Detection of respiratory syncytial virus specific antibody response in saliva of healthy Singaporeans

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NANYANG
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UNIVERSITY

**DETECTION OF RESPIRATORY SYNCYTIAL
VIRUS SPECIFIC ANTIBODY RESPONSE IN
SALIVA OF HEALTHY SINGAPOREANS**

REKHA THIAGARAJAN

SCHOOL OF BIOLOGICAL SCIENCES

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REKHA THIAGARAJAN

SCHOOL OF BIOLOGICAL SCIENCES

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Publication declaration

**Part of the results from this thesis will be used in a manuscript in future by
Danone Nutricia Research- Nanyang Technological University**

Abbreviations

Ig	Immunoglobulin
RSV	Respiratory Syncytial Virus
RNA	Ribonucleic Acid
ILI	Influenza-like Illness
MRC-5	Medical Research Council cell strain 5
HEK	Human Embryonic Kidney
RhMK	Rhesus Monkey Kidney
CPEs	Cytopathic effects
IFA	Immunofluorescence assay
PCR	Polymerase chain reactions
ELISA	Enzyme-Linked ImmunoSorbent Assay
IA	Immunoassays
OIA	Optical Immunoassay
LFIA	Lateral flow immunoassay
HRP	Horseradish Peroxidase
LAMP	Loop-Mediated Isothermal Amplification
M-LAMP	Multiplex Loop-Mediated Isothermal Amplification
RT	Reverse transcription
PCR	Polymerase chain reaction
QD	Quantum Dots (QDs)
Rota	Rotavirus
WHO	WORLD HEALTH ORGANISATION
CONC	Concentration
CV	Coefficient of Variation
OD	Optical density
IRB	Institutional Review Board
NDL	Not Detectable Levels

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Summary

Respiratory Syncytial Virus (RSV) has been established as the major viral cause of acute lower respiratory tract infections in children. However, many healthy individuals in Singapore carry RSV without developing a disease. The gold standard to determine specific responses against RSV is the titer of specific antibodies in plasma. This may impose a challenge of drawing blood from infants, children and the elderly, especially in situations when repeated samplings are required. RSV comes in contact with the host via the mucosal linings in the nostril and lungs, therefore further harm may be eliminated by inactivating them while they still resided in these tissues prior to reaching the bloodstream [3].

Thus, for these infections it would be relevant to measure the local antibody responses in respiratory tract or in the mouth. Hence, measuring RSV specific immunoglobulins (Ig) namely IgA, IgG and IgM in saliva as may give a good reflection of mucosal immunity.

In this study, a standardized method of non-invasive sampling of whole saliva and stool was employed to assess whether RSV-specific antibodies (Igs M, G and A) can be detected. Once the presence of RSV-specific antibodies in saliva was confirmed, their relationship with age, gender and ethnicity was evaluated. In addition, the expression of RSV specific Igs in plasma and saliva was compared in a subgroup. Rotavirus specific antibodies were also measured in some of these saliva and stool samples.

Our findings have shown that RSV Ig M, G and A can be detected in saliva but not in stool while rotavirus antibody responses can be measured in both samples. In addition, the data suggest that measuring RSV antibodies IgM alone in saliva might be sufficient to determine RSV exposure or infection. It is also clear the RSV antibodies in

saliva do not fully reflect systemic antibody response, thus, measuring specific antibodies in saliva cannot fully substitute analysis of plasma. This study has served as a proof of concept on the use of saliva to measure RSV specific immunoglobulins while addressing the ethical and practical problems in blood samplings. Additional studies with bigger sample size or targeted cohort would determine which components in saliva could reflect the responses in plasma.

1. Introduction

1.1 Global issue of RSV infections

Respiratory Syncytial Virus (RSV) was first isolated in a chimpanzee that was displaying flu-like symptoms in 1956. It was then identified as a human pathogen in infants with severe lower respiratory tract infections. Since then, RSV was acknowledged as the primary viral cause of acute lower respiratory tract infections and bronchiolitis in infants and young children under 5 years of age [12, 13]. The global burden of RSV estimates to be 33.8 million new cases of acute lower respiratory tract infections in children under five years old and 3.4 million hospital admissions yearly. In 2010, there were approximately 253,000 deaths due to RSV with most occurring in developing countries [14, 17].

The lack of proper medical care contributes to disease severity and deaths in developing countries. This may be an underestimate as the number of cases and deaths may not be recorded as a result of poor availability or accessibility of local medical organizations. Furthermore, the mortality rates may not be captured accurately due to the lack of proper diagnosis. The RSV Global Estimates Network keeps track of all RSV related cases and the next update will be determined after 2016 [17].

1.2 Virology of RSV

RSV is an enveloped, single negative-strand RNA virus belonging to the family *Paramyxoviridae* and is classified under the genus *Pneumovirus* [13, 15], (Figure 1). The

15.2 kb genome comprises of 10 genes and encodes a total of 11 proteins [13, 16]. The capsid contains three surface envelope proteins, they are the attachment G (major glycoprotein), protein F (fusion) protein and SH (small hydrophobic) protein.

The RSV G protein which is heavily glycosylated is responsible for viral attachment to cells. The antigenic variation of the protein G makes up the major differences between these two subtypes of RSV, A and B. The G protein can differ up to 41% in both subtypes and have up to 20% difference within the same subtype [13, 16]. Studies on the G protein antigenic variability in various regions shows the RSV A strain subtypes are the pre-dominant and prevalent subtypes that cause RSV infections worldwide [18]. There is a 13 amino acid loop-like structure which is a conserved region in all RSV G protein and possibly plays a role in host receptor binding site [15, 16]. It has been shown that recombinant RSV without the G protein are able to infect cells, suggesting that the F protein may also has a role for a host co-receptor attachment. The function of the F protein is to facilitate the fusion of viral and cell membranes, thus aids in the transfer of transfer viral genetic material into the host cells [15]. Both G protein and F protein are of great interest in vaccinology as they are thought to be the major candidates for antibody responses against RSV.

The function of the other capsid SH protein is not clear but a study suggested that it may possibly play has a role in inhibiting the TNF α pathway. In RSV lacking the SH gene, it was demonstrated that SH protein also has a role in inhibiting apoptosis during RSV infection [19]. M (matrix) protein is located in the inner membrane of the capsid and is required to form virus-like particles [20].

The ribonucleoprotein complex consists of the viral genome and nucleocapsid proteins consisting of the N (nucleoprotein), P (phosphoprotein) and L (large RNA-dependent RNA polymerase) protein with the latter necessary for replication and transcription of the

RSV genome [13, 20]. Two non-structural proteins – NS1 and NS2 although expressed, are assumed to be absent in the viral particle. Instead these proteins are associated with counteracting the host innate immune response [21].

Figure 1: Schematic diagram of RSV structure. (Adapted from Lambert, L., A. M. Sagfors, et al. (2014) [13])

1.3 RSV Clinical disease

It is estimated that approximately 80% children have experienced at least one RSV infection by the time they turn two, with 2/3 of these infections taking place within one year of age [22]. Although in most cases, the infants manifest mild upper respiratory tract infections such as rhinitis and cough, or otitis media in some infants, 40% of the infants will end up developing more severe lower respiratory tract infections. Dyspnea,

subcostal recession and difficulties while feeding are classic symptoms of lower respiratory tract infections [13, 15, 22].

During RSV lower respiratory tract infections, the diffusion of inflammatory cells into the airspaces, overstimulation of mucus, shedding of necrotic airway epithelial cells, together with airway wall edema cause the progression of bronchiolitis [13]. All these stages cause the narrowing of airflow in the airway lumen, leading to airway obstruction and impaired gas exchange [13]. While having bronchiolitis, crackles and wheeze are likely to occur with labored breathing and a prolonged expiratory phase. Complications that arise from the air trapping include a rapid respiratory rate, a palpable spleen and liver. Acute respiratory failure or hypoxia varying with severity is observed in severe bronchiolitis cases [13, 15]. There have been several studies linking RSV infections to recurrent wheezing or asthma (26-28).

In one such study, it was revealed that children hospitalized for RSV-induced bronchiolitis before the age of 1 have a higher risk of developing recurrent wheezing subsequently at 30 to 42 months (28.1% vs 13.1%) and 69 to 81 months (22.6% vs 9.6%) compared to the control group. In this cohort, almost 40% developed asthma by age of 7 compared to 20% in the control group [26]. A comparable trend was also observed in a Swedish study consisting of 140 children who had severe RSV infection prior to 1 year of age. Whereby, 30% of them subsequently developed asthma at the age of 7.5 years. Moreover, 28% of RSV-induced bronchiolitis cases developed recurrent wheezing symptoms while this was observed in only 11% of the control group [27]. These children were followed up at 5 years and 10 years. A significant rate of either recurrent wheezing (30% vs 16.3%) or asthma (37% vs 5.4%) at later age was exhibited in children who had severe RSV infection during infancy [28]. It was also noted that children who developed severe RSV infection at an early age are prone to develop allergic sensitization to common aero or food allergens based on measured time points [27, 28].

As allergen sensitization is considered as a potential link to the development of asthma, it is possible that severe RSV infection during early life may contribute to asthma development through common allergens sensitization.

0.5 - 2% of RSV lower respiratory tract infections in children below 5 years of age require hospitalizations [14]. In a birth cohort study conducted in England, it has been observed that the hospitalization due to RSV bronchiolitis peaked at 1 month [23] (Figure 2). Besides being a serious childhood viral disease that adds on to pediatric burden, RSV infections are gradually becoming a significant issue among the elderly over 65 years old with a high mortality rate similar to influenza A infections [25].

Figure 2: Age of bronchiolitis admission in months, for admissions coded as RSV or unspecified bronchiolitis. (Adapted from Murray, J., A. Bottle, et al. (2014) [23])

1.4 RSV Epidemiology and Risk Factors

It is important to understand RSV modes of transmission and how factors like climate changes or seasons; population density may impact the spread of the virus. This can

thus give a better idea of how outbreaks can be controlled and managed.

RSV nosocomial infection is one example of a risk factor. The burden of such hospital infection not only produces significant morbidity in young children and elderly, it poses severe problems in patients with underlying conditions, especially cardiopulmonary and immunosuppressive diseases. The spread of the virus also aggravate pulmonary and cardiac conditions in elderly as well [29].

It has been speculated that RSV spreads by both direct and indirect contact with respiratory secretions instead of inhalation of airborne virus. RSV transmission between health care workers and infants was investigated in a study conducted in a pediatric ward setting. It was observed that RSV spreads by contact with large RSV droplets or self-inoculation after coming in contact with contaminated surfaces such as medical instruments [29, 30]. Hence, educating the health care workers about the impact of RSV nosocomial infections, implementing infection control program and emphasizing good clinical practices like hand washing or proper sanitization of medical apparatus. Thus, these could possibly help to control RSV nosocomial infections.

Apart from RSV nosocomial infections, some risk factors at birth such as prematurity (<37 weeks), small for gestational age, gender in particularly male infants and pre-existing medical conditions also contribute to RSV infection in infants [13, 24, 33]. Moreover, there have been evidence to show maternal health risk factors such as smoking and/or low cord blood vitamin D, increase risks of developing RSV infections in the first year by six-fold in healthy infants [13, 31].

Medical conditions such as congenital heart disease, Down's syndrome and cerebral palsy place them in high risk for the development of RSV infections [23, 33]. An example of rates and risk factors of RSV induced bronchiolitis hospital cases in a birth cohort in England is summarized in (Figure 3).

Risk Group [†]	Number of bronchiolitis admissions (% of infants in risk group)	Total number of infants in risk group (% of whole birth cohort)	Median length of stay in days (IQR)	Rate of bronchiolitis admission per 1000 infants under 1 year (95% CIs)	RR [†] (95% CIs)	Adjusted RR (95%CI)
Born at term	6139 (2·2)	274403 (92·5)	1 (0 to 3)	22·4 (21·8 to 22·9)	–	–
Premature birth	1050 (4·7)	22215 (7·5)	1 (0 to 3)	47·3 (44·4 to 50·2)	2·11 (1·98 to 2·26)	1·89 (1·77 to 2·02)
Cystic fibrosis	11 (6·4)	171 (0·1)	2 (0 to 14)	64·3 (32·1 to 115·1)	2·66 (1·33 to 4·76)	2·45 (1·36 to 4·43)
Congenital heart Disease	272 (12·1)	2239 (0·8)	2 (0 to 5)	121·5 (107·5 to 136·8)	5·17 (4·56 to 5·84)	3·35 (2·92 to 3·84)
Chronic lung disease	282 (5·6)	5016 (1·7)	2 (0 to 4)	56·2 (49·9 to 63·2)	2·37 (2·10 to 2·67)	1·61 (1·42 to 1·82)
Immunodeficiency	7 (11·7)	60 (0·0)	8 (1 to 58)	116·7 (46·9 to 240·4)	4·82 (1·94 to 9·93)	1·69 (0·80 to 3·58)
Nervous system congenital anomalies	42 (8·6)	489 (0·2)	2 (1 to 4)	85·9 (61·9 to 116·1)	3·56 (2·56 to 4·82)	1·73 (1·26 to 2·36)
Down's syndrome	28 (15·4)	182 (0·1)	3 (0 to 9)	153·9 (102·2 to 222·4)	6·37 (4·23 to 9·21)	2·53 (1·72 to 3·72)
Cerebral Palsy	16 (10·7)	149 (0·1)	3 (1 to 5)	107·4 (61·4 to 174·4)	4·44 (2·54 to 7·21)	2·43 (1·48 to 3·99)

[†]Relative risk compared with infants without risk factor of interest.
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Figure 3: Rates and relative risk of a bronchiolitis hospital admission among infants in different risk groups. (Adapted from Murray, J., A. Bottle, et al. (2014) [23])

Siblings of preschool or school age within the same household pose a significant RSV risk factor that accounts for the spread of virus and thus, hospitalization in young children. For the same reason, infants that attend infant-care are also at high risk for RSV infections [24].

Malnutrition increases the risk of infectious diseases especially in early childhood. Poor nutrition affects growth, leaving children underweight, weak and prone to infections [32]. Poor nutritional status has been an important and consistent risk factor for developing acute lower respiratory tract infection. The lack of breastfeeding in infants or infants who receive less than 2 months of breast milk, also face risks in developing RSV infections [13, 24]. It is known that optimal and appropriate nutrition in early life is crucial for the development of proper immune response. Hence, optimal early life nutrition is important to prevent RSV infections.

Low socioeconomic status and parental educational status have also been described to be risk factors for RSV infection related hospitalization [33]. A study from Sweden in 2002 analyzed the role of socioeconomic status with RSV infection and showed a positive correlation with the percentage of immigrants and per capita income

[34]. Low parental education status, in combination with other risks, is positively correlated with RSV related hospitalization. This theory is supported by an early epidemiologic study conducted in Tucson in 1991 [35]. Environmental factors such as crowded living conditions and altitude affect RSV infection rates. As altitude impacts numerous respiratory properties, it is considered an important factor. At higher altitude of 2500m, the chances of children getting RSV infection related hospitalizations are 33% more than those children living at moderate altitude [35, 36].

Climate or season change is a key factor that contributes to RSV epidemics. RSV epidemic curve varies yearly and region to region. RSV infections in temperate climates in the Northern hemisphere usually begin during the winter season, starting from October to December, and ending in between March to May. Thus, RSV infections in these regions are constantly distributed throughout the year. In regions where temperatures remained colder throughout the year, RSV activity is almost continuous throughout the year [39]. In Hong Kong, for example, the prevalence of RSV epidemics is more frequent during the rainy season despite the temperature being high [38]. While in tropical regions that are warm and humid, RSV activity is detected throughout the year with peaks in summer and early autumn [39]. It was noted that weekly RSV activity was linked to temperature in a bimodal style, with peaks observed at temperatures above 24 – 30°C and at 2 – 6°C. Moreover, RSV activity was also highest at 45 – 65% relative humidity [39]. A good understanding and determination of RSV seasonality in different regions of the world is important. One of the current practices for high-risk population is to administer palivizumab monoclonal antibodies during RSV epidemic period based on surveillance data [40]. Hence, by having a predictive model in place, preventative strategies with immunoprophylaxis or future RSV vaccines can be employed [40, 41].

1.5 RSV infections in Singapore

Singapore is geographically located just north of the equator, at latitude 1.5°N and longitude 104°E. The climate is quite uniform with temperatures throughout the year with a minimum of 23°C–26°C and maximum of 31°C–34°C. The relative humidity is 90% and can reach 100% during prolonged heavy rain. There are no distinct wet or dry season, however, maximum rainfall occurs in April and December [44]. These climate characteristics make Singapore a tropical country.

It has been shown that in areas with tropical or subtropical climates and seasonal rainfall, RSV outbreaks are often associated with the rainfall rather than the cold season. In addition, it has been shown that areas with tropical or subtropical climates are often associated with RSV outbreaks, which occur one to two months following the rainy season [45]. However, this is not the case with regions that are close to the equator with constant high rainfall, such as Singapore [46]. These tropical regions have a distinctive pattern of RSV outbreaks occurring, majorly in first half of the year, but not in the other. However, neither temperature nor rainfall appears to be the main factor that determines these RSV outbreaks in Singapore [45] (Figure 4). A similar pattern of RSV outbreaks in Singapore is observed in a vaccine trial study carried out on healthy children displaying influenza-like illness (ILI) (Figure 5) [50]. Rather than rainfall, it was noted that the risk factors of RSV transmission here is linked with higher temperature, lower relative humidity and higher maximal day-to-day temperature variation [47]. This was shown when seasonal trends of viral respiratory tract infections in two hospitals in Singapore between September 1990 and September 1994 were evaluated.

Upper respiratory tract infections and other respiratory diseases, such as asthma and bronchitis, account for the leading causes of diseases seen at primary healthcare clinics in Singapore [47]. Respiratory viral infections have the highest prevalence rates,

with those with influenza and respiratory syncytial virus (RSV) being the most significant ones [51]. A 4-year study revealed that RSV infections made up for more than 70% of respiratory tract viral outbreaks, particularly among children under 2 years of age who require hospitalization. An 8-year study to understand the impact of influenza associated deaths in Singapore was conducted between 1996 and 2003. Samples from infants and adult patients were collected from at KK Women's and Children's Hospital, Singapore General Hospital and other public-sector hospitals, as well as from adult outpatients with influenza like symptoms treated at private healthcare clinics. RSV diagnosis was evaluated for some samples in this study in order to obtain true assessment of influenza [48]. The results of the study show that RSV cases are the highest in patients displaying influenza-like symptoms yearly.

Figure 4: Seasonality of RSV outbreaks in Singapore. Graph depicts the mean monthly rainfall (■), the mean monthly maximum (●) and the mean monthly minimum (○) temperature as lines, and series of cases as bars. Year 1 of study; ■Year 2 of study; ■ Year 3 of study (Adapted from Weber, M. W, et al. (1998) [3])

Figure 5: Monthly distribution of influenza-like illness (ILI) episodes in Singapore in which respiratory syncytial virus (RSV) was detected in healthy children in Singapore. (Adapted from Terry Nolan et al. (2015) [60])

Mortality rates due to RSV infection are low despite RSV infections being the leading cause of respiratory infections. The death rates of RSV in Singapore, over a period of 10 years, from 2000 - 2010 was reviewed by obtaining data from Department of Paediatrics of KK Women's and Children's Hospital. The age group with greatest risk is those of less than 1 year of age (7 out of 11 cases). Existing cardiac disease in children appears to be the greatest risk factor [49]. In an attempt to examine the incidence of Bocavirus in the pediatric population in Singapore, it was revealed that RSV infection was the highest rate (11.8%) among those tested coinfections of respiratory viruses (107).

RSV epidemiology in healthy children between 6 months to 10 years of age, displaying ILI after first vaccination in a pandemic influenza vaccine trial study was evaluated [50]. This study was conducted in 8 countries, including Singapore, between

February 2010 and September 2011. Active surveillance for ILI was carried out over a period of 1 year. All children who displayed ILI had fever and more than 80% of them had runny nose. Nasal and throat swabs from children displaying ILI were collected and analyzed by a multiplex polymerase chain reaction (PCR).

A total of 151 children from Singapore participated in this study. 34 of these children consisting of 55.8% boys displayed 49 ILI episodes. When tested for RSV among those who displayed ILI, 4 episodes were positive for RSV infection. It was noted that 96% of children with ILI associated with RSV developed a new or worsening cough, which is a classic symptom of lower respiratory tract infection. Interestingly, there were no RSV infections in children below 24 months of age. The age ranges for the 4 positive episodes are in 2 cases between those of 24 - 35 months of age, with one between 36 - 59 months and another case above 60 months of age.

This type of study highlights the need to target a healthy cohort and not just hospital or clinic based surveillance. Furthermore, by stratifying the age groups, the variability in RSV incidence and prevalence across a wider age range is better understood as most RSV studies usually target children under 2 years of age and/or the elderly. The limitation of this study, however, is that only children who displayed ILI were tested for RSV. Thus, some healthy children who did not display fever were not tested for RSV and this is not captured. This could also imply that RSV burden is not just limited to those at risk or in malnourished population.

Therefore, in order to understand the burden of RSV in a developed country like Singapore, It would be necessary to conduct a study to target a wider age gap among a healthy population that may/may not display ILI. This not only helps us understand RSV exposure in a healthy cohort, but also the learnings may be useful for future vaccination strategies.

1.6 RSV Detection Methods

Numerous diagnostic methods have been developed ever since RSV was established as an important respiratory human pathogen. The isolation of the virus in cell culture has been the commonly used diagnostic method to determine RSV infections during the 1980s to early 1990s and is considered a golden standard method to diagnose RSV [42]. Virus from infected clinical sample such as nasal aspirate is added to cells (at 75% confluence), such as human lung fibroblasts (MRC-5), human embryonic kidney (HEK) or primary rhesus monkey kidney (RhMK). The infected cells are then observed every few days to observe cytopathic effects (CPEs). However, this method is time-consuming and may take up to a maximum of 2 weeks [42, 43]. Early, reliable and accurate diagnosis of RSV infections is important to reduce or eliminate the use of antibiotic therapy and limit the spread of nosocomial infections [42]. Furthermore infected cells need to be identified by other immunoassays (IA) and this delays time [43]. Hence, the requirement of rapid, sensitive and cost effective IA is important [53]. The following sections describes the principles of IA and table 4 summaries pros and cons of using some of the following techniques.

Immunofluorescence assay (IFA) is an example of an assay with accurate results and comparable to cell culture techniques. This technique requires the use of a fluorescence microscope to directly visualize the detection of RSV proteins on host cells via labeled antibodies [52]. Direct IFA has been a common and standard method used to detect RSV for decades and other RSV detection methods are often compared to this method for evaluating their efficiency [52]. This technique was reliable when tested on time once patient displays RSV symptoms. [52]

Optical Immunoassay (OIA) refers to the direct visualization of antigen-antibody complexes immobilized on a reflective surface. When RSV antigen-antibody complex

forms a thin layer on the surface, the reflective properties of the surface alters making the detection of RSV possible. [54]

Lateral flow immunoassay (LFIA) is a type of IA that employs an immunochromatographic technique for the rapid detection of RSV from nasal washes or nasal aspirates. The lateral flow of RSV antigen-antibody complex on the chromatography strip reacts with the conjugate and forms a visible band to indicate positivity, similar to pregnancy kits [55]. BD Directigen™ EZ RSV is an example of such commercially available kits that use the principle of LFIA. The assay is performed with by adding patients nasal washes or aspirate onto a membrane pad to detect RSV [56].

Enzyme-linked immunosorbent assay (ELISA) is the colorimetric detection of RSV antigen-antibody complexes with an enzyme facilitated method. There are several versions and RSV antigen targets to this method though most of them are target F protein. One of the recent and efficient ELISA methods to detect RSV that is to use high affinity anti-RSV F antibody peptides derived from the vaccine motavizumab [57].

Loop-mediated isothermal amplification (LAMP) is a rapid nucleic acid based detection method used on RNA viruses like RSV. An additional step of cDNA synthesis is required and usually designated as reverse transcription LAMP (RT-LAMP). Specific pairs of primers to cDNA are used and reaction can be monitored by a real-time turbidimeter. The product can used to further distinguish between RSV subtype A and B [59, 60]. Currently, there are some other improved versions of this method such as multiplex LAMP (M-LAMP) assay which can be performed in shorter period and with a higher sensitivity and specificity [62].

RSV can be challenging at times to detect due to low viral titer and antigen-based detection techniques may not be that sensitive [63]. Hence, many PCR based methods

including conventional PCR are developed to detect RSV in adults having respiratory infections [64]. One example is the reverse transcription-nested PCR method targets the F gene of the virus. Real-time PCR (RT-PCR) is another example by far the most sensitive method for the detection and diagnosis of RSV. There are a few RT PCR methods that have been established in clinical samples targeting the N and F gene of RSV. [65-69].

The multiplex PCR is used for detection and subtyping of RSV and human influenza as well as other respiratory viruses [70-71]. This method employs a set of specific primers that helps to distinguish RSV from other viruses.

Nanoparticle Amplified Immuno-PCR or Immuno-PCR, is a combination of ELISA and PCR by using gold nanoparticles technology to detect RSV antigens. [72]. It is possible to track mRNA of host and RSV using Live Cell RNA Imaging [73]. This quantitative technique which employs DNA hairpin structures, allows the detection of multiple mRNA at the same time when scanned for fluorescence. [73].

Quantum Dots (QD) are fluorescent nanoparticles that can be applied in the detection of RSV and very comparable to IFA. [74]. Using confocal microscopy by QDs targeting F and G proteins, the progression of RSV infection in cells can be observed [75]. Flow cytometry by antibody conjugated QDs can be also applied in the detection of RSV. [76].

Gold Nanoparticle Facilitated Microarray is a FDA approved technology that aids detection of microarray based hybridization using gold nanoparticles. This method can be used to detect respiratory viruses including RSV [77]. Surface Enhanced Raman Spectroscopy (SERS) is another technology that combines nanotechnology with spectroscopy to detect RSV [78].

Technique	Advantage	Disadvantage
Fluorescence based assays		
IFA	Easy to perform, rapid	Human error, fading of dyes
QDs	Photostable, inorganic in nature, resistant to metabolic degradation	Toxic and insoluble
Immunoassays		
ELISA	Easy to perform, high sensitivity, specificity	Human error
QIA	Easy, rapid, specificity, cost effective	Negative samples need other confirmation tests
LFIA	Easy, rapid, cost effective, FDA Approved	Sample volume limits detection, qualitative/non quantitative
Molecular methods		
LAMP	Sensitivity and specificity	Semiquantitative, compatible primer required
PCR	Rapid and sensitive	Primer design, possibility of false positives
Real Time-PCR	Rapid, high sensitivity , low limits of detection	Not cost effective
Multiplex PCR	Detection of multiple pathogenic species or strains within one sample, rapid	Complex design, possibility of cross reactivity
Immuno-PCR	Very low limits of detection, improved	Complex design
Microarray	detection compared to ELISA and PCR High sensitivity , large scale identification of multiple pathogens;, targets both protein and nucleic acids	Not cost effective
Biophysical method		
SERS	Rapid method, high sensitivity, lasting detection analytes	Tedious sample preparation

Table 4: Summary of IA and their Pros and Cons. Adapted from reference: [52-78]

1.7 Immune responses to RSV

Following RSV infection, the protection is rather short-lived and this allows re-infection of RSV throughout life. [13]. Most natural infections can take place with different strains though it is still unclear if this is due to host immature immune system or infection history or the type of viral season in circulation [13]. Still, there are some recent longitudinal studies of RSV infections in children over a number of RSV seasons that show evidence that children can be naturally re-infected with the same strain of virus even within the same cold season despite some level of protection [94].

With the absence of cost-effective prophylaxis, RSV will impose a constant risk for various populations, across different age groups, immune statuses conditions and living conditions. It is always going to be challenging to overcome the ethical and practical issues associated with understanding immune responses in young children and elderly [13]. The importance of RSV antibodies and immunity has been demonstrated in both humans and animal models. Hence it would be important to explore it further so that vaccination practices can be implemented.

Placental transfer of antibodies begins at around 28 weeks of gestation in humans. The quantity of maternal antibodies in fetal circulation continues to rise till birth. Hence the levels of total and antigen-specific antibody levels, such as RSV-F or -G titers, are lower in premature infants when compared to full-term infants [82]. In general, the infants with higher RSV antibodies tend to have resistance to severe RSV due to the antibodies derived maternally [79]. In a Bangladesh study, ratio of cord blood to maternal serum collected at birth, revealed RSV neutralizing antibody titers in 149 mother-infant pairs was 1.01 (95% confidence interval [CI] and had a correlation of $R^2=0.7$). The findings demonstrate that the placental transfer of RSV-specific antibodies is an efficient active process [84]. However, the levels of RSV immunoglobulins in infants are found to

be gradually decreasing and are almost undetectable in 6 to 8 months after birth [80]. It has been also demonstrated in some studies that long-lasting protective antibody is not normally generated in children following primary respiratory syncytial virus (RSV) infection, frequently leading to reinfection [81].

In an epidemiological study, it was shown that incidence of breast-feeding among infants (n=8) admitted to hospital with RS virus infection was significantly lower than that among the control children (n=46) [83]. Furthermore, the presence of RSV specific neutralizing antibodies in breast milk was detected. Twenty-one breast milk samples were collected up to 48 hours post-delivery and were tested for neutralizing activity using plaque reduction test. Furthermore, the specificity of the antibodies was tested with an IFA. All samples demonstrated neutralizing activity and it was shown that breast milk contains IgA and IgG, but not IgM. It was also noted that IgA antibody titers in these samples was usually higher and correlated better with neutralizing activity compared to IgG [83]. It was hence demonstrated that breast milk can confer some level of protection against RSV in infants. It was also speculated that ingested and inhaled RSV IgA may play a part in protection. Furthermore, secretory IgA (sIgA) found in breast milk confers mucosal protection against pathogens like RSV [84]. Interestingly, research shows that breast milk is gender biased when it comes to protecting infants suffering from viral respiratory tract infections. Breast milk protected females better than males [85]. This is also strongly observed in premature girl babies. Apparently, breastfeeding activates the internal protective mechanisms in girls better than in boys. But as premature babies do not have any immune memory of any previous exposures, it can be inferred that the breast feeding does not impact adaptive immunity [86].

Often nasal secretion samples such as nasal washing or aspirates are tested mainly for the presence of RSV antigens and sometimes RSV Igs. In a study conducted

in 40 children (6-24 months), serum and nasal wash samples were collected and tested for RSV specific antibodies. The children who were recruited in hospital emergency wards, consisted of 20 wheezing cases, 9 rhinitis and 11 control cases (non-respiratory related). In RSV positive samples, it was noted that the ratio of IgA/IgG antibody to RSV antigens were higher in nasal wash samples than the respective sera samples. These responses may be an indication of local antibody production rather than a leakage from the circulatory system [87]. RSV-specific IgE in these samples were undetectable in all sera samples and was detectable at low levels in four nasal wash samples belonging to children below 12 months. Similar findings were seen in a previous study where RSV-specific IgE in children's sera could not be detected. In contrast, the RSV-specific IgE titers were in high levels in nasopharyngeal secretions in wheezing RSV positive children [88]. At 7-8 years of age, the patients were re-evaluated and there was no correlation with recurrent wheezing and RSV-specific IgE titers [89]. This may indicate that one may expect different immune response at different age of infancy. An old study conducted in 18 subjects (aged between 4-21 months) with primary RSV infection, paired serum and nasal wash samples were collected and measured for RSV antibodies. Half of the younger infants (n=8) between 4 to 8 months failed to achieve an elevated serum or nasal-wash neutralizing antibodies after their first infection making them susceptible for a reinfection. However, elevated antibodies levels were seen in older infants above 9 months of age. It was highlighted that the immaturity of infants' immunity and immunosuppression by maternal antibodies may contribute to this [90].

It would be good to also understand RSV antibody responses in older children and adults as well as to explore immunity in both systemic and mucosal systems. It has been showed that nasal neutralizing antibodies offer more protective than serum antibodies in healthy adults challenged with RSV [91]. Using healthy adult volunteers comprising of males and females ranging from 18–45 years to study preexisting RSV

specific serum and nasal antibodies in individuals and their correlation with infectivity, viral dynamics, and disease severity, it was demonstrated that both preexisting serum and mucosal antibodies interplay in immunity against RSV. It is speculated that higher preinoculation serum neutralizing antibody titers and nasal IgA can confer some protection and can be used to predict RSV infectivity. However, preexisting antibodies in serum and nasal remain ambiguous in predicting viral dynamics or disease severity in infected subjects [92].

Similar results are observed in elderly above 65 years old with natural RSV infections. Low serum neutralizing antibodies and nasal RSV specific IgA contribute to the risk of RSV in this population. (93). The importance of RSV specific IgA was further demonstrated in another study where both humoral and mucosal immunity in RSV infections were assessed. (94). In addition, one recent study shows specific defect in IgA memory may be reason for RSV reinfections, implying that vaccines that induce durable RSV-specific IgA responses may be more protective than those generating systemic antibody alone [97]. Therefore, it is very importance to understand both humoral and local immune response for efficient vaccine development. In this thesis, the measurement of RSV specific antibodies was performed in saliva and stool to evaluate the mucosal response to RSV and it was compared to selected plasma samples in healthy individuals.

1.8 RSV vaccine

RSV vaccination history traces back to 1960s when formalin-inactivated RSV (FI-RSV) vaccine trial was conducted in children. However the vaccine had an negative effect on the children's immune responses, resulting in an large number of children suffering from severe RSV diseases and even two vaccine related deaths [108]. Since

then the development of RSV vaccine has been ongoing. There is no licensed RSV vaccine in market currently. Several vaccine candidates are currently in different clinical phases (109). Antibody therapy such as Palivizumab (Synagis) and Motavizumab (Numax) may reduce RSV severity. (110) Like RSV, measles is an example of paramyxovirus that spread through the respiratory tract and can cause severe diseases in infants. Despite having an effective vaccine, it is only 100% protective in older infants compared to 36% protective in infants below 6 months [95] indicating that the immaturity of the neonatal immune system itself is a barrier to generation of protective immunity despite an effective vaccine. Hence it would be also necessary to consider host factors such as age and presence of comorbidities in determining the immune response to RSV infection or future RSV vaccines. This also brings along the possible need to develop more than one type of vaccine strategy for every targeted population in order to have a successful vaccine [96].

1.9 Saliva as a non- invasive sample

Detecting biomarkers at early stages of diseases can expedite treatment approaches leading to better recovery rates, decreased suffering as well as lessen the burden of reoccurrences. A lot of the current diagnostics requires testing of biomarkers obtained from blood draws biopsies or other painful invasive methods. This often results in discomfort in patients and high medical costs [104]. Hence it is important to explore non-invasive samples and sample collection methods that are efficient, painless and easy to follow.

One example of non-invasive sample is saliva. Saliva is produced in the oral cavity by three major parotid, submandibular and sublingual glands and is made up of

99% of water and many components such as minerals, electrolytes, and enzymes such as amylase, growth factors, cytokines, immunoglobulins (e.g., secretory immunoglobulin A [sIgA]), mucins and other glycoproteins [98]. The mechanisms of how these components are secreted into the oral cavity include transcellular, passive intracellular diffusion and active transport, or extracellular ultrafiltration through the gingival crevice or salivary glands [99]. Apart from early digestion, this is becoming evident that biomarkers in saliva alter in response to certain diseases. Interestingly, salivary biomarkers not only arise in correlation with oral disorders, but also those of distal tissues and organs [100]. This makes oral fluid a good alternative to blood /serum or an even better representative of the mucosal environment that helps to predict/ the onset or presence of disease throughout the body. Saliva is also widely used in the detection of drugs use/abuse. Some drugs such as cocaine or codeine can be measured in saliva and can be well correlated with blood [100]. Saliva can also be used to determine oral cancers and detect cancer in other parts of the body. Saliva can be also used as a prognostic fluid. For example, low copy number of salivary amylase gene helps in the prediction of obesity [101].

In the field of infectious diseases, saliva acts as an excellent bio-fluid in determining infections/ vaccine immune response. In the case of the human immunodeficiency virus (HIV), saliva testing for the presence of HIV antibodies is better than serum and urine with regards to both sensitivity and specificity [102]. Saliva itself may be a good representative of mucosal immunity and reflect disease status better. Saliva collection is relatively easy and quantities for analysis are easily sufficient. However, factors such as rate of salivary, activities such as exercise or time when saliva is collected affects the quality of saliva and all these should be taken into consideration when saliva is being analyzed. Thus, a standardized method for saliva collection needs to be employed [103]. New technologies with validated biomarkers profiles in saliva are constantly being explored for research and clinical applications.

1.10 RSV antibodies in saliva

As previously describe in chapter 2.7, RSV specific antibodies are often measured in serum and nasal samples such as aspirates and washings. Though nasal secretion collections do not require the use of needles, these collection methods can bring about levels of discomfort and pain in patients, especially, in infants and the elderly. Based on earlier studies on the epidemiologic characteristics of RSV, it is possible to measure RSV specific antibodies in saliva samples [105-106].

By measuring RSV specific antibodies in saliva, community studies are made possible by allowing the sample collection out of clinical settings. RSV vaccination strategies to understand immune responses with repeated sampling within the same patients are also made possible by measuring the antibodies in saliva [106]. Furthermore, little expertise is needed for saliva collection and therefore volunteers or untrained personnel can easily use saliva collection kits with minimum supervision. In one study conducted in Leicester, UK, the use of the saliva collection device was described to volunteers aged between 7-10 years who then provided their samples twice with 6 months intervals under the supervision of research workers for the measurement of RSV antibodies in saliva [105]. In this study, the measurement of RSV specific IgG in saliva samples was demonstrated. There was a significant increase of antibodies (P value < 0.01) 1 between October and March anti-RSV IgG and it was acknowledged salivary RSV IgG itself may be a good substitute for blood [105].

The profiles of RSV specific IgG and IgA responses in paired serum and oral-fluid samples from five children from birth to the age of 30 months were compared in an epidemiological study in a rural Kenyan community [106]. The results showed that individually the RSV specific IgG and IgA in saliva were good indicator of infections and

were reliable in identifying infections over a year. It was also suggested that although boosting of RSV antibodies does take place in all infected children, the responses are often transient for both antibody classes. The levels of antibodies upon infections start to decline within 3 months and hence, sampling time plays an important role in identifying infections. It was also recommended that oral fluid samples will make a good alternative to serum in this paper [106].

1.11 Immunoglobulins

The structure of an antibody is related to its function. In human, antibodies are classified into five main classes or isotypes – IgA, IgD, IgE, IgG and IgM. Ig A is an antibody that is primarily involved in mucosal immunity. It is usually a monomer in serum and a dimer known as secretory IgA found in mucous secretions such as tears, saliva and in the gastrointestinal tract (140). It is the main immunoglobulin found in mucous secretions and is often involved in the first line of defense against pathogens (140). As such, viral specific IgA in samples would be helpful in the identification and diagnosis of infections such as Rotavirus or RSV.

IgG is the predominant antibody in the blood and possesses the longest serum half-life of all immunoglobulin isotypes (140). IgG is classified into four sub classes; IgG1, IgG2, IgG3 and IgG4, each having its own functions. IgG directly participates in the elicitation of an immune response such as the neutralization of toxins and viruses, the activation of the complement system (140) and providing extensive systemic immune protection. Although IgA is the primary antibody in mucosal defense, IgG antibodies also participate directly to an immune response including neutralization of viruses (140).

IgM is a pentamer antibody that is the first antibody expressed during B lymphocyte ontogeny and as such is often the first line of defense against invading pathogens (140). Presence of IgM is often associated with a primary immune response and indicates an acute infection (140). Like IgA, IgM also plays a role in mucosal immunity by facilitating the opsonisation of an antigen, activating the complement system for the phagocytosis of the pathogen. The pentameric structure of IgM enables this process to take place efficiently (140).

Though the precise function of IgD is unknown, it is recently noted to be involved in respiratory mucosal immunity (142). The occurrence of IgE in immunity is linked to parasite infections but is more commonly associated with hypersensitivity, allergy and asthma manifestation. (140,141). The role of soluble IgE as a key player at mucosal sites in particularly at respiratory tract and the presence of IgE in nasal lavage fluid has been demonstrated. (143).

1.11 Rotavirus

Rotavirus (RV), being a gut pathogen, affects 95% of children worldwide being affected by the virus by 3 to 5 years of age. It can be easily transmitted from person to person through the oral to faecal route through respiratory secretions or contaminated surfaces and thus is difficult to control via improvements in hygiene or sanitation. (111). RV has been established as the leading cause of severe gastroenteritis and thus RV specific IgA antibodies are developed in the gut lumen (112). In this thesis, the measurement of RV antibodies in saliva and stool samples was used as a control to demonstrate that route of transmission and pathogenesis plays a significant role in immune responses.

1.13 Objectives

The primary objective of this project is to demonstrate the potential use of non-invasive saliva collection for the validation of antibody responses to natural RSV infection or exposure. In this project, methods for saliva collection and the measurement of RSV-specific Immunoglobulins namely IgM, IgA and IgG collected from a local healthy cohort were be established.

The clinical objectives of the project includes,

- a) Writing protocol for sample and information collection, getting an approval by an Independent Ethics committee for the recruitment of healthy subjects in Singapore, within which standardized saliva, stool collection, storage and processing method is evaluated.
- b) Detection of RSV specific IgA, IgG and IgM in saliva samples from healthy Singaporean volunteers aged 1-50 years.
- c) Demonstrate that RSV immune response is mainly limited to the respiratory tract and does not reach the gut by comparing RSV and Rotavirus antibody responses in saliva and stool samples.
- d) To compare expression of RSV specific IgM, IgG and IgA in selected matching saliva and plasma samples.
- e) Determining the reproducibility of the assay in saliva by spiking/recovery and repetition tests.

2. Materials and Methods

The study was approved by the **Parkway** Independent Ethics Committee (**PIEC/2014/036**). A group of 86 healthy volunteers with no known diseases comprising of 38 children (below 19 years old) and 48 adults (>19– 50 years old) participated in this study. Participants were evaluated for the inclusion/exclusion criteria based on current health conditions. Volunteers with any known infectious diseases, such as HIV and Hepatitis B, or with any acute or chronic illness, such as chronic inflammatory bowel disease (e.g. Crohn's disease or ulcerative colitis) or with oral diseases/ulcers were excluded from the study. Healthy volunteers with no existing medical conditions within 50 years fall in the inclusion criteria. Parental/informed consent forms were obtained from participants once they were deemed suitable for the study. A questionnaire was provided to the participants to get information on their demographic data and medical history. Participants could also consent for their blood to be drawn for plasma analysis and this was approved by National Healthcare Group (DSRB 2011/00338). The definition of children's age was based on WHO guidelines. Participants 19 years and below of age were considered as children and participants over 19 years were considered as adults.

2.1 Sample collections:

Participants were given saliva collection kits and one stool container for self-collection together with collection instructions. They were also advised to collect both saliva and stool within the same day or a day apart.

2.1.1 Saliva collection

Saliva collection kit:

Salimetrics saliva swab is made of an inert, non-toxic polymer that features a thin diameter (8mm). Salimetrics swab storage tube is a large outer tube, with a small insert and snap cap used for storing saliva samples collected via swab(s).

Collection method:

The participants were asked to collect morning saliva between 9-11am. Saliva sample was collected by placing one end of a swab under the participant's tongue for 60 seconds. Then the other end of the swab was flipped and introduced under participant's tongue for another 60 seconds. This was repeated thrice to ensure that sufficient saliva was collected. Collected samples were kept frozen up to 72 hours until they were ready for collection to lab.

Sample processing: The swabs were thawed and centrifuged at 1800g for 15 minutes at 4°C. Saliva was collected at the end of the collection tubes. Multiple saliva samples from the same individual were pooled together and total volume was recorded. Flow rate ($\mu\text{l}/\text{min}$) was calculated by dividing total volume with twice the no. of swabs/individual as saliva was collected from 2 sides of each swab. The pooled saliva was aliquoted into 110 μl aliquots, labelled and kept frozen till ready to use for experiments.

2.1.2 Stool collection

Stool collection container:

Stool collection container is a plastic tube that comes with a spoon that facilitates the easy collection of stool.

Collection method:

Participants are to collect their stool immediately after defecation. Participants were instructed to take care to avoid wetting the stool sample with urine or other fluids. The tubes were half filled with stool (about 5 grams) and then capped tightly. Samples were then stored in a freezer until ready for transportation.

Sample Processing:

Stool samples were thawed prior to processing. Approximately 1g of stool was measured and added to a fresh 15ml centrifuge tube. 9 parts of 1xPBS was added to the tube. 10 - 20 1mm glass beads were added to the tube. The tube was capped tightly and placed in a homogenizer and was homogenized for 10 minutes. Next, the solution was centrifuged at 500g for 15 minutes. The supernatant was then collected and aliquoted 1ml into 1.5ml microcentrifuge tubes and stored at -80°C until needed

2.1.3 Blood collection

Collection method:

Blood from healthy adults volunteers was collected into heparin tubes.

Sample processing:

The surfaces of the biosafety safety cabinet were wiped with 70% ethanol. The cabinet was allowed to run for 15 minutes before using the safety cabinet. The blood collection tubes were spun at 2000rpm for 15 mins. Plasma and the pelleted blood were separated. The plasma was aliquoted in 1ml volume into cryovials and labeled appropriately. The cryovials were kept at -80C until needed for analysis.

2.2 Samples transport

Samples are to be transported with ice packs. Upon arrival, the samples are randomly coded to retain confidentiality and stored in a freezer until ready for processing

2.3 RSV ELISA IgA, IgM and IgG (Neo-Scientific)

Standards (anti-RSV IgA for detection of IgA; IgM – for IgM and IgG for IgG, provided in the kit) were serially diluted in assay diluent buffer (provided in kit) to obtain concentrations of 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml and 31.25µg/ml (IgA); 800µg/ml, 400µg/ml, 200µg/ml, 100µg/ml and 50µg/ml (IgM) and 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml and 25µg/ml (IgG). 50ul of each standard and assay diluent (blank) were added to the relevant pre-coated ELISA plate (provided in kit) in triplicate. The identity of the six peptides has been withheld by the manufacturer due to proprietary reason. 50ul of sample were added in duplicate. Saliva samples were added neat and plasma was used at an initial dilution of 1:5. Thirdly, 50 µl HRP (provided in kit) was added into each well. The plate was then sealed and incubated at 37°C in a CO₂ incubator for 60 minutes. After incubation, the plate was washed 5 times using the wash solution (in kit). After washing of the plate, 50 µl of chromogen A was added into each well followed by 50 µl of chromogen B. The plate was then sealed and incubated at 37°C in a CO₂ incubator for 10 minutes in the dark. Then, 50 µl of the stop solution was added. The optical density (OD) was then read at 450nm and the results recorded. The standards were plotted using a 4 Parameter Logistic Curve Fitting graph using Gen5. The concentrations of the samples were extrapolated based on the curve analysis with a CV of within 30%. If the CV was not within 30% then the samples were further diluted and the ELISA repeated until a CV within 30% was obtained.

Figure 2.3: An example of RSV specific Ig standard curve

The X axis represents the concentration of RSV Igs standards in µg/ml The Y axis represents the absorbance of OD 450nm.

2.4 Spiking & Recovery – RSV ELISA IgA, IgM and IgG (Neo-Scientific)

Spiking of the standards was done in non-responsive saliva using Human RSV IgM Neo-Scientific ELISA kits. Firstly, IgM for IgM spiking, IgA for IgA and IgG for IgG standard (found in kit) of known concentration /ml were added to non-responsive saliva in a 1:2 dilution, followed by 2 serial dilutions. Thus, giving concentrations of 400µg/ml, 200µg/ml and 100µg/ml of IgM standard; 200µg/ml, 100µg/ml and 50µg/ml of IgG standard; 250µg/ml, 125µg/ml and 62.5µg/ml of IgA standard. The assay was repeated a second time with similar concentrations except for the IgM standards which were performed at concentrations of 600µg/ml, 300µg/ml and 150µg/ml.

50ul of the diluted standards were added in duplicates in a horizontal direction followed by 50ul of HRP in each well of the RSV IgM/IgA or IgG pre-coated micro titer plate (found in kit). The plate was then sealed and incubated at 37 °C in a CO₂ incubator for 60 minutes. After incubation, the plate was washed 5 times manually with 350ul per well using 1X wash solution (found in kit). Complete removal of the liquid at each step was ensured. Following washing, 50ul of Chromogen A (found in kit) was added to each well followed by 50ul of Chromogen B (found in kit). The plate was sealed and incubated at 37 °C in a CO₂ incubator for 10 minutes away from light. Once the 10 minutes was up, 50ul of stop solution (found in kit) was added. Lastly, the OD was read at 450nm using a micro plate reader and the results recorded. Recovery was calculated using spiked concentration as a percentage of the known concentration.

2.5 Reproducibility Assays

Recollection of 8 saliva samples (1921, 214, 411, 1409, 126, 747, 167, 521, 308) were conducted in September 2016. The saliva collected were ensured volumes of 1.5 – 2.0 ml per sample and aliquoted in 120ul in 1.5ml centrifuge tubes and stored in a -80 °C freezer. In every 1 – 2 weeks, 1 tube of aliquoted saliva was taken to determine the IgA, IgM or IgG using the Neo-Scientific Human RSV IgA/IgG/IgM ELISA kit. By doing so, it was assessed if there were any changes in concentration of the samples over a period of time and reproducibility of the assay.

2.6 Kinetics Study

19 saliva samples were collected in 2015 and were recollected in 2016 for a kinetics study. The saliva samples were tested for Human RSV IgA/IgG/IgM using the Neo-

Scientific Human RSV IgA/IgG/IgM ELISA kits. Their RSV concentrations were measured and concentrations of the same sample ID over the year were compared

2.7 Recombivirus Human Anti-Rotavirus VP6 IgA/IgG/IgM ELISA

The kit was allowed to stabilize at room temperature for 30 mins prior to experiment. The plate provided in the kit is coated with recombinant VP6 antigen. 50 µl of Anti-rotavirus positive and Anti-rotavirus sensitive controls (provided in kit) as well as processed samples were added in pre-determined wells in duplicates. Sample buffer (provided in kit) was diluted 20 times in MilliQ water and was added in duplicates as well. The plate was incubated at room temperature for an hour. Wash buffer (provided in the kit) was diluted 100 times with MilliQ water. After an hour of incubation, the plate was washed 4 times (300ul/wash) using the wash solution. The plate was tapped on fresh paper towels after every wash to remove any residual water. After washing the plate, 50 µl of the Antibody-HRP Conjugate (anti-human IgA HRP, anti-human IgM HRP or anti-human IgG HRP) diluted 100X in sample buffer was added to each wells and incubated in the dark for 30 minutes. After 30 minutes of incubation, the plate was washed 5 times using the wash solution. Then, 50 µl of chromogenic TMB solution (provided in kit) was added and place the plate placed in t a dark place and was for incubated incubation for 15 minutes at room temperature. After Once the 15 minutes is up, 50 µl of the stop solution (sulphuric acid- provided in kit) was added to each well. Lastly, The OD was read at 450nm within 30 minutes of stopping.

3. Results

3.1 Recruitment and the Demographic characteristics of the participants

The protocol was developed according to the objectives of the study. As this is a non - invasive sample collection and the participants collected all the samples at home according to the instructions given, there was no need for medically trained personnel. Thus, the protocol was submitted to Parkway independent ethics committee.

(Table 3.1) describes the following demographic variables of the 86 participants: age; gender; ethnicity; and salivary flow rate.

Participants were aged 0 - 50 years old, with a mean age of 21 years. The ethnic groups comprise 27 Chinese, 3 Malays, 52 Indians and 4 Others /Mixed. There were 38 males and 48 females. The participants exhibited an average saliva flow rate of 644 $\mu\text{l}/\text{min}$ with a range of 100.6 – 1686 $\mu\text{l}/\text{min}$. No participant demonstrated xerostomia (dry mouth condition) as the flow rate for each participant was above 100 $\mu\text{l}/\text{min}$.

3.2 RSV specific Ig M, G and A in saliva of healthy individuals

The term, 'RSV responders', was used on those subjects who have measurable RSV specific Igs in their saliva samples. There are 40 RSV IgA responders, 39 RSV IgG responders and 41 RSV IgM responders among the 86 participants. The standard curve for RSV IgA, IgG and IgM subtype are shown In Figures (3.2A, 3.2C and 3.2E) respectively. The concentration of each RSV Ig subtype in saliva was established based on the corresponding standard curve and are illustrated in Figures (3.2B, 3.2D and 3.2F).

46.5% (40/86) of participants have RSV specific IgA in saliva. The RSV IgA standard curve ranges from 25-500 µg/ml (Figure 3.2A). The average concentration of RSV IgA was 202 µg/ml. The detectable range is 28 - 1300 µg/ml in saliva samples among the responders, (Figure 3.2B).

The RSV IgG standard curve ranges from 20-400 µg/ml (Figure 3.2C). 45.3% (39/86) of participants have detectable RSV IgG in saliva. The detectable range is 23 - 1105 µg/ml in saliva samples in the responders. There was only one individual with a concentration of 1105 µg/ml, who was also the highest among the RSV specific IgA responders. The rest of individuals with positive response fall within the range of 23-336 µg/ml. The average concentration of RSV specific IgG is 139 µg/ml (Figure 3.2D).

RSV IgM standard curve ranges from 50-800 µg/ml (Figure 3.2E). 47.7% (41/86) participants have detectable RSV specific IgM in saliva. The detectable range is 71 - 2594 µg/ml in saliva samples in the responders. The average concentration of RSV specific IgM is 681 µg/ml (Figure 3.2F). The concentration of RSV IgM antibody responses was the highest among all Ig types.

RSV specific IgA, IgG and IgM were not detected in any of the stool samples tested.

3.3 RSV specific Ig A, G and M in saliva of healthy individuals and their age groups

RSV specific Igs were measured in all 86 participants. The 40 RSV IgA responders, 39 RSV IgG responders and 41 RSV IgM responders were categorized according to age. The youngest and oldest participants are 0.6 and 50 years old respectively.

The groups were arbitrary divided into 0-3 years, representing toddlers group, 4-6 years representing preschoolers, 7-10 years representing young children and 11-19 years.

Variables		Number of subjects
Male		38
Female		48
Chinese		27
Malay		3
Indian		52
Others/Mixed		4
Children		38
Adults		48
Parameters	Values	Mean, Median
Age	0 - 50 years	21, 23
Flow Rate (µl/min)	100.6 - 1686 µl/min	644 µl/min, 575 µl/min

Table 3.1: Demographic variable.

The number of participants according to variables including ethnic groups (Chinese, Indian, Malay and Others/Mixed) and genders (Male and Females) are summarized. The table also summarizes the ranges, mean and median of age and saliva flow rates.

Figure 3.2: RSV specific Igs standard curves and ranges in saliva samples.

($\star$) represents every RSV specific IgA (A), IgG (C), IgM (E) standard curve points. The Y axis in IgA (A), IgG (C), and IgM (E) represents the absorbance at 450nm. The X axis in IgA (A), IgG (C), and IgM (E) represents the concentration of each standard point in a log scale.

($\bullet$) represents every RSV specific IgA (B), IgG (D), IgM (F) responders and the corresponding concentration. The figures represent the number of RSV specific IgA (B), IgG (D), IgM (E) responders. The Y-axis represents the concentration of RSV specific IgA (B), IgG (D), IgM (D) in $\mu\text{g/ml}$ in a log 2 scale.

(A): The RSV IgA standard curve has a range of from 25-500 $\mu\text{g/ml}$. (B): There are 40 RSV IgA responders among the 86 participants. The detectable range is 28 - 1300 $\mu\text{g/ml}$ in the saliva samples among the responders. (C): The RSV IgG standard curve has a range of from 20-400 $\mu\text{g/ml}$. (D): There are 39 RSV IgG responders among the 86 participants. The detectable range is 23 - 1105 $\mu\text{g/ml}$ in the saliva samples among the responders. (E): The RSV IgM standard curve has a range of from 50-800 $\mu\text{g/ml}$. (F): There are 41 RSV IgM responders among the 86 participants. The detectable range is 71 - 2594 $\mu\text{g/ml}$ in the saliva samples among the responders.

The rest of the participants were distributed in age ranges of 10 years there forth starting with 20 years of age. The last age range is 41-50 years old. A child 3.5 years old would fall under 0-3 years of age group category. As the number of participants in each age group was not evenly distributed, percentages were chosen to represent RSV antibody responses instead of absolute numbers.

(Table 3.3) captures the summary of the number of RSV responders according to different age groups as well as the gender types within each age group. The number of RSV specific IgA, G and M in each age group is captured. Most of the participants were in the 30-40 years old age group (n=28) and the least number of participants were from the 41-50 years old age group (n=6). The range and median of saliva flowrates ($\mu\text{l}/\text{min}$) of each age group were also classified.

The percentage of each RSV response according to age is depicted in (Figure 3.3A).

The results show that there are less than five responders of RSV saliva responders in early life from 1-6 years of age. There is a slight increase in responders between 7-10 years of age. However, there are less than five RSV incidences in this group. A steady increase of RSV responders is observed after 10 years of age. An increasing percentage in RSV antibody responses is observed from 10-30 years old. The highest peak is at 20-30 years old and the percentage of RSV antibody response starts to decline from 30 years onwards and increases after 40 years of age. It was noted that the mean of saliva flowrate increases with age from 0-30 years of age and decreases then onwards. There is a similar trend of salivary flowrate with the percentage of RSV responders in each age group. It can be established that RSV antibody responses increases with age initially and peaks around 20-30 years of age though it would be relevant to have the same number of participants in each age group to make a fair analysis.

Age (years)	All Subjects (N = 86)	Male (N =38)	Female (N =48)	RSV IgA Pos (N =40)	RSV IgG Pos (N=39)	RSV IgM Pos (N = 41)	Flow Rate (μ l/min) (Mean)
0 to 3	14	8	6	3	2	2	100.6 – 905 (402 μ l/min)
4 to 6	9	7	2	1	1	1	128.1 - 818.8 (440 μ l/min)
7 to 10	9	7	2	3	3	3	275 - 1210 (727 μ l/min)
11 to 19	6	1	5	4	4	4	550 – 1173 (915 μ l/min)
20 to 30	14	2	12	10	10	10	314 - 1613 (986 μ l/min)
31 to 40	28	10	18	15	15	17	121 - 1686 (632 μ l/min)
41 to 50	6	3	3	4	4	4	330 – 1100 (624 μ l/min)

Table 3.3: Summary of RSV responses (absolute number) according to age groups.

The distribution of the absolute number of participants according to different age groups and the number of male and female per age group totaling to 86 subjects is tabulated. The table shows the number of RSV specific IgA, IgG and IgM responders in age group category. The ranges and mean of saliva flowrate for each age group category are displayed in the table.

Figure 3.3: Percentage of RSV antibody response between all the different ages range

Percentage of each RSV antibody response is represented by: RSV specific IgA (■), IgG (■) and IgM (■). responders are stratified according to age groups into seven age groups ranging from 0-50 years old (X-axis). Each (●) stands for the mean salivary flowrate for each age group. The right Y-axis represents the salivary flowrate in millimeters/minute (ml/min).

A: The left Y-axis shows the absolute number of RSV antibody responders. There are a total of 41 RSV antibody responders. Most of the responses ($n=16$) are observed in the age range of 31-40 years and the least is observed in the 4-6 years range.

3.4 RSV IgA, IgG and IgM response with age

There is a positive linear relationship *between* age and RSV specific IgA levels of a slope of 2.802 and a P value of 0.047 (<0.05) indicating that there is significant trend with age in (Figure 3.4.1A). The coefficient of determination, R-squared, of this linear regression

model is 0.046, showing that 4.6% of RSV IgA response variability is determined by age. Similar to that result of RSV specific IgA, a positive linear relationship between age and RSV specific IgG levels of a slope of 2.468 and a P value of 0.013 (<0.05) is observed in (Figure 3.4.1B), indicating that a significant trend of RSV IgG responses with age. The coefficient of determination, R-squared for RSV IgG with age is 0.072. Thus a variability of 7.2% of RSV IgG is determined by age. Again, similar results were observed for the RSV IgM response, with a R-squared of 0.093, indicating a variability of 9.3% of RSV IgM is determined by age. There was a positive linear relationship between age and RSV specific IgM levels of a slope of 11.66 and a P value of 0.004 (<0.05) and hence this is a significant trend, (Figure 3.4.1C).

Hence, it can be concluded that there is a positive relationship between age and RSV Igs and RSV Ig responses increase with age.

These observations have triggered the question whether RSV antibody response is lower in children and further help us understand the immune status between children and adults. The participants have been divided into two groups: children (below 19 years) and adults (19 years and above). As mentioned previously, the age cut off was chosen based on the definitions of children and adults by the WHO (113). It is to be noted that there is no significant difference between genders in both children and adult group. Based on the T-test, it has been found that there is significant difference of RSV specific IgA, IgG and IgM between children's levels and that of adults, (Figures 3.4.2A, 3.4.2B and 3.4.2C).

The P values (Mann Whitney) are determined between children and adults against the levels of RSV specific Igs. The P values (<0.05) are significant with all 3 results: 0.003, 0.0006, and 0.0003 for RSV specific IgA, IgG and IgM respectively. It can be concluded that RSV Igs responses increase with age and also, that the levels of RSV antibody responses are higher in adults than children.

Figure 3.4.1: Age in relation to RSV specific Igs.

(•) represents every RSV specific IgA (A), IgG (B), IgM (C) value (responders and non-responders) according to their age (X-axis). The X-axis represents the age of the responders and non-responders. The Y-axis represents the concentration of RSV specific IgA (A), IgG (B), IgM (C) in $\mu\text{g/ml}$.

(A): There is an upwards slope of 2.802, linear regression analysis gave a P value of 0.047 (<0.05) and a R^2 of 0.046. (B): There is an upwards slope of 2.468 with a P value of 0.013 (<0.05) a R^2 of 0.072. (C): There is an upwards slope of 11.66, there is a P value of 0.004 (<0.05) with linear regression analysis with a R^2 of 0.093.

Figure 3.4.2: Comparison of RSV specific Igs between Children vs Adults.

(●) represents the children (below 19 years old) (A – C), (■) represents the adults (above 19 years old) (A – C). A two tailed T- test was used to analyze RSV Igs between children and adults. The X-axis represents the age of the subjects while the Y-axis represents their levels of RSV specific IgA (A), IgG (B), IgM (C) concentration in $\mu\text{g/ml}$ in saliva samples. (A): P value was determined as 0.003 (<0.05) between children's and adults' RSV IgA response levels. (B): P value was determined as 0.0006 (<0.05) children's and adults' RSV IgG response levels. (C): P value was determined as 0.0003 (<0.05) children's and adults' RSV IgM response levels.

3.5 RSV IgA, IgG and IgM response with gender

Next, we compared males' and females' antibody responses to RSV. RSV specific IgA, IgG and IgM responses were compared among 48 females and 38 males. T-test analysis was done to compare and determine any significant difference with genders and RSV Ig responses. Using Mann Whitney test, it was shown that the P value did not fall below <0.05 for all 3 RSV Igs. Hence, it was concluded that RSV IgA, IgG and IgM responses do not depend on gender illustrated in Figure (3.5 A-C).

Figure 3.5: Comparison between gender and RSV specific Igs levels. (•) represents the males (A – C) and (■) represents the females (A – C). The figure depicts the gender of the subjects (X-axis) and their levels of RSV specific IgA (A), IgG (B), IgM (C) concentration in $\mu\text{g/ml}$ (Y-axis) in saliva samples. (A): Using Mann Whitney test, it was shown that the P value >0.9999 . (B): Using Mann Whitney test, it was shown that the P value 0.8. (C): Using Mann Whitney test, it was shown that the P value 0.97.

3.6 RSV IgA, IgG and IgM response to ethnicity

A total of 27 Chinese, 3 Malays, 52 Indians and 4 mixed subjects participated in the study. The percentage of RSV responders in saliva among ethnic groups in Singapore which comprise of Chinese, Indian, Malay and others are depicted in Table (3.6) and illustrated in (Figure 3.6.1). There are a total of 18 Chinese participants and 21 Indian who are positive for RSV-specific antibodies as well as two Malay participants and one from other ethnicities. As more than half of the subjects are Indians and the number of participants in the Malay and the other ethnic group is too little to make any further analysis, a comparison was done to observe any difference between the 52 Indian and 27 Chinese responders. The average age of Indian and Chinese groups were calculated as 19 and 25 years of age respectively. Using Mann Whitney analysis, P value between Indians' and Chinese's ages was calculated as 0.1181, showing that age differences between these two groups is insignificant. Hence the age in this scenario do not impact the ethnicity based results.

Using non-parametric T-test, the levels of the RSV specific IgA, IgG and IgM in saliva in Indian and Chinese groups were analyzed. It was shown that there is a significant difference with a P value (<0.05) between Indian and Chinese population for all the RSV specific antibody responses. P values of RSV IgA, IgG and IgM between Indian and non-Indian groups were determined using Mann Whitney tests and are 0.0102, 0.0119 and 0.0036 respectively using two tailed P value. These data are illustrated in (Figures 3.6.2 A-C), showing that there are significant differences between Indian and Chinese populations to RSV antibody responses. Based on the results, we speculate that ethnicity may impact the levels of RSV specific antibody responses and needs further validation with a larger cohort to determine this hypothesis.

Ethnicity	All Subjects (N = 86)	Male (N = 38)	Female (N = 48)	RSV IgA Positive (N = 40)	RSV IgG Positive (N = 39)	RSV IgM Positive (N = 41)
Chinese	27	11	16	16	17	18
Malay	3	2	1	2	2	2
Indian	52	24	28	21	19	20
Others/Mixed	4	1	3	1	1	1

Table 3.6: Number of RSV saliva responders in different ethnic group.

The classification of the 86 participants according to the Chinese, Malay, Indian and other/mixed ethnic groups. Each ethnic group is further subdivided into gender groups, males and females. The number of RSV specific IgA, IgG and IgM responders among each ethnic group are also categorized in the table.

Figure 3.6.1 Percentage of salivary RSV specific antibody responders in different ethnic groups.

The Y axis represents the percentage of RSV responders according to Chinese, Indians, Malays and Other/mixed ethnic groups (X axis). Each responder (%) is color coded by RSV specific IgA (blue), IgG (red) and IgM (green).

Figure 3.6.2: The prevalence of RSV specific Igs in saliva among Indian and Chinese ethnic groups.

Chinese (●) (n=27), Indians (■) (n=52) groups displayed (A – C). The figure depicts the values of RSV specific IgA (A), IgG (B), IgM (C) concentration in µg/ml (Y-axis) among different ethnic groups (X-axis). The distribution and mean were used to determine the P value. The statistical test used is non-parametric T-test and it is a two-tailed test. (A): There is a significant difference between Chinese and Indian populations of a P value 0.0102 (B): There is a significant difference between Chinese and Indian populations of a P value 0.0119. (C): There is a significant difference between Chinese and Indian populations of a P value 0.0036.

3.7 The types of RSV IgM, IgA and IgG responses

Next, the relationship between the antibody responses was analyzed. Percentage of

responders was calculated for triple positive (IgM, IgA and IgG positive), double or single positive and non-responders and illustrated in (Figure 3.7). It was observed that 44.2% (n=38) have all 3 RSV responses (IgM, G, and A) while 51.16% (n=44) are non-responders. RSV specific IgM response is observed in 37/38 of all subjects that have RSV response in saliva samples. In one RSV responder where RSV specific IgM is not detected, only RSV specific IgA response is observed. One subject has the presence of only RSV specific IgM, suggesting a recent infection or exposure. Two subjects have RSV IgM but only have one of either RSV specific IgA/G respectively.

Figure 3.7: The Subtypes of RSV responses in saliva.

The X axis represents the combinations of RSV Igs responses including the responders with all three RSV Igs and non-responders in saliva. The Y axis represents the number of subjects. The percentage of each of combination of RSV responders out was determined.

3.8 A paired comparison between plasma and saliva samples

15 healthy adult participants also consented for blood collection for plasma. Thus, the same analysis could be performed for their plasma and saliva to compare the responses. The participants gave saliva and blood on the same day to have a fair assessment of their immune status with regards to RSV responses. The concentration of RSV specific IgA, IgG and IgM in their plasma and saliva were measured, (Table 3.8).

The results revealed the presence of RSV specific Igs in all 15 plasma samples but only in 10 saliva samples. There are five subjects who are non-responders (not detectable levels) in saliva but have results with plasma. In general, the concentrations of RSV specific Igs are higher in plasma compared to saliva samples. Hence the distributions of RSV specific IgM, IgA and IgG in saliva and plasma have been compared. We have plotted the distribution in saliva for all 42 responders and for all 15 plasma tested samples

Out of the 86 saliva samples tested, 42 subjects have RSV antibodies in their saliva samples. Of these RSV responders, 97.6% (n=42) have RSV specific IgM, 95.2% (n=40) have RSV specific IgG and 92.9% (n=40) have RSV specific IgA. The highest concentration was observed for RSV IgM in saliva. The levels of RSV specific IgA and IgG are observed to be somewhat similar in general though some subjects have higher RSV specific IgA levels as compared to RSV specific IgG in their saliva, (Figure 3.8A).

All 15 subjects tested have RSV antibodies in their plasma samples. The range of RSV specific IgA is between 609 - 13667.5 µg/ml, 325 - 8724 µg/ml for RSV specific IgG and 1001 - 14323 µg/ml for RSV specific IgM. The distribution of RSV antibodies in plasma has a similar trend compared to the distribution of RSV antibodies in saliva. Similar to the observation in saliva, RSV IgM had the highest concentration in plasma. Levels of RSV specific IgA in plasma are the next followed by concentration of RSV specific IgG. It was

observed that each of the individual has higher concentration of RSV Igs in plasma compared to saliva, (Figure 3.8B). The 15 paired plasma and saliva samples were plotted as well. It is observed that RSV antibody responses are higher in plasma samples compared to saliva samples (Figure 3.8C).

		Saliva Response			Plasma Response		
Subject ID:	Age (years)	RSV IgA (µg/ml)	RSV IgG (µg/ml)	RSV IgM (µg/ml)	RSV IgA (µg/ml)	RSV IgG (µg/ml)	RSV IgM (µg/ml)
411	24	1218	2722	524	13668	8724	14323
2013	35	NDL	NDL	NDL	4707	5667	9538
1823	36	NDL	NDL	NDL	609	425	1001
910	34	45	25	231	783	459	1223
126	50	1300	1105	2304	4959	3056	6928
308	42	NDL	NDL	NDL	963	488	1425
167	45	NDL	NDL	NDL	789	457	1147
1010	36	145	70	664	1021	620	1744
1921	34	494	110	592	941	543	1508
123	34	115	106	697	830	910	1763
214	40	52	31	268	868	948	1716
747	37	NDL	NDL	NDL	1622	1557	4152
7325	33	499	254	838	5361	6816	11316
DS11	27	147	65	308	735	325	1539
DS16	44	403	352	846	2014	1760	4231

Table 3.8: The concentration of RSV antibodies in 15 paired plasma and saliva subjects.

The table summarizes the results of RSV specific IgA, G and M responses in saliva and plasma samples of 15 adult subjects aged between 24-50 years old. Ten of the fifteen subjects have both saliva and plasma results. The term NDL (Not Detectable Levels) observed in five responders who do not have saliva RSV responses but have RSV Igs response in plasma.

Figure 3.8: Distribution of RSV IgA, IgG and IgM concentration in saliva (A) and plasma (B) and paired plasma and saliva samples in 15 RSV responders (C)

The concentration of each RSV antibody response is annotated as IgA (●), IgG (▲) and IgM (■) (A – B). The Y-axis represents the concentration of RSV antibody response in µg/ml. The X-axis represents the responders for RSV responses.

(A): Types of RSV specific IgA, IgG and IgM are distributed in 42 RSV positive responders' saliva samples.

(B): Types of RSV specific IgA, IgG and IgM are distributed in 15 RSV positive responders' plasma samples displayed.

(C): The concentration of each antibody response in 15 saliva samples is annotated as IgA (●), IgG (■) and IgM (▲) and RSV antibody response in the corresponding plasma samples as IgA (●), IgG (■) and IgM (▲). The Y-axis represents the concentration of RSV antibody response in µg/ml. The X-axis represents the RSV response in 15 paired saliva and plasma samples.

3.9 Correlation of RSV Igs subtypes in saliva and plasma

The levels of RSV specific IgA, IgG and IgM concentration were plotted against each other to understand any correlation/trends in each sample type - plasma and saliva responders. Exploring this method would also give an idea if the measurement of one or two RSV specific Igs might be sufficient for future analysis.

In saliva samples, there is a strong positive correlation between RSV specific IgA and IgG levels of R^2 of 0.8, (Figure 3.9.1A). There is a weak positive correlation between RSV specific IgG and IgM with a R^2 of 0.4 in saliva samples, (Figure 3.9.1B). When RSV specific IgA is compared with IgM, it was observed that there is a weak positive correlation of with a R^2 of 0.3, (Figure 3.9.1C). As the matrix of saliva and plasma are different, the correlation of each antibody response within saliva varies compared to that of plasma. It can be hypothesized that salivary RSV IgM is likely to be local production and hence is in higher concentration in saliva compared to salivary RSV IgA or G

There is a strong correlation of a R^2 of 0.9 and above between all RSV Igs subtypes in all 15 plasma samples. Correlation between levels of RSV specific IgA and IgG reveals that R^2 is 0.9, (Figure 3.9.2A). Levels of RSV specific IgG and IgM have a very strong correlation of R^2 of 0.98, (Figure 3.9.2B). The relationship between RSV specific IgA and IgM shows a R^2 of 0.9 in plasma samples, (Figure 3.9.2C).

In conclusion, there are strong correlations among RSV IgA, IgG and IgM subtypes in plasma, showing that there is a correlation of RSV IgM switching into IgG and IgA in plasma. On the other hand, in saliva there is no correlation of IgM switching to IgA or IgG. However, there is a strong correlation between RSV specific IgA and IgG, suggesting that one of the either measurement might be sufficient to understand RSV Ig responses in saliva.

Figure 3.9.1: RSV specific Igs correlations in saliva.

The point (●) represents the correlation between RSV specific IgA and IgG (A), IgG and IgM (B), IgA and IgM (C) in saliva.

(A): Levels of the RSV specific IgG in $\mu\text{g/ml}$ in saliva (Y-axis) is plotted against RSV specific IgA in $\mu\text{g/ml}$ in saliva (X-axis). The analysis shows there is a strong positive correlation of R^2 0.8 between RSV IgA and IgG in saliva. (B): Levels of the RSV specific IgM in $\mu\text{g/ml}$ in saliva (Y-axis) is plotted against RSV specific IgG in $\mu\text{g/ml}$ in saliva (X-axis). There is a positive correlation of R^2 0.4 between RSV IgG and IgM in saliva. (C): Levels of the RSV specific IgM in $\mu\text{g/ml}$ in saliva (Y-axis) is plotted against RSV specific IgA in $\mu\text{g/ml}$ in saliva (X-axis). There is a positive correlation of R^2 0.3 between RSV IgA and IgM in saliva.

Figure 3.9.2: RSV specific Igs correlations in plasma.

The point (●) represents the correlation between RSV specific IgA and IgG (A), IgG and IgM (B), IgA and IgM (C) in plasma.

(A): Levels of the RSV specific IgG in $\mu\text{g/ml}$ in plasma (Y-axis) is plotted against RSV specific IgA in $\mu\text{g/ml}$ in plasma (X-axis). Like saliva, the analysis shows there is a strong positive correlation of R^2 0.9 between RSV IgA and IgG in plasma.

(B): Levels of the RSV specific IgG in $\mu\text{g/ml}$ in plasma (Y-axis) is plotted against RSV specific IgM in $\mu\text{g/ml}$ in plasma (X-axis). There is a strong positive correlation of R^2 0.98 between RSV IgG and IgM in plasma.

(C): Levels of the RSV specific IgM in $\mu\text{g/ml}$ in plasma (Y-axis) is plotted against RSV specific IgA in $\mu\text{g/ml}$ in plasma (X-axis). There is a strong positive correlation of R^2 0.9 between RSV IgA and IgM in plasma.

3.10 Relationship of plasma and salivary RSV specific IgA, IgG and IgM

Using the results from RSV specific IgA, IgG and IgM in 15 paired saliva and plasma samples, the relationship of RSV responses in these two samples were established, (Figures 3.10A, 3.10B and 3.10C). There is a moderate correlation of 56.5% ($R^2=0.56$) between the plasma and saliva levels for RSV IgA using Pearson analysis. Using linear regression, it was shown that there is a significant difference of P value of 0.001 (<0.05) in RSV IgA in saliva and plasma samples. For RSV IgG, there is a correlation of 49.7% ($R^2=0.5$) between the plasma and saliva levels using Pearson analysis. Using linear regression, it was shown that there is a significant difference of P value of 0.003 (<0.05) in RSV IgG in saliva and plasma samples, similar to the results to that of RSV specific IgA.

However, there is a little correlation ($R^2=0.081$) between the plasma and saliva levels for RSV specific IgM levels unlike RSV IgA and IgG. Using linear regression, it was shown that there is also no significant difference of RSV IgM in saliva and plasma sample with a P value of 0.3 (>0.05). Based on the analysis, we can conclude that there is a linear relationship between plasma and saliva samples. RSV specific antibody responses are in higher levels in plasma compared to saliva. There are significant differences in the levels of RSV specific IgA and IgG between saliva and plasma.

Also, there are correlations of more than 0.5 between saliva and plasma with the levels of RSV IgG and IgA responses. However, there is neither any significant difference nor little correlation with the levels of RSV specific IgM in plasma and saliva.

Figure 3.10: Relationship of RSV Igs levels in paired plasma and saliva samples.

The point (•) represents the levels of RSV specific IgA (A), IgG (B), IgM (C) in $\mu\text{g/ml}$. (A): Levels of the RSV specific IgA in $\mu\text{g/ml}$ in saliva (Y-axis) is plotted against RSV specific IgA in $\mu\text{g/ml}$ in plasma (X-axis). The analysis shows a significant difference with a P value of 0.001 and a R^2 of 0.56 between plasma and saliva results. (B): Levels of the RSV specific IgG in $\mu\text{g/ml}$ in saliva (Y-axis) is plotted against RSV specific IgG in $\mu\text{g/ml}$ in plasma (X-axis). The analysis shows a significant difference with a P value of 0.003 and a R^2 of 0.5 between plasma and saliva results. (C): Levels of the RSV specific IgM in $\mu\text{g/ml}$ in saliva (Y-axis) is plotted against RSV specific IgM in $\mu\text{g/ml}$ in plasma (X-axis). The analysis shows there is no significant difference with a P value of 0.3 and no correlation with a R^2 of 0.081 between plasma and saliva results.

3.11 Kinetics of RSV IgM, IgA, IgG responses in saliva

The kinetics of RSV Ig responses in saliva was studied by collecting and measuring the responses within the same subjects at two time points a year apart (2015 and 2016). This is displayed in (Table 3.11). The 20 saliva samples consisted of samples belonging to eight children (below 19 years old) and 11 adults (above 19 years old). The results show that three of the eight children with RSV responses in 2015 but not in 2016. This suggests that the responses in children's saliva are short-lived. The other five children have no detectable levels in both year and hence considered as non-responders. Four adults, who had results in 2015, had RSV responses in 2016. The concentrations of these responses were almost similar or higher in 2016 than 2015. It can be inferred that salivary RSV antibody responses in adults may be more lasting than children. However, three adults, who did not have response in 2015, had responses in 2016, indicating possibly new infections in 2016.

3.12 Validation of the use of saliva samples in quantitative ELISA kit

The ELISA kit used in this study is quantitative. The ELISA employed the use of RSV anti - antibody coated onto a 96 well microtiter plate. Six RSV peptides were used to raise antibodies that will recognize RSV Igs. The RSV anti -Ig coated on the plate \ captures RSV specific Ig and bind to it. This is in turn detected by anti-human Ig isotype conjugated with HRP, hence making the quantitation of the RSV Igs possible. As these assays are meant for the quantitative measurement of RSV specific Igs n serum/plasma, the use of saliva was optimized on the ELISA kits. Spiking and recovery as well as reproducibility assays were carried out. Furthermore, comparisons were done with another RSV ELISA kit.

Subject ID	Age (2015)	RSV IgM (µg/ml) (2015)	RSV IgG (µg/ml) (2015)	RSV IgA (µg/ml) (2015)	RSV IgM (µg/ml) (2016)	RSV IgG (µg/ml) (2016)	RSV IgA (µg/ml) (2016)
1812	1	399	140	37	NDL	NDL	NDL
118	2	NDL	NDL	NDL	NDL	NDL	NDL
521	4	NDL	NDL	NDL	NDL	NDL	NDL
1113	4	229	147	228	NDL	NDL	NDL
1813	4	NDL	NDL	NDL	NDL	NDL	NDL
2612	4	NDL	NDL	NDL	NDL	NDL	NDL
110	7	NDL	NDL	NDL	NDL	NDL	NDL
1919	7	183	23	66	NDL	NDL	NDL
419	20	NDL	NDL	NDL	327	342	110
7325	32	838	254	499	765	668	981
1921	33	263	124	195	592	110	494
1307	34	NDL	NDL	NDL	NDL	NDL	NDL
2013	34	NDL	NDL	NDL	403	ND	348
316	35	NDL	NDL	NDL	61	ND	46
1823	35	NDL	NDL	NDL	NDL	NDL	NDL
1907	35	NDL	NDL	NDL	NDL	NDL	NDL
1010	36	85	23	ND	664	70	145
747	37	NDL	NDL	NDL	NDL	NDL	NDL
214	40	268	31	52	652	687	698

Table 3.11 Kinetics RSV antibody response.

The table indicates the subject ID, their corresponding age in 2015 and the antibody responses in two time points. There are 19 subjects including eight children and 11 adults. Kinetics of RSV antibody responses was established by measuring salivary RSV antibody at two points - 2015 and 2016. Two sets are RSV IgA, IgG and IgM responses in (µg/ml) (2015 and 2016) for each subject are measured and compared. NDL (not detectable) represent RSV non-responders.

3.12.1 Spiking and Recovery of standards

As saliva is of a different matrix as compared to plasma/ serum, known concentrations of standards were added to three non-responsive saliva samples (#626, #440, #110) to observe the recovery rates percentage and determine if saliva is suitable for use in the kits. Three levels of concentrations of each RSV Ig standards - high, medium and low were selected to validate the spiking and recovery assays. Spiking and recovery was done two rounds to ensure that saliva matrix was not interfering with the measurement of RSV specific Igs levels. The averages of the spiking of each RSV Ig were calculated and plotted in (Figures 3.12.1A, 3.12.1B and 3.12.1C).

The results show that 95 % of RSV IgA standard was recovered at a high concentration. A recovery rate of almost 70% was obtained using at medium concentration. At a low concentration, RSV IgA has a recovery rate of 72.5%. The error bars represent the standard deviation of each concentrations level ($\mu\text{g/ml}$) of RSV Ig (High, Medium and Low) as 12.9, 3.2 and 11.3 respectively. A similar trend like RSV IgA was observed in RSV IgG spiking and recovery assays. An average of 105% of RSV IgG standard recovery was observed at a high concentration. A recovery rate of 75% was obtained using at medium concentration. At a low concentration, RSV IgG has a recovery rate 87%. The standard deviation of each concentrations level ($\mu\text{g/ml}$) of RSV IgG (High, Medium and Low) is 4.3, 5.8 and 0.7 respectively as depicted by the error bar. With RSV IgM spiking, a declining recovery pattern is observed as the standards are diluted. The more the standards are diluted in saliva, the lesser the recovery rates for RSV IgM. The recovery rates are between 77-103 % in all three levels of RSV IgM standard concentrations. The standard deviation of each concentrations levels ($\mu\text{g/ml}$) of RSV IgM (High, Medium and Low), which is represented by the error bars in the figure is 1.3, 19.5 and 9.3 respectively. It is observed that RSV Igs standards have a high recovery rate of

between 70-105% when it is spiked in saliva. Thus, it can be concluded that saliva is a suitable fluid to be used in these kits and saliva does not interfere with assay performance.

Figure 3.12.1: Spiking and recovery of RSV Igs standards in saliva.

Each average point of RSV IgA (A), IgG (B), and IgM (C) spiking and recovery in 3 saliva samples is represented by (●).

The mean recovery percentage % is on the Y-axis and concentrations levels ($\mu\text{g/ml}$) of RSV Ig (High, Medium and Low) were represented on X-axis. The averages of two rounds of spiking and recovery plotted. (I) represents the error bar which shows the standard deviation of each concentration level (High, Medium, Low). (A): The standard deviation of each concentration level (High, Medium, Low) is 12.9, 3.2 and 11.3 respectively. (B): The standard deviation of each concentration level (High, Medium, Low) is 4.3, 5.8 and 0.7 respectively. (C): The standard deviation of each concentration level (High, Medium, Low) is 1.3, 19.5 and 9.3 respectively.

3.12.2 Reproducibility assays

Assays were repeated on eight RSV responders to determine the reproducibility of the assays. The assays were repeated at least thrice to assess the reliability and reproducibility of the assay. The repeatability of the assay also depends on the stability of the kits, samples, suitability of the sample types as well as operators. The Coefficient of Variation percentage (CV)% was calculated to assess the reproducibility in (Figure 3.12.2 A-C).

Six samples that were tested for RSV specific IgA, had a CV% of less than 30%. Two samples had a higher CV value of 40%. Both samples with a high CV% had one occasion where the values differ markedly from the other two results, thus creating a high CV%. Six samples that were tested for RSV specific IgG had a CV% of less than 30%. Thus, RSV specific IgA assay is reproducible in 75% of the samples tested where CV is 30% or below. Like RSV specific IgA, the results of two samples have high CV%. However, only in one of the three assays, the values of the samples were markedly different from the other two assays. It is noted that RSV specific IgG assay is reproducible in 75% of the samples tested.

Seven samples that were tested for RSV specific IgM had a CV% of less than 30%. One sample had a higher value of 50%. However, only in one of the three assays, the result of the sample was different from the other two assays. It is noted RSV specific IgM assay is reproducible in 87.5 % of the samples tested.

Sample #6 analysis is not reproducible with all three RSV specific Igs, implying that the quality of the samples may be compromised during collection or processing as this sample had a high CV% in all 3 RSV specific Igs reproducibility assays. It can be

concluded that this assay is repeatable in 75% of the samples with a reproducibility CV of 30% and below.

Figure 3.12.: RSV Igs reproducibility in saliva.

Each RSV specific IgA (A), IgG (B), IgM (C) response is represented by (●). The RSV specific Igs response in µg/ml of 8 samples repeated on three different occasions are plotted. On the Y axis, RSV specific Ig concentration in µg/ml is represented and on the X-axis, each responder is represented. Each figure has a table that summarizes the Coefficient of Variation CV% based on the results of respective RSV responses on the three occasions of each saliva sample.

3.13 Rotavirus antibodies versus RSV antibodies in saliva and stool samples

As rotavirus is transmitted via oral-faecal route, it is expected to have detectable rota antibodies in both saliva and stool samples. However, as RSV is a respiratory pathogen, it is not unusual not to find any RSV specific antibodies in stool but in saliva samples. Hence to prove this hypothesis, we collected paired saliva and stool samples. In this study, 65 of the 86 participants, aged between 0.6 years to 65 years, gave their stool and saliva samples for analysis for rotavirus and RSV antibodies. As the rotavirus ELISA is a qualitative assay, the results for RSV and rotavirus antibodies are reported as positive and negative in (Table 3.13 A-C). The results show that RSV specific IgA, IgG and IgM are not found in any of the 65 stool samples tested. The number of rotavirus specific IgA responders is the highest in saliva and stool samples. Rotavirus specific IgG responders is the next highest in saliva but not detectable in stool samples. Rotavirus specific IgM is found in saliva samples and only two stool samples which are also positive for rotavirus specific IgA.

53.8% of the participants have RSV specific IgA in their saliva but none of them have RSV specific IgA in their stool. 47.7% of them have rotavirus specific IgA in their stool and also have it in their saliva samples as well. 44.6% of the participants have rotavirus IgA in their saliva only. Neither rotavirus nor RSV specific IgG are detectable in stool samples. 53.8% of the participants have RSV specific IgG. 67.7% of the participants have rotavirus specific IgG. It was observed that all of those who have rotavirus specific IgG also have rotavirus specific IgA in their saliva. As previously described, the highest number of RSV responders in saliva samples are RSV specific IgM responders. No RSV specific IgM is detected in stool samples. Only two samples have rotavirus specific IgM in their stool. 33.8% have rotavirus specific IgM in their saliva. It was noted that those with rotavirus specific IgM have rotavirus specific IgA and IgG in their saliva.

In conclusion, no RSV specific Ig responses were detected in stool samples.

Table: 3.13A

RSV / Rotavirus IgA	Positive responders	Positive %	Sample type
RSV	35	53.8	Saliva
RSV	0	0	Stool
Rotavirus	60	92.3	Saliva
Rotavirus	31	47.7	Stool

Table: 3.13B

RSV / Rotavirus IgG	Positive responders	Positive %	Sample type
RSV	35	53.8	Saliva
RSV	0	0.0	Stool
Rotavirus	44	67.7	Saliva
Rotavirus	0	0.0	Stool

Table: 3. 13C

RSV / Rotavirus IgM	Positive responders	Positive %	Sample type
RSV	37	56.9	Saliva
RSV	0	0.0	Stool
Rotavirus	22	33.8	Saliva
Rotavirus	2	3.1	Stool

Table: 3.13A – C: Rotavirus/RSV Igs in saliva and stool.

The number of Rotavirus/RSV IgA (A), IgG (B), IgM (C) positive responders and the percentage of positive responders among 65 subjects in saliva and stool samples are summarized.

4. Discussion:

As people residing in Singapore get infected with viruses throughout the year, infected healthy individuals often display influenza like symptoms and are not treated appropriately. In a study to detect the incidences of Bocavirus infections in the pediatric population in Singapore, it has been shown that coinfection rate with RSV is significant. Furthermore it was shown that RSV was the most common respiratory virus in Singapore (107). Hence, it is important to assess the incidences of RSV infections among a healthy population in Singapore to allow accurate diagnosis.

In this thesis the detection of the RSV specific Igs in saliva samples of a healthy population is being explored. In order to recruit healthy human volunteers review and approval by an independent ethical board is necessary. Thus, for study conduct and ethical board's approval documents such as consent forms, assent forms, advertisement, study protocol, and saliva and stool collection instructions (see appendix) were designed. Once the ethical approval was received from Parkway Independent Ethical Committee, subjects were approached and recruited. (see Appendix for IRB details, questionnaire and Informed consent forms).

There were a few challenges in the study involving recruitment. Recruitment was done by word of mouth and hence it was easier to recruit more working professionals initially in 2015. However, as adults cannot solely be generalized to represent the whole population and RSV infection being a childhood burden, it became essential to recruit more children. As a result, more children below 10 years of age were recruited in 2016. One of the limitations of most studies is the absence of compliance with established collection protocol occasionally observed in saliva donors. Hence, following cautious steps were taken to ensure every subject adhere to collect samples within the instructed period. Firstly, saliva sample analysis being the main focus in the study, it was important

to standardize saliva collection method. Previously, a study revealed intense exercise immediately impacted immunoglobulin IgA and IgM levels in saliva and recovery occurs within 24 hours (115). Furthermore, timing of the saliva collection may be as crucial as shown by a study that salivary IgA concentration is elevated from midnight to dawn, indicating the reduced mechanism of saliva secretion during sleep impacts oral immune system (116). Hence it was necessary to standardize the time of collection and it was decided to collect only morning unstimulated saliva.

Finally, obtaining blood from our participants has been one of the biggest challenges in this thesis. As the IRB to collect blood was limited to 21 years and above, we were unable to collect any blood samples from our participants below 21 years. In general, participants were not open to blood drawing, thus further limiting comparison of blood with saliva. The use of saliva in this thesis to analyze and evaluate RSV antibody responses may give a better representation of the local mucosal immunity. As RSV infection takes place in the respiratory system, it would be necessary to assess mucosal immune responses in saliva or nasal aspirates/washes rather than the circulatory system. We chose saliva since it represents a non-invasive approach and the collection is more acceptable by the participants.

This ELISA (neo scientific) employs the method use of coating RSV anti-antibody that recognizes RSV specific antibodies. The anti-antibodies were raised using six RSV peptides to recognize RSV specific Igs. This novel method allowed us to quantify RSV antibodies in saliva. Matrix of saliva can affect the results as the interaction of saliva components could reduce the binding effect of the antibody to target. Thus, we performed spiking and recovery experiments to exclude this effect. A spiking and recovery range of 70-130% is usually accepted (130). Similar ranges were observed with the spiking and recovery results in this study. In addition, the assays were repeated three times and similar ranges were obtained. This further confirmed the application of Neo

scientific quantitative RSV ELISA kits in saliva. Hence it can be inferred that the kits from Neo scientific are specific, sensitive and suitable for the detection of RSV specific antibodies in saliva samples.

In a study to understand flow rates of unstimulated saliva in a healthy adult cohort from age 20- > 80 years of age, it was shown that there is a significant decrease of saliva flow rate as age progresses (117). In another study, where morning unstimulated salivary flow rates were compared in young healthy children between the age of four to seven, there was a significant increase of salivary flow rate in the older group of children. (118). Results from both studies show similar results in this thesis where the flow rates increase with age and declines gradually after 30 years of age in a healthy population. In this thesis, there seems to be similar trend in between salivary flow rate and RSV saliva responders where an increasing effect is seen with increasing age and a decline is observed afterwards.

Here, we have shown that RSV specific Igs responses in saliva are age dependent. There is a linear positive relationship between RSV specific Igs and age. Thus, the data was further divided into children and adults groups. The results revealed there are not only more adult RSV responders than children, the levels of RSV specific Igs are significantly higher in adults than children. Three out of the 14 children aged between 0 - 3 years, had RSV specific Igs response. Two of these responders were seven months of age and were being actively breastfed at point of sample collection. Literature shows that similar results with young children indicating that antibodies could have been maternally derived and/or could have been passively transferred from the mother's milk (79, 84). Thus, we cannot exclude that the salivary anti-RSV antibodies in this child was transferred from the mother. Only one other child under 3 years of age showed RSV Ig response suggesting that the antibodies can be acquired after a possible infection. In the pre-school age (4-6 years), there is one responder among 9 children tested. Overall,

there are seven out of 32 healthy (21.9 %) children below the age 10 years with RSV responses in saliva. To note that none of these children displayed any influenza-like symptoms or respiratory issues at point of collection. As part of an influenza vaccine efficacy trial, it was revealed that healthy Singaporean children aged six months to 10 years displaying influenza-like symptoms, having RSV infections (50). Hence, it is very likely that many healthy children in a developed country like Singapore might be not displaying any symptoms of RSV infection and it could have passed off as a case of influenza in many instances. We did not have comprehensively collected information about previous infections or any doctor diagnosis. The participants were deemed healthy if they declared themselves (or their children) healthy at point of sample collection. Our results coincides with a study conducted in Kenya where RSV was detected in 42% of asymptomatic individuals and a decline of infection was observed in adults peaking till 40 years and declining onwards (121). The presence of existing RSV antibodies in their saliva is an indication of past infections.

It has been shown that more than one RSV infection is needed to induce a complete immune response. Furthermore the decay of the antibodies within a short duration upon RSV infection, leads to insufficient protection and hence more prone to re-infections (119). This explains the reason why the RSV responses in saliva are observed more as age progresses. It is evident that multiple exposure of RSV in Singapore leads to better responses in adults than children. This is also supported by a recent study where age profiling was done to understand RSV antibody responses. It was demonstrated that children's antibody responses take months or even years to mature and multiple infections are needed (120). It is to be noted that the older adults/elderly may have a different RSV antibody response and hence we focused on a population below 50 years old. Indeed, a study by Sundaram et al has shed some light on RSV infections in older individuals and demonstrated that RSV infections are common

respiratory illness in that age category and the risk of RSV infections escalates with age (122).

Some studies show that breastfeeding confers more protection against RSV infection in females than males. The protection comes more from an activation of a process rather than the passive transfer of a soluble molecule from the mother. This activation process seemed more pronounced in females (85, 123). In adult population sexual hormones may also influence the immunoglobulin production by B-lymphocytes and however this needs further exploration (124). Yet, there are conflicting results as in some studies comparison of RSV infections or antibody responses among male and females were shown to be statistically insignificant (125, 126). Our results however have shown that gender does not impact RSV antibody responses in a healthy population in Singapore. It would be interesting, however, to look at gender differences in diseased or immunocompromised cohort in Singapore to further understand if same phenomenon would be observed.

Ethnic or race differences in RSV infection may be an important factor to consider. High rates of RSV infection in Māori and Pacific islanders' infants compared to other ethnic groups were observed in a New Zealand study (127). RSV related hospitalization rates were found to be higher in non-Hispanic race compared to other races in a Californian study where factors such as race and geographic locations were studied (128). However, it was shown that ethnicity did not impact the infection rates among the Malays, Chinese and Indians in Malaysia (129). Socioeconomic status could influence the rates of infections and the antibody responses among certain ethnic groups in some studies, understanding such differences might help in diagnosis and treatment of RSV infection. In this study, we show that there are significant differences between Indian and Chinese participants when the levels of RSV Igs were compared. It was observed that levels of RSV Igs responses were generally lower in Indian compared to

Chinese, indicating that there are differences in immune responses among ethnic groups. Even though comparing RSV responses between ethnic groups in Singapore was not an objective of this thesis, the results have revealed some interesting data that might be worth exploring further.

Most studies measure RSV specific IgA and IgG in serum and nasal sample during RSV infections (87, 90). There are not many studies that evaluate RSV antibody responses in saliva. Hence studying and comparing RSV specific Igs in saliva and plasma in healthy volunteers may give further clues on the exposure to the virus as well how the mucosal and systemic humoral immune responses cross-talk. The relationship of RSV antibody responses between paired plasma and saliva samples was evaluated in this thesis. RSV antibody responses were observed in all 15 adults plasma samples. However, only 10 of the 15 subjects (66.7%) have detectable salivary RSV Igs. Preexisting viral specific antibodies at infection site is the most reliable and efficient way to offer protective immunity against infections including that of RSV (134). In the case of infection or vaccination, serum antibodies will rise and can persist for years; however, the mucosal antibody responses are rather short-lived. In most scenarios, short-lived immunity is often linked with localized mucosal infections while systemic infection such as mumps and polio may induce antibodies that exist for a long period of time (135). The exact reason between the differences of the duration antibodies may be unclear. As antibodies produced by plasma cells are first line of defense, it is speculated that the levels of plasma cells and lifespan of plasma cells in serum originating from bone marrow and plasma cells at mucosal sites may differ. Moreover, the microenvironment of the site of plasma cells may play a part in the duration and thus affecting immunity (134). This phenomenon could explain the reason why the salivary RSV responses are not found in all samples despite the presence in plasma samples. In general, memory responses are essential for long lasting immunity and the B cell memory is more efficient in systemic

infection compared to a local mucosal infection (136). In a systemic infection, there is sufficient time for B memory cells to proliferate and differentiate, thus reinfection can be controlled better. Whereas in RSV infection, the duration taken for the virus to cause damage at site of entry at the respiratory tract is shorter; thus B cell memory is unable to fully develop and fully prevent clinical symptoms although re-infections are usually less severe (134). Thus, it probably takes repeated infections to develop lasting antibody responses in mucosal immune system. Therefore, it is not surprising that salivary RSV antibody responses are not detected even though responses in plasma samples (systemic) can be observed.

Levels of RSV IgM are higher compared to RSV IgG and IgA in both plasma and saliva. The presence of high level of RSV specific IgM in serum samples have been previously described in other studies especially as a marker of acute infection in both adult and children (131,132). RSV specific IgM in nasopharyngeal aspirates was found to be a good diagnostic marker of acute respiratory infections in children (133). Hence, it can be inferred that high levels of RSV IgM in saliva is good indication of acute or recent infection. However, it has been also observed that RSV specific IgM and IgA in children's serum are short-lived (132). This could explain the high number of salivary RSV Ig non-responders among the children in this thesis. Moreover, the analysis of the kinetics of RSV responses further supports this as it is observed that RSV antibody responses in children in 2015 do not last a year later in 2016. It is to be noted the RSV antibodies increase with age and with repeated infection (119), explaining the presence of salivary RSV antibodies two consecutive years in adult responders but not children in this study. It can be speculated that the detection of high titre of salivary RSV Igs in healthy volunteers may be a reflection of the Igs being potentially protective. As the assay quantifies the RSV antibody subtypes, it is not known as if the antibodies are neutralizing or confer protection. Hence it would be ideal to measure and compare neutralizing

antibody levels with detectable RSV antibody responses and to observe any correlation between high detection levels with high neutralizing levels

While there are strong correlations of 90% and above among RSV specific IgM, IgG and IgA in all 15 plasma samples, a strong correlation is only observed between salivary RSV IgA and IgG levels. When RSV IgM is compared with RSV IgA and IgG in saliva, only mild correlations of 30 and 40% are observed respectively. Moreover, when salivary IgM, IgG and IgA were compared with the corresponding Igs in plasma, moderate correlation between plasma and salivary RSV IgA and IgG were observed. However, there was no correlation between plasma and salivary RSV specific IgM, suggesting the local production of salivary RSV IgM. It has been previously demonstrated that salivary IgM is produced locally since the plasma cells were found to mainly reside at the salivary glandular stroma and hence the antibodies produced are able to actively diffuse across the epithelial transport system and enter into saliva (137). As IgM is a pentameric form, it is too large for passive diffusion into saliva from blood, thus further confirming that it is present in saliva due to a local production in the mouth (138). Our findings that RSV specific salivary IgM does not correlate with plasma IgM further support the idea the IgM is produced locally. The situation is however different to IgG and IgA. Some of the RSV specific IgG can be serum derived by passive diffusion through gingival crevices while some of them can be transported from salivary glandular, gingival, or tonsillar plasma cells (138). Though dimeric IgA is locally produced in the salivary glands, up to 77% of the monomeric IgA is demonstrated to be serum derived (138). These findings clarify the results of moderate correlation of RSV specific IgA and IgG in saliva and their plasma counterparts but not RSV IgM. With exception to one breastfed infant who had only RSV IgA response, the rest of the RSV antibody responders had RSV specific IgM responses.

The results of this thesis demonstrate that saliva could be used as a potential fluid in detecting RSV specific antibodies. Furthermore, the results propose that measuring RSV specific IgM itself in saliva may be sufficient to determine recent exposure or infection. Therefore, the standardized collection of saliva may provide a simple and non-invasive resource to provide insights to the measurement of RSV specific Igs.

The purpose of measuring and comparing rotavirus Igs with RSV Igs, is to demonstrate that different types of virus explicit varied antibody responses based on their transmission routes. While rotavirus is transmitted by fecal- oral route, RSV is transmitted via respiratory route before they replicate and invade the circulatory system. Although mucosal immune sites are found throughout the body, two factors play an important role. The inductive sites where initial immune responses are activated and the effector sites where plasma cells, viral specific humoral and cell mediated immune responses combating the virus differ at various mucosal sites. (139). Thus, RSV infections bring about the production of antibodies in saliva, lower and upper respiratory tract while rotavirus, being a gut pathogen, induces antibodies in oral (saliva) and the mucosal sites in the gut. Therefore, it is not surprisingly to detect rotavirus specific Igs in stool but not RSV specific Igs.

Understanding the mucosal immunity of viral infection sites will provide useful insights for future mucosal vaccination programs. Therefore, the measurement of the salivary RSV antibodies can be applied to determine vaccination efficacy and assess if subsequent vaccination may be needed. Given that host factors such as age and ethnicity disparities may impact RSV antibodies responses in saliva, it may be important to design studies to evaluate the responses that may in turn impact the development of targeted vaccines to address these issues.

5. Conclusion and future directions

In this thesis, standardized saliva and stool collection methods, approved by an ethics committee, were established. The ELISA method was also evaluated for the use of saliva samples by spiking and recovery methods and repeatability assays. Our findings in this study have shown that salivary RSV Ig M, G and A can be detected and the results propose that measuring RSV antibodies IgM alone in saliva might be adequate to determine RSV exposure or infection. The results further demonstrated that RSV humoral immune response is limited to the respiratory tract and does not reach the gut by the comparison of RSV and Rotavirus antibody responses. It is also clear the RSV antibodies in saliva do not fully reflect systemic antibody response, thus, measuring specific antibodies in saliva cannot fully substitute blood for assessment of humoral immune response to RSV.

Saliva sampling helps to address the challenges of measuring RSV antibodies in certain populations where blood drawing is not ethical or feasible and in surveillance studies where multiple time points of sampling might be necessary. Hence the detection of salivary RSV immunoglobulins has served as a proof of concept while addressing the ethical and practical issues in blood samplings.

For future studies, it would be important to determine if RSV antigens can be also detected in saliva and correlation between viral load and antibodies can be further established. This may give a better understanding of RSV antibodies development in saliva. It might be interesting to also explore if specific RSV antigens can be used to detect salivary antibodies.

Kinetic studies to establish the relationship between viral load and antibodies development will be also useful to further understand mucosal immunity. Future studies

to detect salivary RSV antibody responses in diagnosed infected individuals will shed more light on the development of salivary RSV antibodies. Furthermore, this method of measuring RSV specific antibodies in saliva can be applied in various clinical studies, for example, in longitudinal studies where it has been suggested that RSV bronchiolitis leads to later childhood asthma (27). As IgE-mediated effects are likely to be manifest in the respiratory mucosa, given that specific IgEs have been associated with the development of asthma and rhinitis,(144) it would be relevant to explore the possibility of measuring salivary RSV specific IgE in infected children. This would help to validate the relationship between early life RSV infections and asthma development.

In summary, the data demonstrate that RSV antibodies can be detected in saliva and this can be a non-invasive method that is promising and needs more exploration. This may help to address the challenges of measuring RSV antibodies in certain settings/populations and offer a non-invasive sampling method for clinical studies and vaccination programs.

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Appendix

Ref: PIEC/2014/036

11 February 2015

Dr Elena Sandalova
Danone Nutricia Early Life Nutrition
30 Biopolis Street #05-01B Matrix
Singapore 138671

Dear Dr Sandalova,

PARKWAY INDEPENDENT ETHICS COMMITTEE (PIEC) APPROVAL

Study Title: Understanding innate and adaptive immunological response in stool and saliva samples in healthy donors

We are pleased to inform you that the PIEC has approved the above research project to be conducted in the following study site(s) under expedited review:

- Danone Nutricia Early Life Nutrition:
 - Site PI: Dr Elena Sandalova
 - Co-Investigator(s): Ms Rekha Thiagarajan

Please note that this study can only be initiated after a Clinical Trial Certificate has been issued or the Health Sciences Authority has given a written notification that a Clinical Trial Certificate is not required.

The documents reviewed are:

- a) PIEC Application Form: Dated 23-Dec-2014
- b) Study Protocol: Version 1.1 dated 30-Jan-2015
- c) Saliva and Stool Sample Collection Participant Information Sheet and Informed Consent Form: Version 1.1 dated 21-Jan-2015
- d) Saliva and Stool Sample Collection Participant Information Sheet (Children) and Informed Consent Form: Version 1.1 dated 21-Jan-2015
- e) Child / Participant Assent Form: Version 1.1 dated 21-Jan-2015
- f) Advertisement: Version 1.0 dated 21-Jan-2015
- g) Instructions for Saliva and Stool Collection: Version 1.0 dated 15-Dec-2014
- h) Questionnaire: Version 1.0 dated 15-Dec-2014

- i) Curriculum Vitae: Dr Elena Sandalova, Ms Rekha D/O Thiagarajan
- j) CITI Good Clinical Practice Curriculum Completion Report: Dr Elena Sandalova, Ms Rekha D/O Thiagarajan

The approval period is from **11 February 2015 to 10 February 2016**. The reference number for this study is **PIEC/2014/036**. Please use this reference number for all future correspondence.

Continued approval is conditional upon your compliance with the following requirements:

1. Only the approved Participant Information Sheet and Consent Form should be used. It must be signed by each subject prior to initiation of any protocol procedures. In addition, each subject should be given a copy of the signed consent form.
2. No deviation from, or changes of the protocol should be implemented without documented approval from the PIEC, except where necessary to eliminate apparent immediate hazard(s) to the study subjects, or when the change(s) involves only logistical or administrative aspects of the trial (e.g. change of monitor or telephone number).
3. Any deviation from, or a change of, the protocol to eliminate an immediate hazard should be promptly reported to the PIEC within seven calendar days.
4. Please submit the following to the PIEC:
 - a. All Unanticipated Problems Involving Risk to Subjects or Others (UPIRTSO) should be reported. In order to assist the PIEC, all reports should be accompanied by the PIEC UPIRTSO Reporting Form (Local) or PIEC UPIRTSO Reporting Table Form (Overseas). If necessary, please contact PIEC Secretariat (tel: 6349 5747 or piec@parkway.sg) for all forms and guidelines on reporting.
 - b. Report(s) on any new information that may adversely affect the safety of the subject or the conduct of the study.
 - c. PIEC Study Status Report Form – this is to be submitted at least 4 weeks prior to expiry of the approval period. The study cannot continue beyond **10 February 2016** until approval is renewed by the PIEC.
 - d. Study completion or termination – this is to be submitted using the PIEC Study Status Report Form within 4 weeks of study completion or termination.

Yours sincerely,

Dr Khoo Chong Yew
Chairperson of PIEC

Ref: PIEC/2014/036

26 October 2015

Dr Elena Sandalova
Danone Nutricia Early Life Nutrition
30 Biopolis Street #05-01B Matrix
Singapore 138671

Dear Dr Sandalova,

APPROVAL OF STUDY AMENDMENTS

Study Title: Understanding innate and adaptive immunological response in stool and saliva samples in healthy donors

We are pleased to inform you that the Parkway Independent Ethics Committee (PIEC) has reviewed and approved the amendments submitted for the above research project under expedited review.

The documents reviewed are:

- a) PIEC Study Amendment Cover Note: 29-Sep-2015
- b) Study Protocol: Version 1.2 dated 29-Sep-2015
- c) Saliva and Stool Sample Collection Participant Information Sheet and Informed Consent Form: Version 1.2 dated 12-Oct-2015
- d) Saliva and Stool Sample Collection Participant Information Sheet (Children) and Informed Consent Form: Version 1.2 dated 12-Oct-2015

Yours sincerely,

Dr Khoo Chong Yew
Chairperson of PIEC

SALIVA AND STOOL SAMPLE COLLECTION PARTICIPANT INFORMATION SHEET

Protocol Title: Understanding innate and adaptive immunological response in stool and saliva samples in healthy donors

Protocol Number: BIO-MK-001

1. Introduction

You are being invited to take part in our study. Before you decide it is important for you to understand why the study is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the test if you wish. Do not hesitate to ask the investigators (See section 12) for further clarifications or further information.

2. Why have I been chosen?

You have been invited to participate in the study because you are healthy and below 60 years of age. The study aims to recruit a total of 120 healthy volunteers.

3. What is expected of me and what samples will be collected?

If you are interested to take part in this study, you will be asked to visit the site twice. During the first visit, you will be given a health questionnaire to complete. You will be given saliva and stool collection kits with instructions on sample collection and storage which you need to follow.

You need to collect stool immediately after defecation. Take care to avoid wetting your stool sample with urine or other fluids. Half fill the tubes (about 5grams) with stool and then cap the tube tightly.

Kindly collect the saliva samples within one hour of stool collection. One saliva swab will be given for saliva collection. To collect the saliva sample; you need to place one end of a swab under your tongue for 60-90 seconds. Then, flip and introduce the other end of the swab under your tongue for another 60-90 seconds. Accumulatively 1-2ml of saliva will be collected.

At the second visit, we will collect your saliva and stool samples from you. This will be the end of your participation in our study

In total this study will take maximum one week from the first visit. You will be expected to refrain from alcohol for 12h prior to sample collection.

4. Purpose of the research

Inflammatory cytokines and immunoglobulins are molecules that are released after infection and injury. They have been widely studied for their role in blood. Blood collection is invasive and involves the use of needles. However, these molecules are also present in saliva and stool. Saliva and stool collection do not involve needles and is easy for collection. By measuring these molecules in stool and saliva samples, we will gain knowledge if we could replace blood sampling with saliva and stool sampling.

5. Possible Risks and Side Effects

There are no known risks and side effects for the collection of saliva and stool.

6. Possible Benefits from Participating in the Lab Test

Although there are no immediate benefits for you personally regarding your health or health information, you will help us to obtain more scientific insights in the area of healthcare.

7. Cost and payment

All collection kits will be provided to you at no cost. You will be reimbursed a sum of \$40 for your travel expenses and time.

8. Voluntary Participation

It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign an informed consent form. You are still free to withdraw at any time and without giving a reason.

9. Confidentiality of Study and Medical Records

Your personal information collected in this test will be kept confidential. Your record, to the extent of the applicable laws and regulations, will not be made publicly available.

Your samples will be coded so your name will not appear on any sample or result sheet.
All employees of Danone Nutricia Research involved in this project will have a duty of confidentiality to you as a subject in clinical test.

If you join the study, some parts of the medical records and data will be looked at by the Independent Review Board/Independent Ethics Committee, and will be granted direct access to your original medical records for verification of clinical trial procedures and/or data, without violating your confidentiality, to the extent permitted by the applicable laws and regulations. All will have a duty of confidentiality to you as a research subject and nothing that could reveal your identity will be disclosed outside the research site. By signing the attached Informed Consent Form, you are authorizing such access to your study and medical records.

10. What will happen to the samples I give?

Your samples will be disposed and sent for incineration after completion of the test. Danone Nutricia Research guarantees that your samples will not be used for human genetics research.

11. Who is organizing and funding the research?

The study is organized and financed by Danone Nutricia Research Singapore. The sponsor of this study. With its aim is to bring health to as many people as possible, Danone Nutricia Research conducts studies on medical and foods including nutrition.

12. Who To Contact if You Have Questions

If you have questions about this test and assays performed with your sample, please contact Elena Sandalova (elena.sandalova@danone.com) tel: 68309433 or Rekha Thiagarajan (rekha.thiagarajan@danone.com) tel: 68309412. For questions related to ethical issues, please contact Parkway Independent Ethics Committee at 63495747

INFORMED CONSENT FORM

I confirm that I have been verbally informed about the study as explained in the information sheet, Version 1.2, dated 12 October 2015 for the above sample collection.

Saliva and/ stool sample collection and I have read and understood the information sheet Version 1.2, Dated 12-10-2015 for the above sample collection. I have fully discussed and understood the purpose and procedures of this activity. The purpose of my sample collection has been explained to me in a language that I understand. I have been given enough time to ask any questions that I have about the collection of my samples, and all my questions have been answered to my satisfaction.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason.

Name of Participant

Date

Signature

For participants between 13-20 years, parental/legal representative consent is required.

Name of Mother/Father/
Legal Representative

Date

Signature

I hereby declare to have explained the study to the participant and the mother/father/legal representative of the participant (if applicable).

Name of Person taking consent

Date

Signature

When completed, keep the original version on site and provide one copy to the participant

SALIVA AND STOOL SAMPLE COLLECTION PARTICIPANT INFORMATION SHEET (CHILDREN)

Protocol Title: Understanding innate and adaptive immunological response in stool and saliva samples in healthy donors

Protocol Number: BIO-MK-001

1. Introduction

Your child is being invited to take part in our study.. Before you decide it is important for you to understand why the test is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the test if you wish. Do not hesitate to ask the investigators (See section 12) for further clarifications or further information.

2. Why is my child chosen?

Your child have been invited to participate in the study because your child is healthy and below 60 years of age. The study aims to recruit a total of 120 healthy volunteers including 40children.

3. What is expected of my child and what samples will be collected?

If you are interested to allow your child take part in this study, you will be asked to visit the site twice. During the first visit, your child will be given a health questionnaire to complete. You will be given saliva and stool collection kits with instructions on his/her sample collection and storage which you need to follow.

You need to collect stool immediately after defecation. Take care to avoid wetting the stool sample with urine or other fluids. Half fill the tubes (about 5grams) with stool and then cap the tube tightly.

Kindly collect the saliva samples within one hour of stool collection. One saliva swab will be given for saliva collection. To collect the saliva sample; you need to place one end of a swab under your child's tongue for 60-90 seconds. Then, flip and introduce the other end of the swab under your child's tongue for another 60-90 seconds. Accumulatively 1-2ml of saliva will be collected.

At the second visit, we will collect your child's saliva and stool samples from you. This will be your end of your child's participation in our study

In total this study will take maximum one week from the first visit.

4. Purpose of the research

Inflammatory cytokines and immunoglobulins are molecules that are released after infection and injury They have been widely studied for their role in blood. Blood collection is invasive and involves the use of needles. However, these molecules are also present in saliva and stool. Saliva and stool collection do not involve needles and is easy for collection. By measuring these molecules in stool and saliva samples, we will gain knowledge if we could replace blood sampling with saliva and stool sampling.

5. Possible Risks and Side Effects

There are no known risks and side effects for the collection of saliva and stool.

6. Possible Benefits from Participating in the Lab Test

Although there are no immediate benefits for your child personally regarding health or health information, your child will help us to obtain more scientific insights in the area of healthcare.

7. Cost and payment

All collection kits will be provided at no cost. Your child will be reimbursed a sum of \$40 for travel expenses and time.

8. Voluntary Participation

It is up to you and your child to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign an informed consent form. You and your child are still free to withdraw at any time and without giving a reason.

9. Confidentiality of Study and Medical Records

Your child's personal information collected in this test will be kept confidential. Your child's record, to the extent of the applicable laws and regulations, will not be made publicly available.

Your child's samples will be coded so his/her name will not appear on any sample or result sheet. All employees of Danone Nutricia Research involved in this project will have a duty of confidentiality to your child as a subject in clinical test.

If your child joins the study, some parts of the medical records and data will be looked at by the Independent Review Board/Independent Ethics Committee, and will be granted direct access to original medical records for verification of clinical trial procedures and/or data, without violating your child's confidentiality, to the extent permitted by the applicable laws and regulations. All will have a duty of confidentiality to your child as a research subject and nothing that could reveal your child's identity will be disclosed outside the research site. By signing the attached Informed Consent Form, you are authorizing such access to your child's study and medical records.

10. What will happen to the samples give?

Your sample will be disposed and sent for incineration after completion of the test. Danone Nutricia Research guarantees that your sample will not be used for human genetics research. The result obtained from your sample will give us an idea to levels of immune markers in saliva and stool and this can be used to assess immune status. Results will not be correlated with the identity of the individual.

11. What will happen to the samples I give?

Your child's samples will be discarded after completion of the test. Danone Nutricia Research guarantees that the samples will not be used for human genetics research.

12. Who To Contact if You Have Questions

If you have questions about this test and assays performed with your sample, please contact Elena Sandalova (elena.sandalova@danone.com) tel: 68309433 or Rekha Thiagarajan (rekha.thiagarajan@danone.com) tel: 68309412.

For questions related to ethical issues, please contact Parkway Independent Ethics Committee at 63495747

INFORMED CONSENT FORM

I, Mr/Mrs/Mdm (name-surname of parent/ legal representative, in block letters) who has a relationship asof boy/girl (name-surname of participant) have read the attached participant information sheet version 1.2, Dated 12-10-2015 and I voluntarily allow my child to participate in this lab test.

I confirm that I have been verbally informed about the above saliva and stool sample collection and read and understand the information sheet version 1.2 dated 12-10-2015 for the above sample collection. I have fully discussed and understood the purpose and procedures of this lab test. The purpose of my child's sample has been explained to me in a language that I understand. I have been given enough time to ask any questions that I have about the collection of my child's samples, and all my questions have been answered to my satisfaction.

I understand that my child participation is voluntary and that I am free to withdraw at any time, without giving any reason.

Name of Child

Date of birth

Name of Mother/Father/
Legal Representative

Date

Signature

I hereby declare to have explained the study to the participant's mother/father/legal representative.

Name of Person taking consent

Date

Signature

When completed, keep the original version on file and provide one copy to the participant

CHILD/ PARTICIPANT ASSENT FORM
For Children/ Participants 6 through 12 years

Protocol Title: Understanding innate and adaptive immunological response in stool and saliva samples in healthy donors

Protocol Number: BIO-MK-001

PI Name: Elena Sandalova

You are being asked to be in a research study. This is because you are suitable to help us. This study will look at a new and safe method to help us understand children's health. We want to see how well it works and that is why you have been asked to join.

If you say yes, you will have to do certain things, like to giving us your poop and saliva.

The study will help other people. You can choose if you want to be in this study or not. It is okay if you decide not to be in the study, no one will be angry with you.

ASSENT

This research study has been explained to me and I want to be in this study.

Child/ Participant Name for Assent

Date

Name of Person Conducting Assent Discussion (Print)

Signature of Person
Conducting Assent Discussion

Date

Protocol Title: Understanding innate and adaptive immunological response in stool and saliva samples in healthy donors

Protocol Number: BIO-MK-001

Questionnaire:

This questionnaire is to understand your medical condition that will help us to decide your suitability for this study. All your details will be kept strictly confidential.

Personal particulars:

Name:

Date of birth:

Gender:

Anthropometric measurement:

Height:

Weight:

Section 1: Medical and social history:

Do you smoke? Y/N

Have you EVER:

(a) used any habit forming drugs or narcotics or been treated for drug habits? Y/N

(b) consumed alcohol excessively or been treated for alcoholism? Y/N

Have you have any diabetes, thyroid disorders or any other endocrine disorders? Y/N

Do you have any of these illness/ conditions? Y/N

▪ Major Cancers	▪ Human Immunodeficiency Virus (HIV)
▪ Heart disease	▪ Muscular Dystrophy
▪ Kidney Failure	▪ Parkinson's Disease
▪ Multiple Sclerosis	▪ Aplastic Anaemia
▪ Hypertension	▪ Bacterial Meningitis
▪ Alzheimer's Disease/Severe Dementia	▪ Brain Tumour
▪ Hepatitis/liver disease	▪ Encephalitis
▪ Terminal Illness	▪ Motor Neurone Disease

Section 2: If your answer is yes, kindly list down the condition/s

Do you have a history/existing

(a) any lung diseases as tuberculosis, bronchitis or asthma?	Y/N
(b) any allergies , atopic dermatitis or eczema	Y/N
(c) any intestinal disorder such as irritable bowel syndrome or celiac disease?	Y/N

You have come to the end of the questionnaire. Thank you for your participation.