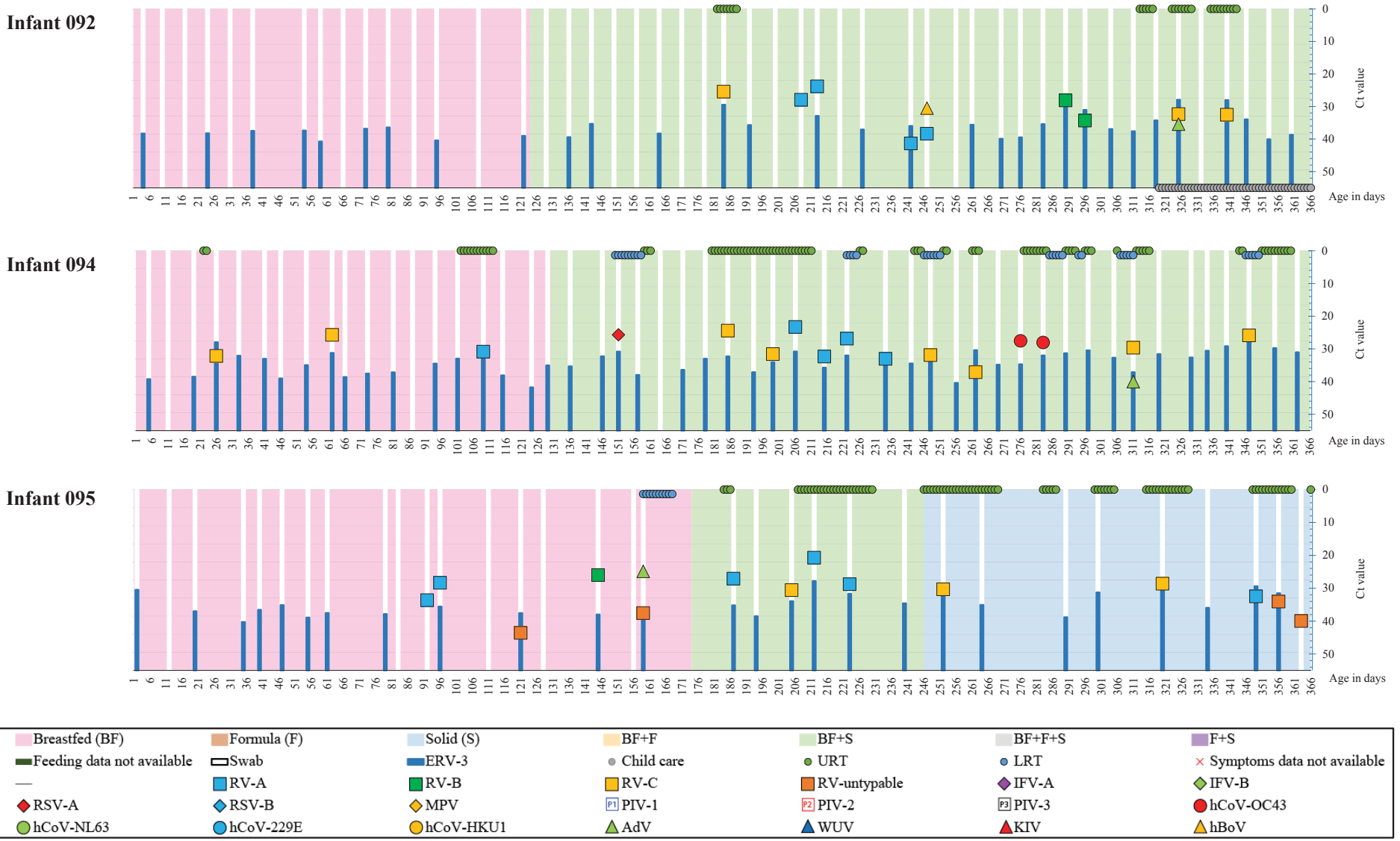
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Appendix-2

Publications during candidature

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Nasal swab samples and real-time polymerase chain reaction assays in community-based, longitudinal studies of respiratory viruses: the importance of sample integrity and quality control

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Abstract

Background: Carefully conducted, community-based, longitudinal studies are required to gain further understanding of the nature and timing of respiratory viruses causing infections in the population. However, such studies pose unique challenges for field specimen collection, including as we have observed the appearance of mould in some nasal swab specimens. We therefore investigated the impact of sample collection quality and the presence of visible mould in samples upon respiratory virus detection by real-time polymerase chain reaction (PCR) assays.

Methods: Anterior nasal swab samples were collected from infants participating in an ongoing community-based, longitudinal, dynamic birth cohort study. The samples were first collected from each infant shortly after birth and weekly thereafter. They were then mailed to the laboratory where they were catalogued, stored at −80°C and later screened by PCR for 17 respiratory viruses. The quality of specimen collection was assessed by screening for human deoxyribonucleic acid (DNA) using endogenous retrovirus 3 (ERV3). The impact of ERV3 load upon respiratory virus detection and the impact of visible mould observed in a subset of swabs reaching the laboratory upon both ERV3 loads and respiratory virus detection was determined.

Results: In total, 4933 nasal swabs were received in the laboratory. ERV3 load in nasal swabs was associated with respiratory virus detection. Reduced respiratory virus detection (odds ratio 0.35; 95% confidence interval 0.27-0.44) was observed in samples where the ERV3 could not be identified. Mould was associated with increased time of samples reaching the laboratory and reduced ERV3 loads and respiratory virus detection.

Conclusion: Suboptimal sample collection and high levels of visible mould can impact negatively upon sample quality. Quality control measures, including monitoring human DNA loads using ERV3 as a marker for epithelial cell components in samples should be undertaken to optimize the validity of real-time PCR results for respiratory virus investigations in community-based studies.

Keywords: Nasal swab, Respiratory virus, Real-time polymerase chain reaction, Quality control, Mould, Community-based study

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Background

Acute respiratory infections (ARIs) caused by viruses are the most common illnesses experienced by all age groups. ARIs are particularly important during early life as infants have the highest infection rates and they can transmit infectious agents to other household members [1]. Recently introduced molecular-based diagnostic techniques have much improved sensitivity compared with previous classical culture and phenotypic-based methods and have led to the discovery of new respiratory viruses [2]. However, contemporary studies employing these new techniques have often used convenience samples obtained from patients admitted to hospital or attending Emergency Department clinics [3-5]. Understanding more fully the ARI disease burden in the community is important for developing public health interventions, such as vaccination programs [6], and for understanding the role respiratory viruses may play in the pathogenesis of certain chronic pulmonary disorders, such as asthma [7-9]. This has led to the instigation of community-based studies. Such studies do however have some logistical challenges, particularly concerning respiratory sample collection and transport. Most studies have relied upon clinic or home visits by trained healthcare workers to collect specimens during an ARI episode, which imposes restrictions upon busy families and may lead to biased disease estimates and specimen availability [10-12]. Cost and feasibility of using healthcare workers are also important when large longitudinal, community-based cohort studies, involving frequent specimen collections, are planned. To help address these limitations, we and others have begun testing parent-collected, anterior nasal swab specimens that have been transported to the research laboratory using the standard mail [13-16]. This approach is considered to be safe, convenient and cost-effective [17].

Importantly, when using highly sensitive polymerase chain reaction (PCR) assays the detection rates for respiratory viruses are similar in both anterior nasal swab specimens and samples collected by the more traditional method of nasopharyngeal aspiration [18,19]. Building on this information, later studies have also shown that PCR testing for respiratory viruses provided similar results for parent-collected anterior nasal swab specimens and either nasal swab or nasoparyngeal aspirates collected by healthcare professionals [16,17]. Other studies examining sample transport have also shown that mailing swabs at ambient temperature has limited or no impact on respiratory virus detection by PCR [14,20,21], although investigating further the effects of transporting samples for extended periods and at higher temperatures was highlighted in one study [20].

The observational research in childhood infectious diseases (ORChID) project is a longitudinal, community-based, dynamic birth cohort study, which seeks to describe the nature and timing of respiratory viruses detected in Australian children during the first 2-years of life [22]. The study commenced in late 2010 and involves parents collecting and mailing nasal swabs weekly to the research laboratory for PCR-based respiratory virus screening. During the first year mould was seen in some samples as they arrived in the laboratory and we became concerned about the impact of this contaminant upon sample integrity. Therefore, as part of the ORChID study, we undertook a broader investigation of sample quality, examining collection and transportation, and how these impact on respiratory virus detection. Our objectives were first to determine the quality of specimen collection by testing for the presence of human DNA (endogenous retrovirus3; ERV3) and then to investigate the effects of sample quality and the presence of visible mould in samples reaching the laboratory upon PCR performance.

Methods

The cohort

Briefly, as part of ORChID, families expecting a healthy term baby were recruited antenatally at either the publically funded Royal Brisbane and Women's Hospital or the North West Private Hospital, in Brisbane, Australia, a subtropical city of more than 2 million inhabitants [22].

Ethics statement

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. Parents/caregivers of each baby provided written, informed consent at the time of enrolment into the study.

Sample collection

Parents were asked to record from birth a daily symptom diary and to collect anterior nasal swab samples every week until their infant's second birthday. Instructions on sample collection were provided at the initial visit by research staff who also demonstrated the technique by undertaking the initial nasal swab specimen shortly after delivery of the newborn baby. In addition, parents were given written instructions on how to collect nasal swab specimens. They also received regular text messages, emails or telephone calls as means of research staff keeping in contact with participating families. Regular supplies of sterile rayon swabs (Virocult, MW950, Medical Wire & Equipment, England) were provided, which were rotated against the internal anterior walls of both nostrils and then placed in the provided transport tube that contained a viral transport media-soaked foam pad in the base. Parents were instructed to squeeze the foam pad to release the fluid and bathe the top of the swab. Ideally within 24-hours of collection, the nasal swabs were then sent by

regular postal mail (in accordance with Australia Post regulations [23]) at ambient temperature to our research laboratory where they were stored at −80°C until analysis.

DNA extraction and quality control measures

Nasal swabs were vortexed in 2 mL of phosphate buffered saline from which 200 μL was spiked with 5 μL of equine herpes virus-1 (EHV1) culture supernatant, which served as an extraction and inhibition control agent, before nucleic acid was extracted using the CAS1820 XtractorGene automated system (Qiagen-Australia) according to the manufacturer's instructions. The final volumes of specimen extracts were 150 μL/specimen eluted in 96 well racks (Matrix, Thermo Scientific, Australia). For each run (96 extracts/run), extracts were tested using a duplex real-time PCR assay for EHV1 and ERV3 in the following reaction compositions; 10pmoles of each primer, 4pmoles of each probe (Table 1), 10 μL of SensiMix II Probe PCR Mix (Bioline, Australia) and 2 μL of extract in a 20 μL final reaction. Cycling conditions used for amplification were: initial hold at 10 min at 95°C; followed by 45 cycles of 30 sec at 95°C and 60 sec at 60°C. The EHV1 component was performed as an extraction and inhibitor control as described previously [24], while ERV3 was used as a marker to evaluate the quality of nasal swab sample collection [25]. Briefly, the samples were considered to have failed the EHV1 component (ie. failed extraction or possessed PCR inhibitors) if the EHV1 real-time PCR cycle threshold (Ct) results for individual samples were more than two standard deviations from the mean value of all samples, which for this study was calculated to be approximately 30 cycles [24].

Respiratory virus screening:

Samples that passed EHV1 DNA extraction quality control testing were screened for respiratory viruses using previously optimized and described PCR and reverse transcriptase PCR assays. Virus testing assays included: rhinovirus (RV) [26], influenza viruses (A and B) [27], respiratory syncytial viruses (A and B) [28], parainfluenza viruses (1–3) [29], human adenoviruses [22], human metapneumovirus [30], human coronaviruses (OC43, HKU1, 229E, and NL63) [31,32], human bocavirus [33] and human polyomaviruses (WUPyV and KIPyV) [34]. For all viruses, except RV, samples were tested in a 10 × 10 pooled format. Briefly, aliquots of the sample extracts were pooled using the CAS-1200 liquid handling system (Qiagen-Australia) and pools tested for the presence of respiratory viruses. For positive pools, individual sample extracts were then tested to confirm positivity. RV screening was performed on individual sample extracts, and not on the pooled extracts, as the number of expected positive samples was considered too high for there to be any benefits from pooling.

Table 1 Oligonucleotide primers for equine herpes virus-1 (EHV 1) and endogenous retrovirus 3 (ERV3) used for samples quality control

Name	Sequence	Reference
EHV1-F	GATGACACTAGCGACTTCGA	[24]
EHV1-R	CAGGGCAGAAACCATAGACA	
EHV1-TM	Quasar-670-TTTCGCGTGCCTCCTCCAG-bhq2	
ERV3-F	CATGGGAAGCAAGGGAACTAATG	[25]
ERV3-R	CCCAGCGAGCAATACAGAATTT	
ERV3-TM	Fam-TCTTCCCTCGAACCTGCACCATCAAGTCA-bhq1	

Sequences are listed 5` to 3`.

Fungal testing

During the initial phases of the study, mould was observed growing on a small number of nasal swabs at the time of their arrival at the Laboratory. In light of this observation, before extraction all swabs were inspected visually for mould and were assigned a semi-qualitative score according to a sliding scale (0 to 3), whereby 0 = no mould observed, 1 = low, 2 = medium, and 3 = high levels of visible mould present. DNA sequencing was used to identify the type of fungi present on a subset of swabs exhibiting varying degrees of visible mould growth (10 swabs where no mould was seen, and 20 each where low, medium and high levels, respectively, of mould contamination was present).

PCR amplification of a fungal internal transcribed spacer (ITS) region was performed using 10 pmoles of forward and reverse primers (ITS1 forward primer TCCGTAGGT GAACCTGCGG and ITS4-reverse primer TCCTCCGC TTA TTGATATGC [35], 25 μL of Qiagen SYBR master mix (Qiagen, Australia) and 5 μL of template in a total 50 μL reaction mix. Cycling was performed using the following conditions: 95°C for 15 min, 45 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 60 sec and a melting step of 60-95°C at the end of the thermal cycling. PCR products were examined by gel electrophoresis using a 2% agarose gel and sent to the Australian Genome Research Facility (The University of Queensland, Brisbane) for automated sequencing.

Exclusion criteria

For this study, samples that failed EHV1 criteria or were not inspected for mould growth were excluded from the analysis (Figure 1).

Data analysis

The association between variables of interest and binary outcomes was investigated using mixed effects logistic regression models, with participants included as a random intercept to account for the possibly correlated outcomes within each infant. The association with continuous

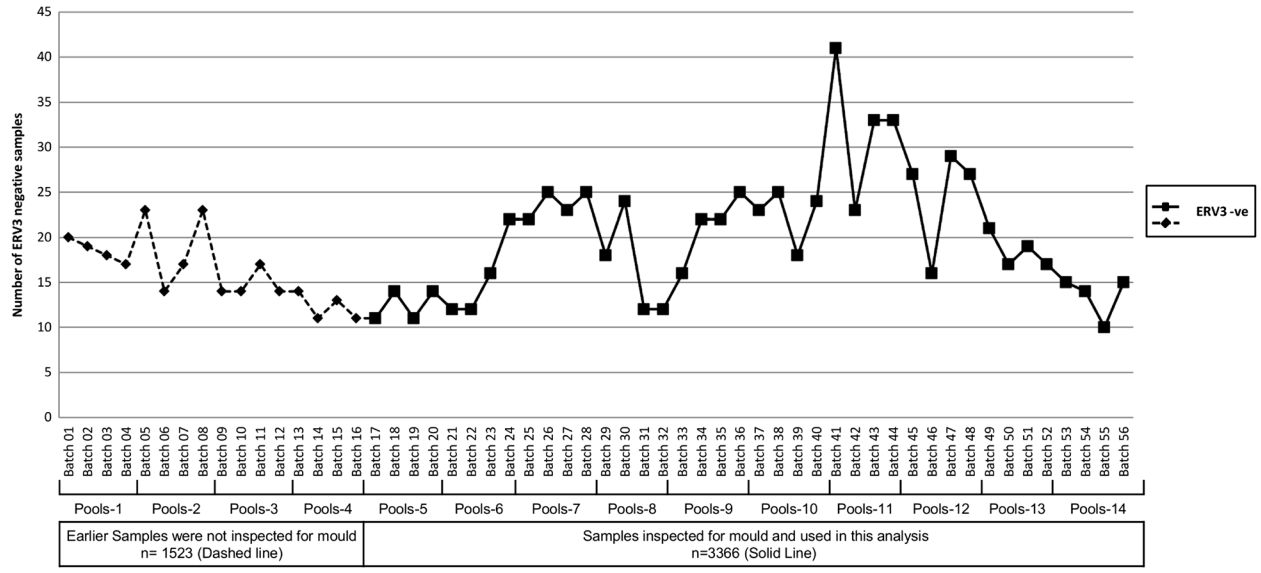

Figure 1 Number of samples negative for ERV3 during screening of each sample extraction batch (92 samples per batch). A total of 56 extraction batches were performed in the first 20-months of the study. Each batch was tested for quality control (EHV1/ERV3 PCR), following which every four batches were pooled and pools were screened for respiratory viruses other than rhinovirus. The batches used in the analysis are indicated by the solid line.

outcomes was investigated using mixed effects linear regression. When examining the association of mould level with sample quality and respiratory virus detection we conducted both univariate and multivariate analyses, with multivariate analyses adjusting for the potential confounders of the child's age, gender, relationship of collector to participant (e.g. father, mother or others), season specimen collected, and time from specimen collection to being frozen in the laboratory. Analyses were conducted using Stata statistical software v.11.0 (StataCorp, College Station, TX, USA).

Results

Swab samples

Between September 2010 and July 2012, 152 infants were recruited into the study. All participants lived within the greater Brisbane metropolitan area and none were from rural communities. One-hundred and twenty-five recruits remained active study participants up until the date of this analysis. Of the 27 withdrawals, four had moved out of the study area, two others were later deemed ineligible, ten withdrew for personal reasons and eleven were ineligible because they could not fulfill sampling requirements. For the active families, swab return rates were >90% for almost 35,000 child-days of observation. In total, 4933 weekly nasal swab specimens (~510 nasal swabs/month) were batched in 56 (96 well) racks, extracted and tested. The median time from collection to swab arrival in the laboratory was 2 (interquartile range 2–4) days; however 10.9% of swabs were received more than 7-days after their collection.

Excluded samples:

For EHV1 extraction and inhibition testing, 42 (0.81%) DNA extracts failed the EHV1 criteria. The initial 1525 samples were not inspected for mould growth during the early stages of the study and therefore were excluded from further analysis.

ERV3 detection

Of the remaining 3366 samples, there were 2718 (80.7%) samples positive for ERV3 with PCR amplification Ct values ranging from 23–45 (median 36) cycles. Overall, ERV3 was not detected in 649 (19.2%) samples. During the first 8-months of batching and screening conducted in the laboratory, the number of ERV3 negative samples ranged from 11 to 25 in each 92 extraction run with a median of 17 negative samples per run (Figure 1). However, following a cluster of samples negative for ERV3 (Figure 1; batches 41, 43, 44) we contacted parents and reminded them of the optimal swab collection technique they had been shown at enrolment of their baby. After this feedback the numbers of ERV3 negative samples declined.

Respiratory viruses detected

At least one respiratory virus was detected in 885 (26.2%) samples. Dual or multiple virus detections were observed in 105 (2.14%) samples. RV was the most

common virus detected, being present in almost 20% of specimens, followed by human bocavirus, human polyomavirus KIPyV, respiratory syncytial viruses and human adenoviruses (Table 2).

Mould

Of 3366 swab samples visually inspected for mould, 99 (2.9%) had high, 252 (7.5%) medium and 411 (12.2%) had low levels present, while 2604 swabs (77.4%) had no visible signs of mould. The mean (standard deviation) time from collection until being frozen in the laboratory for samples with no observed mould was 2.9 (3.0) days. In comparison for low level mould it was 4.9 (3.6) days (crude mean difference compared with no mould; 95% confidence interval (CI) = 1.7; 1.4 – 2.1 days), for medium level mould it was 7.4 (4.9) days (3.9; 3.4 – 4.3), and for high level mould 11.4 (10.7) days (7.1; 6.4 – 7.8). The mean difference in time from collection until being frozen between each mould group and the no mould group was statistically significant ($P < 0.001$ for each comparison). A significant association was also observed between mould and season. In specimens collected in summer, mould was observed on 28.2% of swabs. In comparison mould detection rates were 31.0% in spring (crude odds ratio (OR); 95% CI = 1.08; 0.87 – 1.34), 15.8% in autumn (0.47; 0.37 – 0.59) and 13.7% in winter (0.40; 0.29 – 0.53). When considering samples that contained mould, there was no statistically significant association between season and level of mould.

Fungal identification was achieved for 48 of 70 swabs subjected to PCR and DNA sequencing (Table 3). A diverse range of species was observed with *Epicoccum nigrum* and *Cladosporium cladosporioides* the most prevalent.

ERV3, visible mould and respiratory virus detection

Of the 2718 samples that were ERV3 positive, 810 (37.2%) had at least one respiratory virus detected by PCR. In contrast, the respiratory virus detection rate in ERV3 negative samples was significantly lower (75/649, 11.5%; crude odds ratio (OR) = 0.35; 95% CI 0.27-0.44) when ERV3 was absent in swab specimens. We also observed that among ERV3 positive swabs, the average ERV3 Ct value for samples positive for any respiratory virus (32.8 cycles) was significantly lower (indicating greater ERV3 load) than the average Ct value (35.4) in samples negative for all viruses (crude difference = 2.0, 95% CI 1.4 – 2.6; Figure 2). Moreover, there was a significant difference in ERV3 Ct values ($P = 0.001$) in samples that

Table 2 Results for respiratory viruses screening from 3366 parent collected nasal swab specimens between July 2011 and July 2012 and fulfilling the EHV1 criteria

Virus	No. of infants	No. of samples	% of all samples
RV	105	726	21.57
HBoV	26	46	1.37
KIPyV	17	41	1.22
HAdV	23	30	0.89
RSV-A	26	30	0.89
WUPyV	13	28	0.83
HCoV NL63	12	16	0.48
IV-B	11	11	0.33
HCoV 229E	3	6	0.18
PIV 1	6	6	0.18
HMPV	5	5	0.15
PIV3	3	3	0.09
HCoV HKU1	3	3	0.09
IV-A	2	2	0.06
RSV B	2	2	0.06
HCoV OC43	1	1	0.03
PIV 2	0	0	0

HAdV, Human adenovirus; HBoV, Human bocavirus; HCoV, Human coronavirus; HMPV, Human metapneumovirus; IV, Influenza virus; PIV, Parainfluenza virus; PyV, Polyomavirus; RSV, Respiratory syncytial virus, RV, Rhinovirus.

Table 3 Species detected in 70 samples with different levels of fungal growth

Species	Number detected	(high; medium; low; no visible mould)
Epicoccum nigrum	15	(7,2,4,2)
Cladosporium cladosporioides	7	(3,3,1,0)
Aureobasidium pullulan	4	(1,1,2,0)
Cryptococcus flavescens	3	(1,2,0,0)
Alternaria alternata	2	(1,1,0,0)
Alternaria tenuissima	1	(0,0,1,0)
Aspergillus westerdijkiae	1	(0,0,1,0)
Candida parapsilosis	1	(0,1,0,0)
Cladosporium silenes	1	(0,0,0,1)
Cladosporium tenuissimum	1	(0,1,0,0)
Cladosporium uredinicola	1	(1,0,0,0)
Cochliobolus lunatus	1	(0,1,0,0)
Curvularia brachyspora	1	(0,1,0,0)
Curvularia trifolii	1	(0,1,0,0)
Leptosphaerulina australis	1	(0,1,0,0)
Paraphaeosphaeria sp	1	(1,0,0,0)
Penicillium fellutanum	1	(0,0,1,0)
Penicillium oxalicum	1	(0,1,0,0)
Penicillium polonicum	1	(0,0,0,1)
Penicillium spinulosum	1	(0,0,1,0)
Phoma herbarum	1	(0,0,1,0)
Rhodotorula slooffiae	1	(0,1,0,0)
Total	**48**	

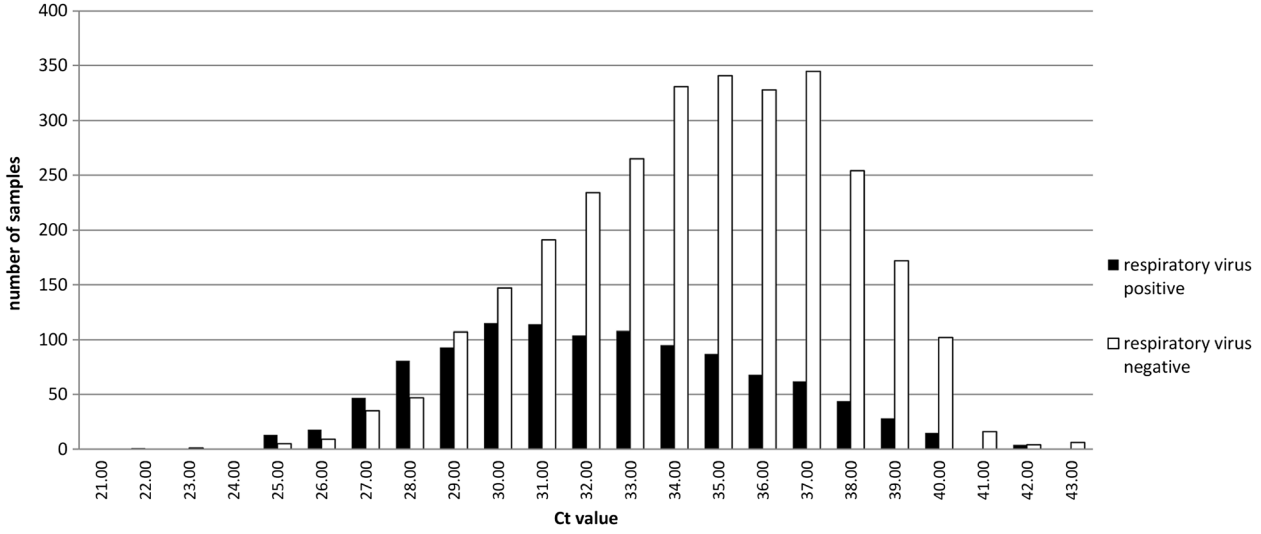

Figure 2 Comparison between average ERV3 cycle threshold (Ct) values in respiratory virus positive (dark bars) versus negative (light bars) samples. In ERV3-positive samples, the average ERV3-Ct values (32.8) in samples positive for any virus was significantly lower than the average ERV3-Ct values (35.4) in samples negative for all viruses (difference = 2.6, 95% confidence interval 2.3-2.9).

had single respiratory virus detection (average = 33.01) comparing with samples that had multiple respiratory viruses detection (average = 31.27).

Of the 762 samples with visible mould, 529 (69.4%) were positive for ERV3, which was significantly lower than rates in samples without visible mould (84.0%; crude OR = 0.35, 95% CI 0.28-0.43). The proportion of samples with visible mould and positive respiratory virus testing (178/762; 23.4%) was significantly lower than that of samples without mould (707/2606; 27.1%; crude OR = 0.70, 95% CI 0.57-0.86).

Table 4 examines the association between ERV3 and respiratory virus detection and potential explanatory and confounding variables. ERV3 positive sample rates increased with age, varied by season and declined with increasing mould levels and time taken for samples to reach the laboratory and to be frozen. Similarly, respiratory virus detection rates increased with age, specimen collection outside the summer months, and time taken to reach the laboratory, while decreasing as visible mould levels in samples reaching the laboratory increased.

Discussion

The ORChID project is an ongoing comprehensive community-based study using PCR assays to detect respiratory viruses in anterior nasal swab specimens taken weekly by parents from their infants throughout the first 2-years of life. This requires parents following a standardized protocol of obtaining swabs regularly and mailing them promptly to our laboratory. However, we have observed that suboptimal sample collection as determined by ERV3 detection and presence of visible mould in swab samples reaching the laboratory can negatively affect sample quality and potentially respiratory virus detection.

The data from the first 20-months of our longitudinal study indicate that respiratory virus detection is associated with the ERV3 load in nasal swab specimens. Swabs negative for ERV3, presumably from sub-optimal collection, had reduced respiratory virus detection rates compared with samples containing ERV3. Furthermore, in those specimens positive for ERV3, a higher ERV3 load was associated with a higher likelihood of respiratory virus detection. Overall, this shows the importance of measuring human DNA as a marker for epithelial cells in swab samples, which if tested and monitored in real time during the study, can identify problems associated with collection that can be addressed quickly. This is illustrated in the current study when a sudden increase in ERV3 negative samples was observed. Parents were contacted and reminded about sample collection protocols following which there was a decline in ERV3 negative sample rates towards baseline levels.

We were also concerned at finding mould on some samples, which occurred despite the commercial swab tubes containing antifungal agents. Most fungal species identified in the swabs were saprophytic, and the most common fungus found, *Epicoccum nigrum*, is a known contaminant of clinical specimens [36]. The relationship between fungal airspora counts and meteorological conditions is complex and impacts at the species level [37]. In Brisbane, *Cladosporium and Alternaria* airspora are detected commonly throughout the year, but as with *Epicoccum*,sp their levels peak during the warmer, humid months. Other factors, such as rainfall and wind speed,

Table 4 ERV3 and respiratory virus positive samples detected by polymerase chain reaction assays in 3366 parent collected nasal swab specimens

Variable		No. samples (%)	ERV3 Positive			Respiratory virus positive		
			No. samples (%)	Univariate OR (95% CI); P value	*Multivariate OR (95%); P value	No. samples (%)	Univariate OR (95% CI); P value	*Multivariate OR (95%); P value
Age (months)	***< 6***	1293 (38.4%)	995 (77.0)	1	1	208 (16.1)	1	1
	6- <12	1295 (38.5%)	1061 (81.9)	1.20 (0.94-1.53); 0.15	1.28 (0.98-1.68); 0.07	411 (31.7)	2.59 (2.07-3.24); <0.001	2.38 (1.89-3.01); <0.001
	≥12	778 (23.1%)	662 (85.1)	1.49 (1.06-2.10); 0.02	1.93 (1.27-2.93); 0.002	266 (34.2)	2.98 (2.26-3.92); <0.001	2.16 (1.57-2.99); <0.001
Gender	***Male***	1647 (48.9%)	1335 (81.1)	1	1	461 (28.1)	1	1
	Female	1719 (51.06%)	1383 (80.4)	0.81 (0.54-1.22); 0.32	0.87 (0.58-1.29); 0.48	424 (24.7)	0.82 (0.60-1.12); 0.21	0.83 (0.61-1.12); 0.23
Collector	***Mother***	2845 (84.5%)	2307 (81.1)	1	1	766 (26.9)	1	1
	Father	441 (13.1%)	342 (77.6)	0.91 (0.66-1.27); 0.60	0.87 (0.62-1.22); 0.42	109 (24.7)	0.94 (0.70-1.26); 0.67	0.88 (0.65-1.19); 0.41
	Research staff	45 (1.3%)	40 (88.9)	2.71 (1.00-7.36); 0.05	1.76 (0.65-4.81); 0.27	3 (6.7)	0.24 (0.07-0.79); 0.02	0.36 (0.11-1.21); 0.10
	Other	35 (1.0%)	29 (82.9)	1.31 (0.49-3.51); 0.59	1.39 (0.46-4.16); 0.56	7 (20.0)	0.72 (0.30-1.74); 0.47	0.87 (0.35-2.13); 0.76
Season	***Summer***	926 (27.5%)	729 (78.7)	1	1	178 (19.2)	1	1
	Autumn	1059 (31.5%)	802 (75.7)	0.90 (0.71-1.13); 0.37	0.74 (0.58-0.96); 0.02	304 (28.7)	1.99 (1.59-2.49); <0.001	1.74 (1.38-2.20); <0.001
	Winter	541 (16.1%)	482 (89.1)	2.63 (1.87-3.70); <0.001	2.41 (1.67-3.49); <0.001	198 (36.6)	3.06 (2.36-3.97); <0.001	2.63 (2.01-3.45); <0.001
	Spring	840 (25.0%)	705 (83.9)	1.39 (1.07-1.79); 0.01	1.50 (1.13-1.99); 0.005	205 (24.4)	1.27 (1.00-1.61); 0.05	1.43 (1.11-1.84); 0.005
Mould	***None***	2604 (77.4%)	2189 (84.1)	1	1	707 (27.2)	1	1
	Low	411 (12.2%)	308 (74.9)	0.47 (0.36-0.62); <0.001	0.69 (0.52-0.93); 0.01	97 (23.6)	0.73 (0.56-0.95); 0.02	0.81 (0.61-1.07); 0.14
	Medium	252 (7.5%)	163 (64.7)	0.27 (0.20-0.37); <0.001	0.47 (0.33-0.66); <0.001	60 (23.8)	0.70 (0.50-0.96); 0.03	0.70 (0.49-0.99); 0.05
	High	99 (2.9%)	58 (58.6)	0.20 (0.13-0.33); <0.001	0.40 (0.24-0.66); <0.001	21 (21.2)	0.57 (0.34-0.96); 0.04	0.53 (0.31-0.93); 0.03
Time to reach Laboratory (days)	***0-3***	2281 (67.8%)	1983 (86.9)	1	1	587 (25.7)	1	1
	4-7	723 (21.5%)	513 (71.0)	0.32 (0.25-0.40); <0.001	0.39 (0.30-0.50); <0.001	187 (25.9)	0.96 (0.78-1.18); 0.69	1.03 (0.82-1.29);0.80
	>7	362 (10.8%)	222 (61.3)	0.17 (0.13-0.24); <0.001	0.24 (0.17-0.34); <0.001	111 (30.7)	1.16 (0.89-1.52); 0.28	1.42 (1.05-1.94); 0.02

*Adjusted for all variables in the Table.

can also influence fungal airspora composition [37,38]. In our study, mould was associated mainly with longer time intervals between taking swabs and their arrival at the laboratory. However, this was especially evident during the warm, humid spring and summer months, which leads us to speculate that fungal contamination occurred during sample collection and was influenced by the aforementioned environmental factors. Unfortunately, we could not explore this further as it was beyond the scope of the present study. In addition, while mould growth proved to be an issue in the subtropical climate of Brisbane, this may be less of a problem in more temperate climates with lower temperatures and humidity levels.

We now remind parents regularly to mail swabs promptly after collection. Of interest however, was that respiratory virus detection rates were not affected by prolonged transport times, but in fact appeared to increase with time taken to reach the laboratory. While the observed increase was unexpected and may have occurred simply by chance, it is plausible that viral nucleic acids were protected to some extent by being encapsulated within the viral capsid, and by using viral transport medium in the swabs.

Fungi were found to be associated with both reduced ERV3 detection and, at high levels, reduced significantly respiratory virus detection. At least three points emerge from this study. First, although swabs may contain antimicrobial agents, the risk of fungal and potentially bacterial contamination may still arise. Second, the times between swab collection and laboratory arrival should be monitored and feedback provided if delays occur. Finally, if delays are expected swabs should be placed in the household refrigerator until mailed to the laboratory [20].

Conclusion

We found that ERV3 as a marker for human DNA and epithelial cells was also an important indicator of sample quality for our study. For community-based investigations similar to our own, real-time sample processing and ERV3 detection can facilitate rapid interventions to maintain sample quality and to optimize respiratory virus detection. Indeed, this may have broader implications since nasal swabs are beginning to replace the traditional, but more invasive nasopharyngeal swab or aspirate sampling techniques in hospitals and clinics, especially following the 2009 influenza pandemic [17]. Thus, similar ERV3 testing strategies could be used by diagnostic laboratories to improve or monitor sample collection quality for optimal respiratory virus detection. Finally, the potential problem of visible mould contamination of swabs taken during community-based studies can be minimized by ensuring samples are transported promptly to the laboratory.

Abbreviations

ARI: Acute respiratory infection; Ct: Cycle threshold; DNA: Deoxyribonucleic acid; EHV1: Equine herpes virus 1; ERV3: Endogenous retrovirus 3; ITS: Internal transcribed spacer; OR: Odds ratio; ORChID: Observational research in childhood infectious diseases; PCR: Polymerase chain reaction; PyV: Polyomaviruses; RV: Rhinovirus.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

Conceived and designed the experiments: AA, DW, SB, SL, TS, KG. Performed the experiments: AA, DW, SB. Analyzed the data: RW, AA, SL. Contributed reagents/materials/analysis tools: AA, DW, SB, SL, TS, KG. Wrote the manuscript: AA, DW, SL, KG. Approved the final manuscript: AA, DW, SB, SL, RW, MN, TS, KG. All authors read and approved the final manuscript.

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A Retrospective Performance Evaluation of an Adenovirus Real-Time PCR Assay

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Human adenoviruses (AdVs) cause a wide range of diseases. To date, there are at least 60 known human AdV types and, as these exhibit high levels of genetic variation this could impact potentially upon their detection by polymerase chain reaction (PCR)-based technology. Here, the sensitivity of a pan-AdV real-time PCR assay (AdV-PCR) used widely for testing clinical samples was determined retrospectively. An in silico analysis was performed initially using the 370 AdV sequences available on the Genbank database. To investigate for potential false-negative results, two additional AdV-PCR assays were used to re-evaluate 779 respiratory samples submitted for virus testing and 1,012 nasal swab samples collected as part of an ongoing community-based study. The results were then compared to those obtained by AdV-PCR. In silico analysis showed the presence of mismatches in the AdV-PCR primers and probe for most AdV sequences available on Genbank. Notably, 215 of the 370 (58%) sequences had at least three mismatches with the AdV-PCR forward primer. Of the 779 clinical samples, 88 were identified as AdV-positive, of which 84 were positive by the AdV-PCR. The four samples providing false-negative results in the AdV-PCR had high cycle threshold values in the other methods suggesting that sampling at low load, rather than sequence variation, was responsible for the negative results. No false-negative AdV-PCR results were observed for the community-based study samples. Reassuringly, the results show that despite the high level of sequence variation in the AdV-PCR assay oligonucleotide targets, the assay remains suitable for routine detection of human AdV strains. ***J. Med. Virol. 86:795–801, 2014***.

KEY WORDS: diagnosis; mutation; DNA; primer; sequence variation

INTRODUCTION

Human adenoviruses (AdVs; Genus *Mastadenovirus*, Family *Adenoviridae*), which were first isolated from the respiratory tract by tissue culture [Rowe et al., 1953], are known to cause a wide range of human diseases, including respiratory, gastrointestinal, ocular, urinary, and central nervous system infections [Lynch et al., 2011]. Although AdV infections are typically acute, self-limiting and not fatal, they may have severe consequences for both immunocompromised patients [Zahradnik et al., 1980; La Rosa et al., 2001] and on rare occasions previously healthy infants [Mistchenko et al., 1998; Henquell et al., 2009]. AdVs can also cause localized epidemics in small and crowed populations, such as schools and military facilities [Caldwell et al., 1974; Yu et al., 2013].

Nucleic acid amplification tests (NAATs), such as polymerase chain reaction (PCR) assays, provide high levels of sensitivity and specificity compared with cell culture and serology. With reduced turnaround time, NAATs are also superior to other viral detection methods for routine applications and in large epidemiological studies. PCR has been used for AdV detection in clinical samples since the 1990s [Allard et al., 1990; Pring-Akerblom and Adrian, 1994; Echavarria et al., 1999]. However, designing a diagnostic PCR assay that is comprehensive for all human AdV types can be problematic. The human AdVs are subgrouped into seven species (A to G) that include

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at least 60 different types of human AdVs [Robinson et al., 2011; Matsushima et al., 2013]. Notably, there are considerable genetic differences between AdV types, and these differences are likely to impact upon the performance of diagnostic PCR assays, relying as they do upon highly conserved target sequences.

In 2003, a real-time PCR method was first developed for pan-AdV detection [Heim et al., 2003]. Since then, this AdV-PCR assay has been used widely by many laboratories for routine detection of AdV infections [Lambert et al., 2008; Kwofie et al., 2012; Tsou et al., 2012; Ayoub et al., 2013]. The original description of the method considered the extent of AdV genetic variation known at the time, and importantly, showed that the assay was able to detect a broad range of AdV types despite known mismatches [Heim et al., 2003]. However, in view of the negative impact that genetic variation may have upon PCR assay performance [Whiley and Sloots, 2005, 2006], concerns were raised about potential false-negative results arising from the appearance of new AdV types or variants. These concerns are reinforced by the more recent publication of newer pan-AdV PCR methods [Bil-Lula et al., 2012; Alkhalaf et al., 2013]. It was for these reasons and as part of standardizing PCR assays for a large longitudinal, community-based study of respiratory viruses in young children [Lambert et al., 2012] that a retrospective performance evaluation of the AdV-PCR assay was conducted. An in silico analysis of recent AdV sequences data was also undertaken to determine sequence target stability. As further validation, two additional adenovirus PCR assays were then used to retest respiratory samples collected for clinical purposes.

METHODS

Sequence Analysis of AdV-PCR Oligonucleotides

To investigate the extent of sequence variation in the AdV-PCR primer and probe targets, AdV hexon gene sequences from the Genbank database were downloaded and analyzed. Briefly, these comprised representative sequences of all 68 known human AdV types, excluding types 57 and 60 for which sequence data for the AdV-PCR targets were not available. Sequences were aligned using BioEdit software (version 7.0.4.1) and the number and location of mismatches in each oligonucleotide target identified (Figs. 1–3).

Assays Design

Based on the above sequence data, two additional assays (defined as Mod1-PCR and Mod2-PCR) were designed. For Mod1-PCR assay, identical sequence targets as the AdV-PCR were used, except that degenerate bases were incorporated at appropriate positions to accommodate sequence mismatches; three forward primers, three reverse primers and two probes with degenerate bases in various positions were designed subsequently (Table I). The Mod2-PCR assay was designed with the aid of Primer Express (Applied Biosystems, version 2.0; Foster City, CA) and targeted sequences flanking those of the above AdV-PCR and Mod1-PCR methods. Similar to the Mod1-PCR, multiple oligonucleotides with degenerate bases in various positions were used to accommodate the observed sequence variation; two forward primers, two reverse primers, and one probe (Table I).

Real-Time PCR Reaction Mix and Cycling Conditions

The real-time PCR reactions for all three assays were performed using the Qiagen Quantitect Probe PCR kit (Qiagen; Doncaster, Australia). The reaction mixes consisted of a total reaction volume of 20.0 μl, including 10.0 μl of Qiagen Quantitect Probe mix (Qiagen; Doncaster, Australia) and 2.0 μl of sample extract or control. For the AdV-PCR, 8.0 pmoles of the previously described forward and reverse primers and 2.0 pmoles of probe were used [Heim et al., 2003]. For

TABLE I. Oligonucleotides Used for the Mod1-PCR and Mod2-PCR Methods

Designation	Oligonucleotide sequence (5′–3′)	Position[a]
Mod1-PCR assay		
Mod1-P1	TCGG**R**GTACCT**S**AG**T**CCGGGTCTGGTGCA	18401–18430
Mod1-P2	TCGGAGTACCTGAG**C**CC**S**GG**K**CTGGTGCA	
Mod1-F1	GCC**S**CA**R**TGGGCATACATGCACATC	18362–18387
Mod1-F2	GCC**G**CAGTGG**K**C**K**TACATGCACATC	
Mod1-F3	GCC**C**CAGTGG**K**C**K**TACATGCACATC	
Mod1-R1	GCCACTGTGGGGTTTCTAAA**Y**TT	18471–18494
Mod1-R2	GCCAC**S**GTGGGGTT**Y**CTAAACTT	
Mod1-R3	GC**T**ACGGT**R**GG**A**TTTCTAAACTT	
Mod2-PCR assay		
Mod2-P1	CTGGTGCAGTT**Y**GCCCG**Y**GC**M**AC	18422–18446
Mod2-F1	ACATGCACATCTCGGGCCAGGA	18376–18398
Mod2-F2	ACATGCACATC**GC****C**GG**R**CAGGA	
Mod2-R1	CGGTC**S**GTGGTCACATC**R**TGGGT	18498–18521
Mod2-R2	CGGTCGGTGGTCAC**R**TCGTG**S**GT	

[a]Position according to adenovirus-D type 16, Genbank accession number JN860680.

the Mod1-PCR, 2.6 pmoles each of the three forward primers (mod1-F1, F2 and F3; Table I), 2.6 pmoles each of the three reverse primers (mod1-R1, R2, and R3; Table I), and 2.0 pmoles of each probe (Mod1-P1 and P2; Table I) were used. Similarly, the Mod2-PCR, comprised 4.0 pmoles each of the two forward primers (mod2-F1 and F2; Table I), 4.0 pmoles each of the two reverse primers (mod2-R1 and R2; Table I), and 2.0 pmoles of probe (Mod2-P1; Table I). All three methods were cycled on the Rotorgene-Q (Qiagen; Doncaster, Australia). Cycling was performed under the following conditions: initial activation at 95°C for 15 min and 45 cycles of 95°C for 15 sec and 60°C for 1 min.

Control Isolates

The performance of the three PCR methods was assessed initially using eight AdV isolates of various types (Table II). Briefly, 10-fold dilutions of DNA extracted from each isolate were tested in all three methods and the detection limits and associated cycle threshold value data compared.

Clinical Performance

The performance of the AdV-PCR was retrospectively investigated by retesting clinical samples using both the Mod1-PCR and Mod2-PCR assays. All respiratory specimens were provided by Pathology Queensland, Royal Brisbane and Women's Hospital (Brisbane, Queensland) and collected during the year 2012. These included 79 samples that had tested positive by the AdV-PCR assay previously at Pathology Queensland as well as an additional 700 de-identified respiratory specimens. The latter 700 samples were pooled (10 samples per pool) for testing. All samples from pools providing positive results by any method were then tested individually by all three assays (AdV-PCR, Mod1-PCR, and Mod2-PCR) in parallel and the results compared.

Community-Based Respiratory Samples

Assay performance was also investigated using 1,012 nasal swab samples collected between September 2010 to April 2012 as part of a longitudinal, community-based study of respiratory viruses in young children [Lambert et al., 2012]. Briefly, samples were pooled and tested as per above, except that only the AdV-PCR and Mod1-PCR assays were used, the Bioline SensiMix II Probe Kit (Bioline; Sydney, Australia) was used instead of the Qiagen Quantitect Probe mix and cycling was performed on an ABI7500 (Applied Biosystems; Melbourne, Australia). The decision not to use the Mod2-PCR assay for this testing was based on the similar results observed for the Mod1-PCR and Mod2-PCR methods when used to test the above control isolates and clinical samples.

AdV-PCR Sequence Target Analysis

DNA sequencing was used to investigate sequence variation in the AdV-PCR targets in all control isolates, as well as clinical samples providing discrepancies between the three PCR methods. Briefly, a sequence of 489 bases (based on AdV-E type 4; genbank accession number EF371058) was amplified by PCR using primers flanking the above assays (ACTCTGAACAG-CATCGTGGGT, this study; and CAGCACGCCGCGG ATGTCAAAGT; Allard et al., 1990), and submitted for DNA sequencing to the Australian Genome Research Facility (The University of Queensland, Brisbane, Australia). AdV genotyping was also performed on the discrepant clinical samples, based on the high variable region 7 (HVR7) in the hexon gene as described previously [Sarantis et al., 2004].

Additional AdV Genotyping

To further investigate circulating AdV genotypes in the local population, 20 randomly-selected AdV-positive clinical samples (from the 2012 sample set above) were also subject to AdV genotyping.

RESULTS

Sequence Analysis of AdV-PCR Oligonucleotide Targets

Overall, 334 (90%) of the 370 AdV hexon gene sequences available on the Genbank database had at least two mismatches with the AdV-PCR forward primer and 215 (58%) sequences had at least three mismatches (Fig. 1). Likewise, 254 (69%) of the sequences had at least two mismatches with the AdV-PCR reverse primer (Fig. 2). The AdV-PCR probe was the most conserved with 304 (82%) of sequences having either one or no mismatches (Fig. 3). In general, the highest level of variation was observed amongst the AdV B species; B1 and B2 (data not shown). This variation involved predominantly the forward primer, with three mismatches observed typically.

Control Isolates

Testing 10-fold dilutions of the control isolates revealed similar detection limits between the three assays (within 1- to 10-fold dilution) for all isolates (data not shown). However, for some isolates, significant differences were observed between assays with respect to cycle threshold (Ct) values (Table II). Notably, isolates 1 and 2 (AdV types 34 and 11 respectively; both B species) showed significantly lower C_t values in the Mod1-PCR and Mod2-PCR assays by 10 and 5 cycles, respectively compared with the AdV-PCR (Table II). In contrast, C_t values for all other control isolates (3 C, 1 E, 1 F, and 1 B species) differed by only one to two cycles between assays.

TABLE II. Cycle Threshold Values for Adenovirus (AdV) Eight Control Isolates and 13 Selected Positive Clinical Samples When Tested by the AdV-PCR, Mod1-PCR, and Mod2-PCR Assays

		C_t values			AdVPCR Oligonucleotides		
Sample info.	AdV type (species)	AdVPCR	Mod1PCR	Mod2PCR	FPrimer (5′–3′) GCCCCAGTGGTCT TACATGCACAT	Probe (5′–3′) TCGGAGTACCTGAGCC CGGGTCTGGTGCA	RPrimer (5′–3′) GCCACGGTGGGGT TTCTAAACTT
Control isolates (2000)							
1	34 (B2)	26.72	16.21	15.24	......A...G.A..........	.............T..............	..T.....A..A..........
2	11 (B1)	21.04	16.2	15.22	......A...G.A..........	.............T..............	..T........A...A.......
3	04 (E)	21.81	22.55	20.43	..........G.G..........	.............T..............	.....C.........C........
4	14 (B2)	20.29	20.51	19.22	..........G.A..........	.............T..............	..T........A..........
5	40 (F)	17.51	18.35	18.78	...G..A................	....................C.......	..................G.....
6	06 (C)	21.25	21.69	20.88	...G...................	................C..G........	.....C.................
7	02 (C)	25.24	25.51	24.94	...G...................	................C..G........	.....C.................
8	05 (C)	20.63	20.78	20.56	...G...................	................C..G........	.....C.................
Clinical samples (2012)							
1	03 (B1)[a]	24.19	20.2	18.39	......A...G.A..........	.............T..............	.....T..............T..
2	03 (B1)[a]	16.99	12.17	11.65	......A...G.A..........	.............T..............	.....T..............T..
3	n/a	38.09	nd	nd	n/a	n/a	n/a
4	n/a	35.41	nd	33.47	n/a	n/a	n/a
5	n/a	35.35	37.16	nd	n/a	n/a	n/a
6	02 (C)	36.33	36.7	nd	n/a	n/a	n/a
7	02 (C)[a]	nd	38.52	33.95	n/a	n/a	n/a
8	02 (C)[a]	nd	36.23	32.6	n/a	n/a	n/a
9	01 (C)[a]	nd	38.9	35.57	n/a	n/a	n/a
10	n/a	nd	37.97	35.49	n/a	n/a	n/a
11	03 (B1)[a]	19.29	13.51	12.88	......A...G.A..........	.............T..............	.....T..............T..
12	n/a	41.69	34.76	33.05	n/a	n/a	n/a
13	03 (B1)[a]	41.91	33.49	32.75	n/a	n/a	n/a

Sequence alignment of the Adv-PCR oligonucleotides with target sequences are also provided where available. Dots indicate matching bases. nd, not detected; n/a, not available.
[a]Based on genotyping of the high variable region 7 in the AdV hexon gene.

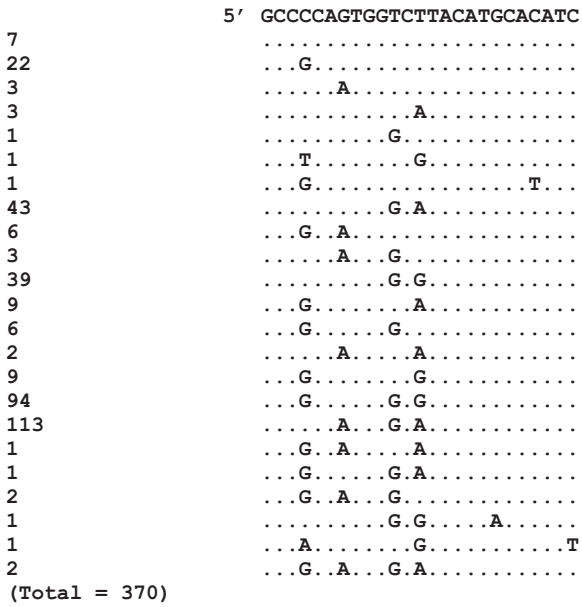

Fig. 1. Sequence alignment of the Heim forward primer with 370 adenovirus sequences from the Genbank database. Dots indicate a match whereas capitalized bases indicate mismatches with the primer sequence.

DNA sequencing of the AdV-PCR targets for the control isolates (Table II) revealed mismatches for primer and probe targets for all isolates. However, the most number of mismatches were observed for control isolates 1 and 2 (B species), with three mismatches present in both the forward and reverse primers for both isolates.

Clinical Samples

For the 79 AdV-positive specimens from Pathology Queensland, the three methods were positive and provided similar Ct values (i.e., <3 cycles difference between assays) for 67 samples (12–38.8 cycles in the AdV-PCR; mean of 27 cycles). A further two samples were positive by all three methods, but provided earlier Ct values (>3 cycles difference) in both the Mod1-PCR and Mod2-PCR assays (samples 1 and 2; Table II). One sample was positive by the AdV-PCR only (sample 3; Table II), one sample positive by both the AdV-PCR and Mod2-PCR assays only (sample 4; Table II), two samples were positive by AdV-PCR and Mod1-PCR only (samples 5 and 6; Table II) and there were two samples that were negative by all three methods. Of note, was that there were four additional samples negative by the AdV-PCR, but positive by both the Mod1-PCR and Mod2-PCR assays (samples 7–10; Table II). To further investigate the latter, all four samples were retested in duplicate in the AdV-PCR; two samples provided positive results in both replicates, and two samples were each positive in one of the two replicates.

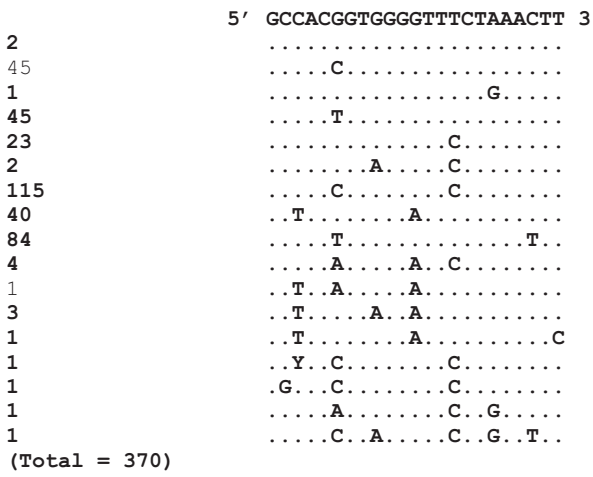

Fig. 2. Sequence alignment of the Heim reverse primer with 370 adenovirus sequences from the Genbank database. Dots indicate a match whereas capitalized bases indicate mismatches with the primer sequence.

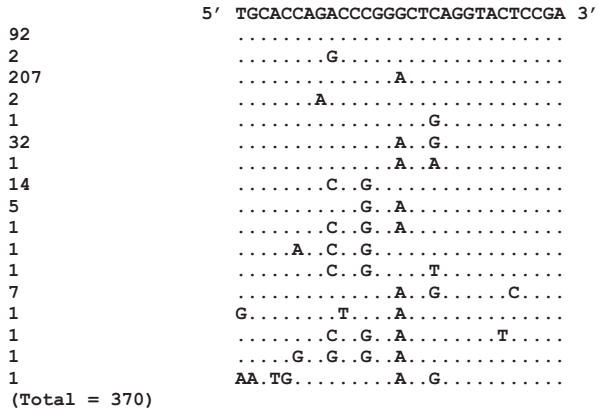

Fig. 3. Sequence alignment of the Heim probe with 370 adenovirus sequences from the Genbank database. Dots indicate a match whereas capitalized bases indicate mismatches with the probe sequence.

The additional testing of 70 respiratory pools (from 700 respiratory virus extracts) revealed 10 positive pools in all methods and an additional two pools positive by the Mod2-PCR only (total of 12 positive pools for this method). The 120 individual samples from these 12 pools were tested subsequently with all three assays and 12 samples were found positive by all three methods (i.e., one positive sample/pool). Of the 12 positive samples, nine provided similar C_t values in all three methods (23.7–34.8 cycles in the AdV-PCR; mean of 29.8 cycles). Three samples provided earlier C_t values in the Mod1-PCR and Mod2-PCR assays compared to the AdV-PCR (samples 11–13; Table II).

DNA sequencing of the AdV-PCR targets and AdV genotyping was attempted for all the clinical samples providing discrepancies (samples–13; Table II). Of these, the AdV-PCR sequence targets could only be

sequenced for three samples (samples 1, 2, and 11; Table II) being the samples with the highest viral load (based on the PCR C_t values), whereas HVR7 genotyping was successful for eight samples (Table II). Similar to the results of the control isolates, samples 1, 2, and 11 had a total of five mismatches in the primers, were members of the B1 species, and correlated with the late C_t values observed in the AdV-PCR.

Additional AdV Genotyping

HVR7 genotyping of the 20 randomly selected AdV-positive clinical samples revealed four types from three species; nine samples comprised AdV type-1 (C species), eight samples had AdV type-2 (C species), two samples typed as AdV type-3 (B species) and only one sample was typed as an AdV type-4 (E species).

Community-Based Respiratory Samples

The testing of the 1,012 community-based nasal swab samples provided near identical results in both the AdV-PCR and Mod1-PCR assays; 53 samples were positive by both methods with similar C_t values (i.e., <3 cycles difference between assays) for 51 samples with Ct values ranging from 23.1 to 41 cycles (mean of 34 cycles) in the Mod1-PCR. A further two samples were positive by both methods, but provided earlier C_t values in the Mod1-PCR (34 and 35.2 cycles, respectively) compared to the AdV-PCR (39.1 and 41.4 cycles, respectively).

DISCUSSION

The primary goal of this investigation was to determine whether the AdV-PCR remains suitable for routine defection of AdVs in clinical samples, and was instigated because of concerns over the age of the assay and the potential for false-negative results arising from new AdV types or variants [Bil-Lula et al., 2012; Matsushima et al., 2013; Alkhalaf et al., 2013]. The in silico sequence analysis showed a high level of variation in the AdV-PCR oligonucleotide targets, particularly for the primers, and is consistent with initial concerns. However, the variation observed was similar to that described originally [Heim et al., 2003]. In addition, the experimental data here showed that despite considerable variation in the AdV-PCR targets, few false-negative results were observed in the AdV-PCR. The samples that were negative by AdV-PCR, but positive by either Mod1-PCR or Mod2-PCR had typically low viral loads (as indicated by their respective C_t values; clinical samples 7–10; Table II), and so low load sampling may explain these results. This explanation is supported by observing that these samples provided positive results upon repeat testing in the AdV-PCR assay. In addition, other samples positive by AdV-PCR at high C_t values were negative by one or both of the other two methods (clinical samples 3–6; Table II).

Overall, these results suggest an unexpected tolerance of the AdV-PCR assay for mismatches, further confirming Heim et al.'s original data. The explanation for this tolerance is likely to be due to variation being largely absent from the extreme 3′ end of the primers (Figs. 1 and 2), which is the critical region for primer performance. It should however be noted that false-positive results in the AdV-PCR could otherwise explain the failure of the Mod1-PCR and Mod2-PCR assays to confirm the AdV-PCR results, particularly for sample 3 (Table II). The most logical way to explore the potential for false-positive results here would be the use of DNA sequencing. However, we did not attempt this as the real-time C_t values involved were very high, making this very difficult and prone to error. The main problem being that failure to obtain a clear AdV sequence could lead us to determine incorrectly AdV was absent, whereas a small amount of specific AdV-CR product might still be present, but mixed with the various non-specific products that occur typically towards the end of PCR cycling.

While the results indicate the AdV-PCR remains suitable for routine detection of AdVs, the data suggest that variation does have some impact upon amplification, insofar as Ct values can be delayed. For example, control isolates 1 and 2 (AdV types 34 and 11) had three mismatches in both the forward and reverse primers that led to significant delays in C_t values (5–10 cycles) compared to the Mod1-PCR or Mod2-PCR methods. Similar issues were observed for the clinical samples. While not affecting *qualitative* detection, these results do suggest however, the AdV-PCR assay would not be suitable for *quantitative* purposes and that the Mod1-PCR or Mod2-PCR assays may be more suitable for quantitative purposes.

Notwithstanding the above, consideration needs to be given to biases that may exist for Genbank sequence data as well as the limited genotypic diversity of AdVs observed in the sample population studied here. Likewise, AdV genotypes may vary over time and between geographical locations. Hence these data do not preclude the possibility that other AdV variants exist. Nonetheless, despite the high level of sequence variation in the oligonucleotide targets, these results support the ongoing use of the AdV-PCR for qualitative AdV detection.

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