QATAR UNIVERSITY

COLLEGE OF HEALTH SCIENCE

MAGNITUDE OF RSV FUSION PROTEIN-SPECIFIC ANTIBODIES IN INFANTS AND CORRESPONDING MOTHERS

BY

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A Thesis Submitted to the Faculty of

the College of Health Science

in Partial Fulfillment

of the Requirements

for the Degree of

Masters of Science

in

Biomedical Sciences

June 2018

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ABSTRACT

TALEB SARA, Masters of Science: June : 2018, Biomedical Sciences

Title: MAGNITUDE OF RSV FUSION PROTEIN-SPECIFIC ANTIBODIES IN INFANTS AND CORRESPONDING MOTHERS

Supervisor of Project: Hadi M. Yassine.

INTRODUCTION: Respiratory syncytial virus (RSV) is a threatening agent causing lower respiratory infections (LRI) among children, where no vaccine is available. RSV expresses three surface glycoproteins, of which the attachment (G) and fusion (F) are targets for neutralizing antibodies (Ab). Being essential for viral entry and highly-conserved, the F protein is a potential vaccine candidate. To overcome the irreversible transition from a metastable Pre-F to a stable Post-F conformation, cavity-filling-mutations have been designed and introduced to stabilize the F protein structure which harbor most of the neutralizing epitopes. This stabilized Pre-F is being considered to vaccinate pregnant females and to induce high-level of the maternal Abs that can be transferred to infants and protect them during their first critical months of life. **Aim:** To evaluate the level of maternal Abs in RSV-hospitalized children and to investigate their correlates to protection ability. **METHODS:** 65 blood samples and nasal aspirates were collected from RSV-infected children at the Pediatric Emergency Center (PEC) at Hamad Medical Corporation (HMC) in Qatar, along with blood samples from their corresponding mothers. Both maternal and infants' sera were screened for the presence of Pre-F and Post-F Abs using ELISA and neutralization procedures. Further, the

circulating RSV subgroups in Qatar were identified by PCR on nasal aspirates, followed by sequencing analysis. **RESULTS:** Children involved in this study raged in age between 0 and 6 months (mean: 1.7 ± 1.2). Relatively, low levels of anti-F Abs were detected in hospitalized children. Endpoint Ab titers to Pre-F in children ranged between 0.03 x 10^3 and 2.81 x 10^3 (mean: 0.97 x 10^3). Anti-F Abs titers in children showed positive-correlation with maternal Abs titers, and negative-correlation with infants' age. Only 14% of maternal Abs were detected in infants, with an average of 0.97 x 10^3 and 1.12 x 10^3 Pre-F and Post-F end-point-titers, respectively. Unlike Post-F, Pre-F-adsorption diminished almost 80% of binding and neutralizing Ab-titers from both maternal and infants' sera, indicating higher levels of Pre-F Abs. Molecular analysis showed interchangeable circulation of RSV subgroups throughout 2016/2017 and 2017/2018 winter seasons. There was an inverse correlation between infants' anti-Pre-F Abs titers and virus titers in nasal swabs. F gene sequence analysis revealed site Ø and site II located mutations, some of them were never reported and probably need confirmation and others indicated resistance to Palivizumab. **CONCLUSION:** Our results indicate low-levels of maternal Abs in RSV-infected-infants, which partially explain children's susceptibility to the disease. Vaccination of pregnant females in the last trimester of pregnancy with Pre-F-protein would guarantee the delivery of high levels maternal RSV Pre-F-specific-Ab-titers to neonates and thus protect then against RSV-infection during their first critical months of life.

ACKNOWLEDGMENTS

To begin with, I am thankful to Allah for blessing me with this opportunity, with patience, strength, and welfare to work on this project. No doubt, I am grateful to my supervisor Dr. Hadi Mohamed Yassine, his continuous support and guidance throughout my Masters' project. Working with him and his lovely team (Maria Smatti, and Shilu Mathew) maturated my personal and professional skills, helped enormously to complete this work, and opened new opportunities. I appreciate the provision of my committee members; Dr. Gheyath Nasrallah, Dr. Khalid Alansari and Dr. Asmaa Althani. Special thanks to the Biomedical Research Center and Pediatric Emergency Center teams for contributing in this project in every way possible. And to finish with, my sincere appreciation goes to my family and friends for their encouragement, prayers, cheerfulness and trust in my capabilities.

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I- INTRODUCTION

Respiratory syncytial virus (RSV) is an enveloped non-segmented negative-strand RNA virus belonging to the Paramyxoviridae family. It is the most leading cause of lower respiratory tract infections (LRTI) in young infants worldwide, with about 33 million cases and about 160,000 -190,000 deaths annually (Gilman et al., 2016; Ngwuta et al., 2015; Sastry et al., 2017). According to a recent study, 6.7% of all deaths in less than 1 year infants is due to RSV infection (Nair et al., 2010). RSV hospitalization is common throughout the first 5 years of age, however, it peaks among 2-3 months old infants (Graham, 2017). By the age of 2-3 years, most of the children get infected with this virus (Widjaja et al., 2016), then reinfected repeatedly for lifetime as the host immunity to RSV diminishes over time (Varga & Braciale, 2013). Infection with RSV is manifested by airway obstruction, runny nose, shortness of breath, wheezing, hypoxia, and in severe cases, pneumonia and bronchiolitis (Graham, 2017). Adding to that, development of asthma has been highly associated to RSV (Sawadkohi et al., 2012).

RSV genome contains more than 15,000 nucleotides coding for at least 11 proteins (Collins, 1991; Collins et al., 1986; Graham, Modjarrad, & McLellan, 2015). (G), (F) and (SH) are surface proteins (Kingsbury, 2012). As the name signifies, attachment protein (G) helps attaching the virus to host cells, and Fusion protein (F) is responsible for viral fusion and syncytium formation. Small Hydrophobic (SH) protein, not-well understood, seems to improves virus entry to the host cell (Tripp, Jorquera, & Tripp, 2016), while the Matrix (M) protein serves as the inner envelope protein

(Kingsbury, 2012). Four nucleocapsid-associated proteins symbolize viral replication machinery: Nucleoprotein (N), Phosphoprotein (P), Large (L) and (M2-1) proteins (Li et al., 2014; Nair et al., 2010). M2-2 protein, encoded by downstream open reading frame of M2, is responsible for RNA synthesis during virion assembly (Collins, 1991). NS1 and NS2 are non-structural proteins and are suspected to play a role in IFN release inhibition from infected cells (Barik, 2013) (Chin et al.) [Figure 1].

RSV strains are classified into 2 main subgroups: RSV-A and RSV-B. Despite sharing comparable infectivity and epidemiology profile, both subgroups are antigenically distinct with about 47-50% genetic differences in the G glycoprotein and 8-10% differences in the F glycoprotein (Chamat et al., 1999; Fuentes, Coyle, Beeler, Golding, & Khurana, 2016; Mufson, Örvell, Rafnar, & Norrby, 1985). Like other respiratory viruses, RSV infections tend to increase in cold weather, low temperature and following rainfall. In temperate climate countries, RSV circulates throughout winter season, but peaks during December or January (Aamir, Alam, Sadia, Zaidi, & Kazi, 2013). In tropical countries, RSV causes outbreaks in hot, humid, rainy days during June to November period (Al-Toum, Bdour, & Ayyash).

For RSV treatment, Ribavirin is given to severely infected patients. Further Palivizumab, the only FDA approved monoclonal antibody, is recommended by the American Academy of Pediatrics (AAP) for infants at high risk (e.g. immune-compromised, premature, congenital heart- or chronic lung-diseased) (Diseases, 2009; Simoes, 1999; Turner et al., 2014). These treatments are limited in use among adults. Hence, vaccination would be the optimal method to protect immune-compromised

adults from RSV illness and complications. Still, no vaccine has been licensed, specifically after the failure of Formalin-inactivated RSV vaccine which resulted in enhanced disease illness in the vaccinated group (Modjarrad, Giersing, Kaslow, Smith, & Moorthy, 2016; Simões et al., 2015).

G glycoprotein, a target for neutralizing Abs, is antigenically variable making it challenging to create a broadly protective vaccine. On the other hand, the F glycoprotein, which is another target for neutralizing Abs, is highly conserved among circulating strains. Nonetheless, F-protein is presented on the virion surface in two forms: one metastable structure, called pre-fusion (Pre-F), which is disposed to switch unpredictably to another stable post-fusion (Post-F) structure (Mousa, Kose, Matta, Gilchuk, & Crowe Jr, 2017). Despite the fact that G and F glycoproteins provoke neutralizing Abs elicitation, F is the major vaccine development target since it is essential for viral entry, is more conserved, and presents more epitopes targeted by neutralizing Abs (Beeler & van Wyke Coelingh, 1989; Magro et al., 2012; Ngwuta et al., 2015). To date, six antigenic sites for neutralization have been identified on Pre-F and Post-F conformations. Site I, a weak neutralizing epitope discovered by an escape mutation (P389), binds to 2F and 44F monoclonal Abs (Calder et al., 2000; Ngwuta et al., 2015). Site II binds to Palivizumab, the only FDA approved prophylactic monoclonal antibody provided for RSV-infected infants at high risk (Rogovik, Carleton, Solimano, & Goldman, 2010). Site IV, a moderate neutralizing epitope, binds to 101F and 19 monoclonal Abs (Magro, Andreu, Gómez-Puertas, Melero, & Palomo, 2010; G Taylor, Stott, Furze, Ford, & Sopp, 1992). Site V, is a quaternary epitope found on the

Pre-F only and it binds to AM14 monoclonal antibody (Killikelly, Kanekiyo, & Graham, 2016; McLellan et al., 2010). Most importantly, site Ø, another Pre-F specific epitope, binds to D25, 5C4 and AM22 monoclonal Abs which have 100-times more neutralizing activity than Palivizumab (McLellan, Chen, Leung, et al., 2013). Other Pre-F specific monoclonal Abs were discovered such as MPE8 which binds near site II (Corti et al., 2013; Gilman et al., 2015; Ngwuta et al., 2015). Because of Pre-F structure's high potency in neutralization, researchers have recently established a stabilized Pre-F form by protein engineering to be used as putative RSV vaccine (Gilman et al., 2016; Krarup et al., 2015; McLellan, Chen, Leung, et al., 2013) [Figure 2].

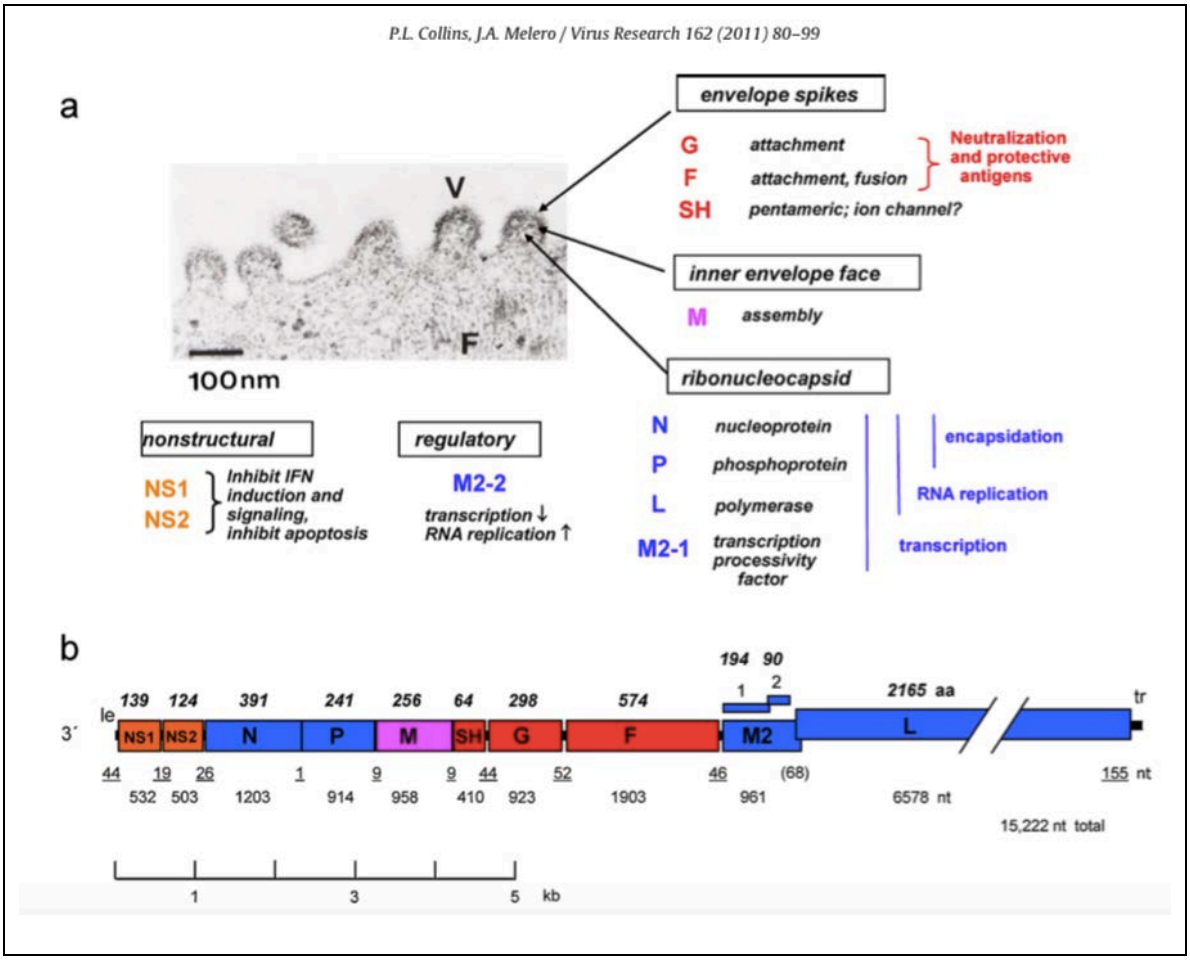

Figure 1: RSV proteins (a) and genetic makeup (b). (a) Location of 11 RSV proteins at an electron microscope image of the virus [V: virion and F: filamentous cytoplasm], and their corresponding functions. (b) Map of the negative sense RNA genome (RSV strain A2) where (nt) and (aa) indicate nucleotides and amino acid lengths, respectively. (le) and (tr) represent leader and trailer, respectively. Intergenic regions are underlined, and the length of the gene overlap is in parentheses. Adopted from (Collins & Melero, 2011).

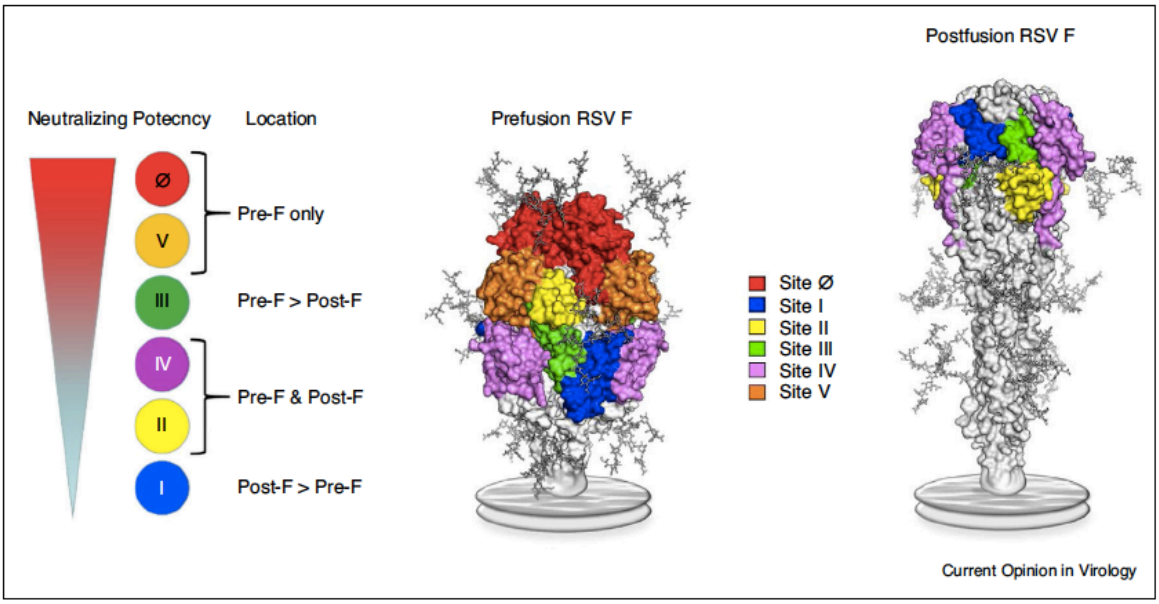

Figure 2: Location of six antigenic sites on Prefusion (Left) and Postfusion (right) structures of RSV F glycoprotein and their neutralizing potency. Adopted from (Graham, 2017).

AIM:

Considering the failures in the previous vaccine trials, an alternative strategy to protect young infants from RSV infection could be through maternal Abs transfer. Accordingly, one potential strategy that is being considered is to vaccinate pregnant women in their last trimester with stabilized Pre-F protein to boost their immune responses to RSV. This will ensure the delivery of high RSV-Abs titers to their babies, which will protect them from infection during the first critical months of life. However, the role of Pre-F maternal Abs in few months old infants has not been investigated so far. Therefore, this project aims to determine the magnitude of maternally-derived RSV-Abs against Pre-F and Post-F conformations in infants in comparison of their mothers, and consequently define the outcome of introducing a stabilized Pre-F prospective vaccination to pregnant woman.

OBJECTIVES:

1. Measuring binding Pre-F specific Abs in sera from infants and pediatrics admitted to PEC with RSV infection.
2. Measuring binding Post-F specific Abs in sera from infants and pediatrics admitted to PEC with RSV infection.
3. Measuring binding Pre-F specific Abs in sera from mothers correspondent to infants and pediatrics admitted to PEC with RSV infection.
4. Measuring binding Post-F specific Abs in sera from mothers correspondent to infants and pediatrics admitted to PEC with RSV infection.

5. Determine the correlation between infants' Abs against Pre-F and Post-F proteins and demographics, disease severity and viral load/subgroups.
6. Determine the correlation between magnitude of maternal Abs against Pre-F and Post-F proteins in hospitalized infants and manifestation of the disease.

II- LITERATURE REVIEW

2.1 RSV molecular biology

RSV genome is a negative sense ssRNA containing more than 15000 nucleotides and encodes for 11 proteins. F, G and SH are the only glycoproteins expressed on the surface of the virion (Kingsbury, 2012).

F protein is a class I fusion protein composed of 574 amino acid (aa). With a molecular weight of a 50 kDa and two subdomains, C-terminal fragment F1 and a 20 kDa N-terminal fragment F2, the protein acquires a trimer of heterodimers (Graham et al., 2015). At amino acid positions 109 and 136, two furin cleavages take place. This feature releases a glycopeptide and thus reveals the hydrophobic site at F1 fragment. F1 and F2 are linked by a cysteine-rich region at two positions: between aa70 and aa212, and between aa37 and aa439. Other F-related features involve N-glycosylation in F1 at aa position 500, and in F2 at aa positions 27 and 70. F protein is highly conserved, with only 25 aa differences between RSV subgroup A and B (Graham et al., 2015).

G protein is a type II integral membrane protein composed of 298aa with a molecular weight of 90kDa. It is highly glycosylated and is expressed in two forms, secreted and membrane-anchored, called Gs and Gm, respectively. Gs is linked to neutralization inhibition, while Gm is related to viral attachment. This virus-host-membrane attachment is mediated by heparin sulfate proteoglycans receptor interaction. The antigenic variation is located in the mucin domain of G protein at both C- and N-terminal ends. N- and O-glycosylation enables the protein to mature and enhances immune escape mechanisms. Other feature includes a central conserved region (CX3C

motif) which is responsible for CX3CR1 binding to diminish inflammatory cytokines release (Graham et al., 2015; Kingsbury, 2012; Tripp et al., 2016; Wertz et al., 1985).

2.2 RSV pathogenesis

Studies regarding pulmonary immune cells in young infants are difficult to implement due to ethical and technical obstacles. Instead, different animal models have been used to study RSV pathogenesis and immune responses to RSV infection (Openshaw, 2013). Further, most researches study immune responses to RSV infection in adults more than neonatal models. For that reason, RSV pathogenesis in infants remains incompletely understood (Ruckwardt, Morabito, & Graham, 2016).

RSV is the most common agent for LRTI among children below the age of five. Most of those acquire mild to moderate disease manifestations, and 2-3% progress to severe illness resulting in hospitalization (Graham, 2017). Factors contributing in RSV pathogenesis and disease severity could be environmental, viral and host factors (Watkiss, 2012).

2.2.1 Environmental factors:

Exposure to smoke, beside its negative effect in general, has been reported to increase the risk of RSV infection, bronchiolitis and disease severity (Bradley et al., 2005; Karr et al., 2009). Cigarette smoking directly effects primary airway epithelial cells, resulting in necrosis which induces inflammation and enhances viral load (Groskreutz et al., 2009). Cold weather is another favorable factor for RSV spread and infection. It has been found that this virus circulates more in cold months, where the virus is highly

sensitive to high temperatures and get inactivated within few hours on furniture and surfaces at room temperature (Hambling, 1964).

2.2.2 Viral factors:

RSV has various mechanisms to enhance its replication and pathogenesis. The main cell targets of this virus are type I alveolar cells and superficial airway epithelial cells (J. E. Johnson, Gonzales, Olson, Wright, & Graham, 2007). RSV infection results in disruption of ciliated epithelial cells, followed by mononuclear cell infiltration, mucosal edema, and syncytia formation (Wright et al., 2005). One of the important alternation that the virus cause inside the host cell is in cell-cycle-regulatory proteins, resulting in accumulation of G0/G1 cell population and thus enhanced viral replication (Wu et al., 2011). Infectivity, disease severity and increased cytopathology are directly linked to viral load as well as to the strain/isolate specificity (DeVincenzo et al., 2010). For instance, studies revealed that infection with subgroup A-strain 19 causes elevated IL-13 excretion, airway hypersensitivity and mucus production in mice (Moore et al., 2009). A2001/2-20 isolate, on the other hand, initiates more severe complications than strain A2, strain 19, Long, and A2001/3-12, including epithelial desquamation and bronchiolitis (Stokes et al., 2011). Further, RSV disguises and manipulates the host immunity through different mechanisms. For example, NS1 and NS2 proteins are known to block the release of type I IFN (IFNa and IFNb) from infected epithelial cells via JAK/STAT pathway or toll-like receptor (TLR), resulting in interrupted dendritic cells recruitment and the subsequent adaptive immune response cascade (Barik, 2013). Further, NS1 and NS2 proteins downgrade cell apoptosis through PI3k pathway

activation to prolongate infected cells survival for more viral replication (Bitko et al., 2007). G glycoprotein is another source of immune escape. It is able to limit the function of CX3CL1-mediated CXCR1 leukocytes recruitment (NK, CD4+ and CD8+ T cells) (Tripp et al., 2001), decoy neutralizing Abs and delay antigen recognition because of its high genetic variation and glycosylation. Gs, a secreted form of G glycoprotein, plays the role of binding to RSV Abs and diminishing neutralization of the virus (Bukreyev et al., 2008). Moreover, this short form acts as a TLR antagonist to downregulate early inflammatory responses released from infected cells through TLR-2, -4, -9 (Polack et al., 2005) (Lukacs, Smit, Schaller, & Lindell, 2008).

2.2.3 Host factors:

RSV-induced LRTI severity is influenced by several host factors. Any damage or incomplete development of to the respiratory tract enhances the pathogenesis of the virus. Reports showed an association of vitamin D deficiency in early life to LRTI severity (Belderbos et al., 2011). In general, RSV infection is more prevalent at age extremity, among premature babies, males, those who have congenital heart disease or chronic lung disease or immunodeficiency, and in case of low birth weight and low maternal Abs titer (Watkiss, 2012). Host genetic variation plays a key part in any infection severity and response, including RSV. The reported genetic polymorphisms involve: innate and adaptive defense genes, surfactant protein genes, host cell receptor genes, Th1/Th2 response genes (Watkiss, 2012). Imbalances or defects in cytokines release from infected cells, macrophages or dendritic cells, influence down-stream activation of adaptive immunity. Knowing the importance of cellular adaptive

components in viral clearance, proper T cell activation and differentiation guarantees controlled RSV spread and infectivity. For that, Th1 to Th2 imbalance or Th2-biased immunity could result in an enhanced respiratory diseases as seen in the previous vaccination trails using FI-RSV (Littel-van den Hurk, Mapletoft, Arsic, & Kovacs-Nolan, 2007) [Figure 3].

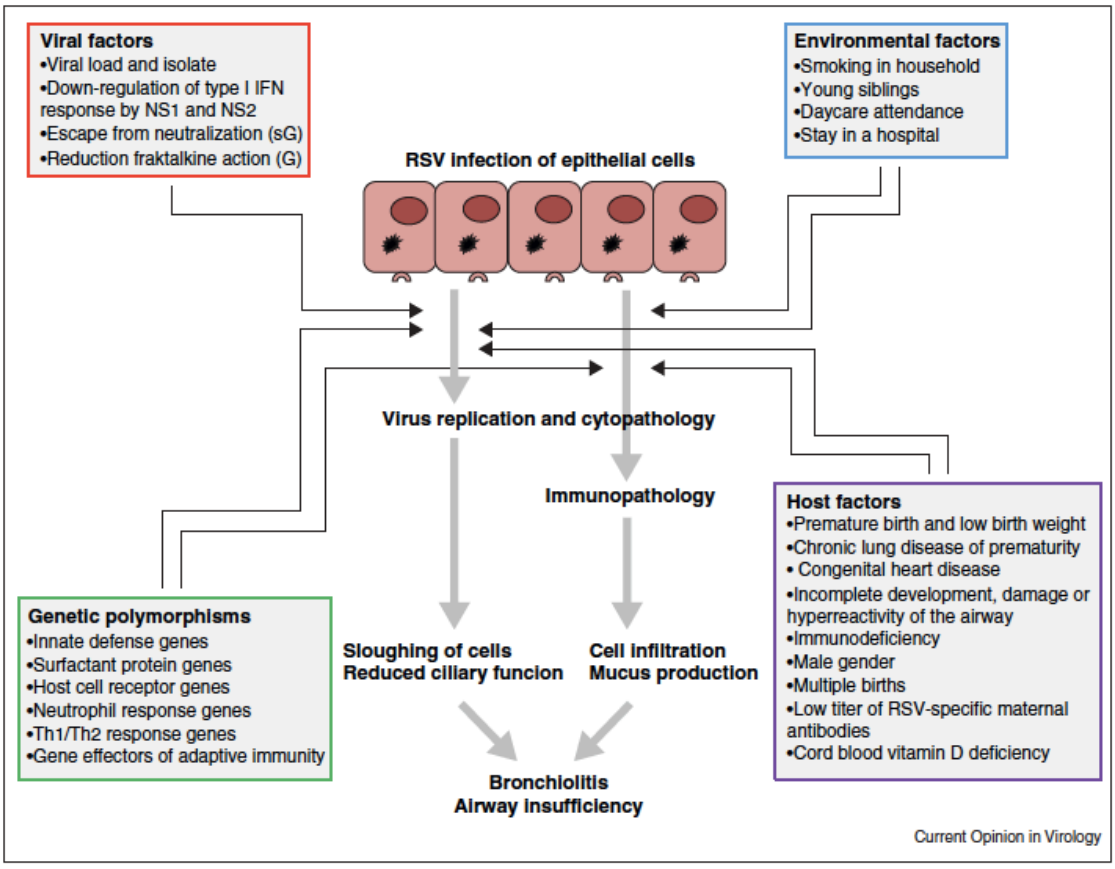

Figure 3: Environmental, viral, and host factors involved in RSV pathogenesis. Adopted from (Watkiss, 2012).

2.3 Immunity to RSV

2.3.1 Innate immune system responses

Early life immunity can be described as suppressive. Due to organs/systems development and the presence of circulating maternal immunoglobulins in few months-old babies, the immune system is designed to be tolerant and defective in quality and quantity (Gervassi & Horton, 2014; Ruckwardt et al., 2016). For example, fetal CD4+ T cells were revealed to differentiate into more T regulatory (Treg) cells as a mechanism of immune tolerance towards maternal Abs and developing organs, rendering the immunity adaptive to early life needs rather than defective (Mold et al., 2010). Nevertheless, these features make young infants more susceptible to infections compared to older children and adults. Among suppressed immune components, natural killer (NK) cells were found to be modulated through transforming growth factors beta (TGFβ) secreted by Treg (Ndure & Flanagan, 2014). Neutrophils as well were found to have reduced cytokines release and phagocytic function (Dowling & Levy, 2014). Dendritic cells (DC) and subtypes, essential parties to link innate to adaptive immunity, were found limited in number and function such as recognizing, processing, and presenting foreign antigens to T cells. These deficiencies tend to stimulate the immune system towards Th2 differentiation (Cormier et al., 2014; Marr et al., 2014; Ruckwardt, Malloy, Morabito, & Graham, 2014). Up to the present time, immunomodulation remains an incompletely discovered process, and further investigation is required to understand early life immunity, hoping to find the appropriate methods for young infants' protection from RSV infections, for instance.

Activation of adaptive immunity depends on efficiency of innate immunity. Antigen presentation is the critical point to induce adaptive defenses and thus the following up functions. Viral clearance and protective antibody production are the expected results from T cell and B cell contributions. However, the early life alternations in innate defense influence critically the whole process from adaptive immunity activation to antibody production and viral clearance (Malloy, Falsey, & Ruckwardt, 2013).

2.3.2 Adaptive immune system responses

As mentioned above, DC trigger T cells to activate and differentiate into T cytotoxic. This role is disturbed by every limitation occurring in DC at early life resulting in disturbance/imbalance of immune system efficacy (Iwasaki & Medzhitov, 2015). CD4+ T cells were found to lose the balance to differentiate toward Th1 and Th2 in response to infectious diseases at early stage of life, confirming that immunomodulation resulted in Th2-derived immune response (L. Lambert, A. M. Sagfors, P. J. Openshaw, & F. J. Culley, 2014a). Previous studies have confirmed the association of enhanced disease illness resulting of formalin-inactivated RSV (FI-RSV) to Th2-driven pathology (Karron, Buchholz, & Collins, 2013). CD8+ T cells play an essential role in protection from viral infections (J. Liu et al., 2016). They participate in all situations of viral clearance once the infection is striking (Morabito et al., 2017). Cases of RSV infections with decreased CD8+ levels such as in severe combined immune-deficiency (SCID), bone marrow (BM) or lung transplantation have led to death (Ruckwardt et al., 2016). At early life, CD8+ T cells are also lacking, resulting in severe RSV infection that could

be fatal (Welliver et al., 2007). Another limitation at early life which have been documented is antibody production. The importance of Abs manifests in some Fc-accelerated auxiliary processes of viral clearance, and young children's protection from severe RSV illness, particularly neutralizing Abs which reduce the number of infected cells and delay the virus from reaching to lower respiratory tract (Groothuis, Simoes, Hemming, & Group, 1995). Therefore, decreased antibody production and responses may contribute in RSV severity. This antibody inadequacy raises from B cells shortage and inability to promote somatic mutation (Basha, Surendran, & Pichichero, 2014). According to one study, RSV neutralizing immunoglobulins are unlikely to develop after a natural RSV infection during the first four months of life. That is, B cells start to increase and mature gradually at 4-6 months old, resulting in significant increase of neutralizing Abs after 6 months of age (Sande, Cane, & Nokes, 2014). These findings confirm the ineffectiveness of vaccination prior four months of age since both innate and adaptive immune system are suppressive, and instead empower the need of alternative source of protection to compensate neonatal immunity. [Figure 4]

2.3.3 RSV Mechanisms of immune escape

The ability of RSV reinfections in individuals throughout life is attributed partially to the weakening of host immunity to this pathogen over time. Further, the virus evolved various mechanisms to avoid immune system despite its limited genetic variation. Three main classes of immune escape are exerted by the virus: 1. location-related infectivity, 2. conformational avoidance from neutralization/neutralizing antibody, and 3. functional change of the immunity. Infecting the ciliated epithelial cells (superficial) of the

respiratory airways saves the virus from getting exposed to dendritic cells, responsible for transporting RSV antigen to lymph node to recruit immune reactions (J. E. Johnson et al., 2007; Zhang, Peeples, Boucher, Collins, & Pickles, 2002). To avoid neutralizing Abs, the structure of F protein, responsible for virus-host and cell-cell fusion, changes conformation from a metastable to stable form so called Pre-F and Post-F, respectively. Since Pre-F is the required part for viral fusion and the target for more potent neutralizing Abs, RSV balances between both functions by transitioning to Post-F in a smartly calculated manner to enter the cell (remain infectious) and escape neutralization by Pre-F-specific Abs (Hambling, 1964; Killikelly et al., 2016). Lastly, modulating host immunity initiates when RSV targets young infants. Because they have naive immune system (immature dendritic cells, deficiency of B cell somatic hyper-mutation) (Lambert et al., 2014a), the virus inhibits type I IFN through NS1 and NS2 proteins (Barik, 2013), manipulates dendritic cell signaling through G glycoprotein (T. R. Johnson, McLellan, & Graham, 2012), and tricks antibody neutralizing responses through secreted form of G glycoprotein (Bukreyev et al., 2008).

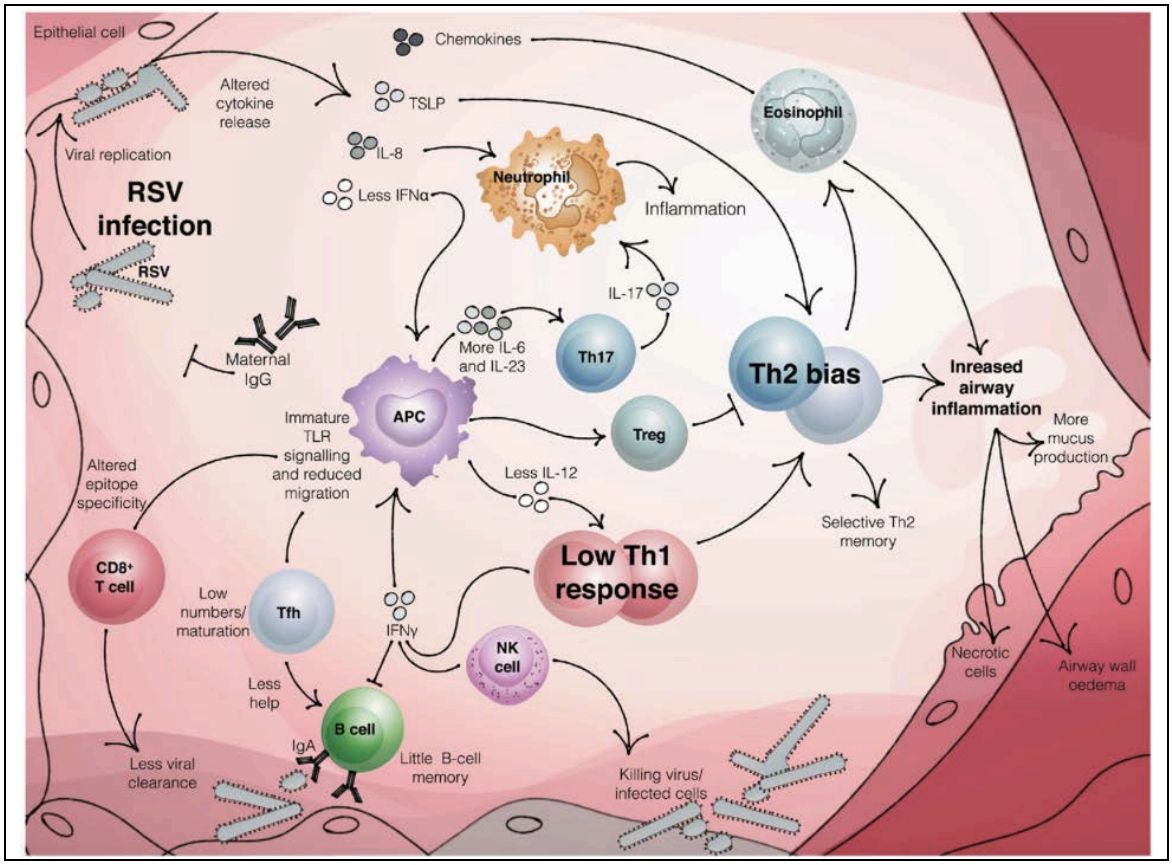

Figure 4. Early life immunity responses towards RSV infection. Neonatal innate response following RSV infection is weak: low levels of cytokines, such as interferons, diminished TLRs signals, altered antigen presenting function, and reduced activation of regulatory T cells. This results in a skewed adaptive response toward Th2 and low CTL activation through Th1. Impaired Tfh activation, little or no B cell memory and inhibition of antibody production by IFNγ, results in low titer and low affinity Abs. Consequences of suppressive immune response lead to bronchiolitis in susceptible infants. Adopted from (L. Lambert, A. M. Sagfors, P. J. M. Openshaw, & F. J. Culley, 2014b)

2.4 RSV vaccine history and enhanced respiratory illness

Although RSV disease is believed to be an ancient agent, the virus was first identified in 1955 as “Chimpanzee Coryza Agent”, then a year later was linked with bronchiolitis in young children (Morris, Blount Jr, & Savage, 1956). Few years later, a vaccine attempt was done using formalin-inactivated RSV given to young children. The idea of producing a formalin-inactivated RSV vaccine was inspired from previous successful trials such as in Polio (KIM et al., 1969). In four separate studies, the FI-RSV vaccine was given at a 3-dose regimen (0, 1, 4 months) for 2-7 months old infants (Killikelly et al., 2016). Unfortunately, the experiment failed and resulted in enhanced respiratory diseases following a natural RSV infection, in which 16/20 (80%) of the vaccinated children were hospitalized, compared to 5% of non-vaccinated children, and 2/20 (10%) passed away (KIM et al., 1969). Enhanced respiratory disease in FI-RSV was characterized by development of antibodies directed more towards Post-F non-neutralizing epitopes. Those antibodies, following a natural RSV infection, induced Th2-derived immune responses (Connors et al., 1992) (Connors et al., 1994). Induced binding-Abs were found to have a low neutralizing activity (Murphy & Walsh, 1988), and were associated with immune complex deposition and complement activation in airways (Killikelly et al., 2016; Ruckwardt et al., 2016). Eosinophilia was another characteristic of FI-RSV, causing hypersensitive peri-bronchiolar inflammation regulated by IL-4, IL-5, IL-13 and IgE (Knudson, Hartwig, Meyerholz, & Varga, 2015), in addition to low CD8+ T cells activation, following RSV infection in FI-RSV vaccinated individuals (Polack, 2015). These immunological features (humoral and

cellular) involved in FI-RSV enhanced diseases explain the reason behind this vaccine failure.

According to recent studies, Pre-F conformation provoke stronger neutralizing Abs as compared to Post-F. Yet, based on one study, the expression of Pre-F antigens on the surface of the virus was almost lost within the 72 hours of formalin fixation period (Killikelly et al., 2016). Therefore, to develop an efficient vaccine without complications such as enhanced illnesses, it is critical to consider the consequences of viral inactivation and formalin concentration (McLellan, Chen, Joyce, et al., 2013; Moghaddam et al., 2006). Viral inactivation alters antigenicity and immunogenicity. Formalin, a chemical fixative/preservative to inactivate viruses, creates different outcomes using different concentrations where high concentration of 1% destroys viral infectivity via inter- and intra-protein cross-linkage, and while lower concentrations preserve antigenic epitopes differently (Clemens et al., 1995; Furesz, Scheifele, & Palkonyay, 1995).

Following FI-RSV vaccine experimentation, other trials of live-attenuated virus vaccine were established. Side-effects were also observed with such vaccines and therefore were not approved for infants because of their failure to preserve antigenicity and immunogenicity (Karron et al., 2013; Ruckwardt et al., 2016).

2.5 F protein-based neutralization

Structures of Pre-F, Post-F proteins and their corresponding epitopes are depicted in figure 2. Several recent studies have demonstrated the relationship between Pre-F and

Post-F binding Abs and their neutralizing activities. Structural and immunological analysis demonstrated that Pre-F-specific Abs are at least 8-fold more potent than shared (Pre-F and Post-F cross-reactive) Abs and 80-fold more potent than Post-F-specific Abs (Gilman et al., 2016; McLellan, Chen, Joyce, et al., 2013). Adsorption of sera of individuals aged between 7 to 93 years with stabilized Pre-F protein removed >90% of neutralizing activity, and diminished binding Abs to both F conformations. On the other hand, sera adsorbed with Post-F retained most of the neutralizing activity (>70%) as well as the binding Abs to Pre-F conformation (Ngwuta et al., 2015). These findings demonstrate a positive correlation between Pre-F binding and virus neutralization (Zhao M. et al. (2017)), while the correspondence of Post-F binding and neutralization was not significant (Graham et al., 2015). The study further investigated neutralization activity at the epitope level to compare well-identified antigenic sites present on Pre-F and Post-F conformations. 60% of highly potent neutralizing Abs were targeted to site Ø and V, which are Pre-F specific antigens. Meanwhile, site III and IV, shared between Pre-F and Post-F, represented targets for moderate neutralizing Abs, followed by Site I and II, which represented targets for weak neutralizing Abs (Widjaja et al., 2016). These differences in neutralization towards both F conformations could possibly be explained by the uniqueness, the accessibility and the approach angle of each antigenic site (Gilman et al., 2016).

2.5.1 Palivizumab and recently discovered RSV antibodies

Palivizumab is the only licensed humanized monoclonal antibody and is designed to identify a shared antigenic epitope between Pre-F and Post-F, site II, in the hope of

increasing its target spectrum. Determination of Pre-F structure in complex with D25 antibody (McLellan, Chen, Leung, et al., 2013) and subsequent stabilization of the protein with cavity-filling hydrophobic substitution mutations (McLellan, Chen, Joyce, et al., 2013), enabled better understanding of the immune response to the two forms of this metastable protein. It was found that Pre-F preserve more antigenic sites compared to Post-F, which are the target for more potent neutralizing Abs than Palivizumab's antigenic site. Various studies have compared the neutralizing potency of site II to particularly Pre-F-specific epitopes. Palivizumab or 1129 antibody showed comparable binding affinity to its appropriate antigenic site on both forms of the protein. However, site Ø-specific antibody 5C4 expressed 50-fold more neutralizing activity than 1129 in mice, and decreased RSV titers by 1000-fold more than Palivizumab, suggesting that site Ø elicits more protective Abs from RSV infection than site II (Zhao et al., 2017). Site V, another Pre-F-specific site, was also investigated against Palivizumab-site II. AM14, a quaternary Pre-F-specific antibody, neutralized all tested RSV strains, with IC50s of: 13.6 ng/ml for strain A Long, 12.4 ng/ml for strain A2, 30.8 ng/ml for subgroup B strain 18537 and 4.6 ng/ml for subgroup B strain 9320, compared to Palivizumab which neutralized the same strains with IC50s of 300 ng/ml, 320 ng/ml, 380 ng/ml and 120 ng/ml, respectively. These data suggested that Abs to site V are 100-fold more potent than site II directed Abs (Gilman et al., 2015). Recently, a new Pre-F specific epitope was discovered and is referred to as site VIII. This site was shown to reside between site II and site Ø. mAb 90, specific to this new site, neutralized 3 tested RSV strains of A2, 18537 B, and Long with IC50s of 4 ng/ml, 10 ng/ml, and 35 ng/ml,

respectively, compared to Palivizumab IC50s of 1900 ng/ml, 1300 ng/ml, and 212 ng/ml, of the same RSV strains. These findings as well confirm 1000-fold more potency of site VIII than site II (Mousa et al., 2017). The main conclusion from this comparison is to emphasize the important role of Pre-F-specific Abs in RSV infection prevention, and the potential use of Pre-F antigen as an effective vaccine. Particularly that Palivizumab is at high cost, given only to high-risk premature infants, restricted for adults with immunodeficiency or elderly people, and has low in neutralizing activity.

2.6 RSV vaccine approaches

The reverse outcomes of FI-RSV vaccine and virus-attenuated trials invigorated researchers to develop and test new vaccine concepts that avoids side effects and complications. The nature RSV infectivity, the host immune responses at early life including the impact of imbalanced Th1 to Th2 immunopathology, and the viral mechanisms to escape immunity are all decisive points towards developing RSV vaccine. Since infection with RSV is more dramatic in infants less than three months of age, and implementing an efficient primary vaccination at this stage is challenging, alternative sources of protection shall be considered to support their immune system during this critical period. Passive immunity towards RSV can reduce RSV burden, if enough protective maternal Abs titers are delivered to the neonates (Lambert et al., 2014b). Further, the use of Palivizumab for therapy indicate the importance of neutralizing Abs in protection from severe RSV infection. Characterizing the stabilized Pre-F protein as a main target for neutralizing Abs, particularly those to site Ø which

are 100-1000fold more potent than the licensed monoclonal antibody, has opened the door towards using it as a putative vaccine (Graham, 2017; Mousa et al., 2017; Ngwuta et al., 2015; Zhao et al., 2017). On the other hand, the selection of appropriate adjuvant influences immunogenicity or/and immunogenicity. One recent study assessed the impact of different adjuvants on vaccine efficiency and immune responses. It was found that an oil-in-water adjuvant, Sigma adjuvant system (SAS) plus Carbopol, induced the highest RSV neutralizing antibody responses, followed by Alum, SAS alone, AdjuPlex, and Poly (I:C). TLR4 agonist MPLA, Alum plus MPLA or AddaVax generated moderate responses. All these findings were compared to DS-Cav1 (stabilized Pre-F protein) alone without adjuvant which induced much lower response. When elderly mice with pre-existing immunity against DS-Cav1 were tested for an immune boost with DC-Cav1 plus an adjuvant, results showed that SAS plus Carbopol enhanced immune responses 2- to 3-fold, while Alum enhanced immunity by 5-fold (Sastry et al., 2017).

One of the main strategies is to vaccinate pregnant women prior delivery to boost their pre-existing Pre-F-specific Abs (Graham, 2017). Transferred maternal Abs would protect young infants for up to 6 months, enough for the early immunity to maturate and develop naturally anti-RSV Abs following upcoming RSV infections (Lambert et al., 2014b). This potential vaccine could also be administrated to children once their immune system is stable and adequate. As a delivery method, other strategies described the use of vectors (Geraldine Taylor et al., 2015) and nucleic acids (Pardi & Weissman, 2017). A study has recently claimed a preclinical development of RSV F vaccine by

expressing a stabilized Pre-F via human parainfluenza type 1 (HPIV1) recombinant vector. RSV F was expressed as a full-length protein or as a chimeric form with HPIV1 F transmembrane and cytoplasmic tail (TMCT) domains. Results showed that full-length was more immunogenic in terms of neutralization and protection following RSV challenge, compared to the chimeric form with TMCT modification which had reduced immunogenicity (Liu et al., 2017). Another study used human parainfluenza type 3 as a vector to deliver the vaccine and codon-optimized Pre-F gene was incorporated. Optimized-codon with low CpG enabled the virus to better express Pre-F protein, reduce IFN release and replicate efficaciously *in vivo*. This bivalent RSV/HPIV3 vaccine (DS-Cav1 Pre-F stabilization, reduced CpG content, and vector packaging combination) improved F immunogenicity, induced higher complement-independent Abs titers and resulted in better protection following RSV challenge in hamsters (Liang et al., 2017).

All these approaches aim to develop the most appropriate RSV vaccine candidate, preserving Pre-F expression for an enhanced immunogenicity and protective immune responses. Antigen-naïve population is the main target for these vaccine trials, yet, older children, immunocompromised adults and elderly people are potential candidates for RSV vaccination.

III- METHODS AND MATERIALS

3.1 Study design:

Stabilization of the Pre-F protein has opened new doors to understand the pathogenicity and immune response to RSV infection. Considering the propose of Pre-F vaccine trials in pregnant women, we sought to determine the correlation between the F-specific maternal Ab responses to RSV in infants and manifestation of the disease. This cohort study was initially designed to enroll 100-200 patients admitted with RSV infection to Pediatric Emergency Center (PEC) of Hamad Medical Corporation (HMC). Ethical approvals to collect blood samples (from infants and their mothers) and nasal swabs/nasopharyngeal aspirations (from RSV-infected infants only) were obtained from HMC (HMC-IRB 16196/16) and QU (QU-IRB 890-E/18). Because of the nature of RSV seasonality, we were able to collect samples from only 65 RSV-positive children and their corresponding mothers. Enrollment criteria consisted of the following: 1. Infected infants should be hospitalized at PEC, 2. Infants should be RSV-positive only (tested at PEC), 3. RSV-infection should be a primary infection, 4. Infants' age should be maximum 6 months old, 5. Samples from enrolled infants should be collected before receiving any treatment. Once RSV-infection was confirmed by PEC through PCR, all corresponding guardians/mothers signed consent forms before the collection of samples. Blood and nasal aspirate samples were transported to Biomedical Research Center (BRC) – Qatar University (QU) for further analysis. Blood samples were used to separate sera and measure RSV-F binding and neutralizing Abs titers in infants and corresponding mothers. Further, nasal swabs/aspirations were used to determine RSV

subgroups, Fusion (F) glycoprotein sequences, and viral load. All 65 infants were 0-6 months old, previously healthy, and hospitalized with RSV primary infection. Clinical data, like presenting signs and symptoms, length of stay, birth history, and bronchiolitis score at admission and discharge were extracted from patients' file by PEC research team. Molecular and comparative analysis were done to find correlation between obtained finding and disease manifestation in RSV-infected infants.

3.2 Viruses and cells:

Virus stocks (kindly provided by Viral Pathogenesis Laboratory, Vaccine Research Center, National Institute of Health, USA) were propagated in HEp-2 cells (ATCC® CCL-23™) cultured in 10% minimum essential medium (MEM). As described in (Ngwuta et al., 2015), at 80% confluency in T75 flask, cells were infected with 1 ml of RSV A2 and incubated for 1-hour at 37^{o}C. 2-3 ml of 10% MEM were then added, then infected cells were incubated at 37°C for 4 days. When substantial syncytia had formed, cells were scraped loose from the flask, transferred to 50 ml tubes for sonication (50% amp. six 1s bursts) and centrifuged at 4°C and 1300 rpm for 15 min. These were added to the original supernatant containing virus, aliquoted into dram vials, and stored at −80°C (Graham, Perkins, Wright, & Karzon, 1988).

3.2.1 Virus quantification by plaque assay:

Serial dilutions of generated virus stock were prepared from 1:10^{1} to 1:10^{7} in complete media (10% MEM). At 80% confluency of HEp-2 cells, seeded a day before at a concentration of 2.5 x 10^{5} in 1 ml of 10% MEM per well using 12-well plate, 50 µl of

the virus at different dilutions were added to each well in triplicate. After 1-hour incubation, rocking, at 37^{o}C, 1 ml of methyl cellulose (autoclaved 3.75g of methyl cellulose in 500 ml of complete media) was added to cover each well, then incubated at 37°C for 4-5 days. 1 ml of 10% formalin was added to each well to fix the cells, then incubated at RT for at least 1-hour. All wells were washed with water, stained with 150 µl of crystal violet for 20 minutes, then washed again with water. Plaques were counted after an overnight air dry in triplicate and plaque forming units per ml (PFU/ml) were determined using the following formula: average of plaques in one dilution / (dilution factor x volume of inoculum in ml [0.05 ml]).

3.3 Proteins production and purification:

Pre-F (DS-Cav1) and Post-F RSV proteins and vectors for protein expression were kindly provided by Viral Pathogenesis Laboratory, Vaccine Research Center, National Institute of Health. Further protein expression was done as previously described (Ngwuta et al., 2015). Plasmids were amplified in *Escherichia coli* competent cells and purified using PureLink™ HiPure Plasmid Maxiprep Kit (Cat # K210006, Invitrogen, USA). Plasmids were transfected into Expi293F cells using 293Fectin™ Transfection reagent (Cat # 12347019, Invitrogen, USA). The culture supernatants were harvested 5 days after transfection and centrifuged at 10,000 rpm to remove cell debris. Supernatants were then sterile-filtered, and RSV F glycoproteins were first purified by affinity columns using Ni2+–nitrilotriacetic acid (Cat # K95001, Novex, USA) and HisPur™ Ni-NTA Resin (Cat # 88221, Thermo Fisher Scentific, USA). Relevant

fractions containing the RSV F variants were pooled, concentrated, and subjected to size-exclusion chromatography. Fractions corresponding to the trimer peak were concentrated, and frozen at −80°C.

3.4 RSV Pre-F- and Post-F-Abs separation by serum adsorption assay:

Each serum sample (300 ul) was diluted 1:9.7 in 1× phosphate-buffered saline (PBS) and split into three parts. 20 ul of Pre-F or Post-F proteins (0.5 mg/ml) were added to 970 ul of diluted samples, whereas the positive control, 970 ul of diluted unadsorbed serum, were further diluted 1:10 with 1X PBS. All samples were then incubated at RT for 2-hours, and positive control samples were stored at 4°C until further use. To F-treated samples, 10 ul of reconstituted NWSHPQFEK Strep-tag II mAb at 0.5mg/ml (Cat # A01732-100, GenScript Biotech, USA) were added, and the mixture was placed on a 360° rotator at 4°C for 2 hours. 80 ul of sheep anti-mouse immunoglobulin G Dynabeads (2mg/ml) (Cat # 11206D, Invitrogen, USA) were washed twice with wash buffer [1% bovine serum albumin (BSA), 2M EDTA in 1X PBS (pH 7.4)]. The wash buffer was separated from the beads using a DynaMag-2 magnet (Cat # 12321D, Invitrogen, USA). The Sera-RSV-F-strep-tag II mAb mixture was added to corresponding labeled tubes containing Dynabeads and incubated for 1-hour at RT on a rotator. The beads were separated from supernatant using DynaMag-2; the supernatant was stored at 4°C (Ngwuta et al., 2015). Unadsorbed and adsorbed sera were then used in ELISA, and neutralization assay.

3.5 RSV Abs detection by enzyme-linked immunosorbent assay (ELISA):

Plates (96-well) were coated with 100 ul of stabilized Pre-F (1 mg/ml) or Post-F (1 mg/ml) diluted in 1X PBS (pH 7.4) and incubated overnight at 4°C. Plates were washed with wash buffer (0.2% Tween 20 in 1X PBS) and blocked with 200 ul of 5% milk (in 1X PBS) per well for 1-hour at RT. 100 ul of unadsorbed serum, serum adsorbed with Pre-F, and serum adsorbed with Post-F were added to Pre-F– and Post-F–coated wells in serial dilutions (1:100 to 1:72900), incubated for 1-hour at RT, washed, and coated with 100 ul of goat anti-human IgG-HRP (1:500, Cat # AHI0404, Invitrogen, USA). After 1-hour of incubation, the plates were washed, and 100 ul of TMB KPL SureBlue^{TM} substrate (Cat # 95059-282, SeraCare, USA) were added to each well, then incubated for 20-30 mins. The reaction was stopped by 100 ul of 2M H2SO4 per well. An ELISA plate reader (Epoch 2, Biotek, USA) was used to read signals at 450-nm wavelength. Using GraphPad Prism, endpoint titers (EP) were calculated from non-linear curve fitting of data from individual patients, corresponding to the OD reading at highest dilution four times above the background. Results are expressed as the inverse of logarithm to base 10 (log10) (Hackett, Zhang, Stefanescu, & Pass, 2010).

3.6 Molecular analysis:

3.6.1 RNA extraction:

Extraction of viral RNA from 400 ul nasal aspirate samples was performed using a QIAamp Viral RNA Extraction miniKit (Catalog # 51106, QIAGEN, Germany) according to the manufacturer's instructions. Extracted RNA were quantified using

NanoQuant microplate reader (Infinite Pro M200, Tecan, Switzerland) and stored -80 ^{o}C until used for quantitative and nested RT-PCR.

3.6.2 Viral quantification by qRT-PCR:

To measure RSV load in infected infants, extracted RNA was detected using OneStep reverse transcriptase quantitative real time PCR kit (Luna® Universal One-Step RT-qPCR Kit, NEB, US) as per the manufacturer's recommendations. The primers included in the PCR amplification reaction were as following: RSV-AB-1 (5'-GGCAAATATGGAAACATACGTGAA-3'), RSV-AB-2 (5'-TCTTTTTCTAGGACATTGTAYTGAACAG-3') and RSV probe (FAM-CTGTGTATGTGGAGCCTTCGTGAAGCT- BHQ-1) (Aamir et al., 2013). The standard curve was created using extracted RNA from the generated RSV stock (10^9 PFU/ml) in serial dilutions to quantify viral RNA load in all the tested samples (copies/reaction equivalent to PFU/ml)

The qRT-PCR reaction was carried out in QuantStudio™ 6 Flex Real-Time PCR instrument (Applied Biosystems, USA) using the following cycling conditions: reverse transcription at 50°C for 30 min; heat activation at 95°C for 15 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 2 min; and a final extension step of 72°C for 10 min.

3.6.3 RSV subtyping by nested RT-PCR:

RSV subgroups were detected from extracted RNA using a OneStep RT-PCR kit (Catalog # 210210, QIAGEN, Germany). The first amplification cycle detecting G gene of both subgroups utilized RSV-AB-F (5'-GTCTTACAGCCGTGATTAGG-3') and

RSV-AB-R (5’- GGGCTTTCTTTGGTTACTTC-3’) primers as described (Abels, Nadal, Stroehle, & Bossart, 2001). To distinguish between both subgroups, the products of the first PCR cycle were used in the second cycle by using RSV-A-F (5’-GATGTTACGGTGGGGAGTCT-3’), RSV-A-R (5’-GTACACTGTAGTTAATCACA-3’) primers for group A viruses and RSV-B-F (5’ AATGCTAAGATGGGGAGTT-3’), RSV-B-R (5’-GAAATTGAGTTAATGACAG-3’) primers for group B viruses. PCR reactions were prepared in a final volume of 50 µl using: 0.25 µl of HotStarTaq DNA Polymerase (Catalog # 203203, QIAGEN, Germany), 10 µl of 5X Q-Solution, 5 µl of 10X PCR buffer which already contains 15mM MgCl2 (all from QIAGEN kit), 1 µl of 10mM dNTP's (Catalog # N0447S, New England Biolabs, USA), in addition to 1 µl of 10µM/µl of the primers mentioned above. Both nested RT-PCR reactions were carried out in Biometra TAdvanced Twin 48G thermocycler (Analytik Jena, Germany) using the following cycling conditions: reverse transcription at 50°C for 30 min; Taq polymerase activation at 95°C for 15 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 60 s, and extension at 72°C for 2 min; and a final extension step of 72°C for 10 min. PCR products (first cycle product: 836bp for RSV A and B; and second cycle products: 334bp for RSV A and 183bp for RSV B) were detected by gel electrophoresis using 1.5% agarose and with ethidium bromide staining [Table 1].

3.6.4 F gene sequencing:

F gene was amplified from all enrolled RSV-infected nasal aspirates (n=65) by nested RT-PCR according to the cycling conditions described in this article (Tapia et al., 2014) using forward and reverse primers targeting RSV-A and B F1 subunit of F gene [Table

1]. Amplified cDNA product was visualized by gel electrophoresis where RSV-A F1 subunit is 1047bp and RSV-B F1 subunit is 1065bp. Furthermore, the amplified product was cleaned-up following PCR purification kit's procedure (Catalog # 28104, Qiagen, USA). Samples were sent to Molecular Cloning Lab (MCLAB) for DNA sanger sequencing services. Sequenced data analysis was performed using FinchTV software version 1.5 for chromatography reading and using MEGA version 7.0 for sequence translation and alignment. Visualization of 3D structure of Pre-F protein was performed using Pymol Molecular Graphics System to localize antigenic sites of interest, amino acid residues and detected mutations.

3.7 Statistical analysis:

Comparisons between unadsorbed and adsorbed serum samples were done using Two-way ANOVA and multiple t-tests (paired and unpaired). Correlations between continuous variables or/and discrete variables were assessed using Pearson's correlation (in case of parametric data), and Spearman' (in case of non-parametric data) tests. To test for the data's normality, D'Agostino & Pearson normality test was performed. All P-values were compared to a two-sided level of 0.05. All statistical analyses were done with GraphPad Prism v7.

IV- RESULTS

4.1 Sample collection

Sixty-five patients diagnosed with RSV-bronchiolitis at PHC-HMC were enrolled in this study from February 2017 to February 2018. Blood samples along with nasal aspirates were collected from all infected infants following admission and before any intervention or treatment. Additionally, blood samples were collected from recruited patients' mothers for comparative analysis. As expected, RSV infections followed a seasonality-based trend, where the virus barely circulated during hot months (May 2017 to September 2017), then dramatically increased in activity during cold months (October 2017 to January 2018) [Figure 5].

4.2 Demographic characteristics of enrolled patients

The main objective of this study is to determine binding and neutralizing antibody titers in children diagnosed with primary RSV infection. Accordingly, all RSV-infected children in this study were below the age of six months, of which, 89.2% were below three months old [Figure 6a]. 55.3% of patients were male, with female to male ratio of 1:1.24 [Figure 6b]. Most observed nationalities were Qatari, Pakistani, then Yemeni representing 30.7%, 20%, and 12.3% of total patients' number, respectively [Figure 6c]. At the time of admission, clinicians at PEC examined RSV-bronchiolitis severity among infected children and gave it a score according to the disease manifestation. Few patients were not rated at the time of submission and/or at the time of discharge, resulting in total of 62 and 51 rated patients, respectively. Most patients had a score between 4 and 6 (77.4%) at admission, and a score of 3 (88.2%) at discharge [Table 2].

4.3 RSV Abs' binding activities towards Pre-F and Post-F proteins

The levels of RSV Ab titers in infants and their corresponding mothers were tested by ELISA to both conformational structures of F glycoprotein. Sera samples were adsorbed with Pre-F and Post-F proteins prior to ELISA testing in order to quantify Pre-F- and Post-F-specific Abs. Binding of maternal unadsorbed sera in serial dilutions to both proteins revealed equivalent Ab end-point titers to Pre-F and Post-F with an average of $7.33x10^3$ and $7.75x10^3$, respectively. In infants, serially diluted unadsorbed sera showed binding to Pre-F and Post-F proteins with an average of $0.97x10^3$ and $1.12x10^3$ end-point titer, respectively. These results confirmed the transfer of maternal RSV Abs to young infants, yet they represented approximately only 14% of total the maternal RSV Pre-F- and Post-F-Abs. In infants, Pre-F adsorbed sera lost 82% of binding capacity to Pre-F, and 77% of binding capacity to Post-F ($p<0.05$). Similarly, adsorption with Pre-F in mothers removed 87% binding capacities to Pre-F and to Post-F ($p<0.05$). On the other hand, adsorption of infant sera with Post-F removed 49% and 74% of the binding capacity to Pre-F and Post-F, respectively ($p<0.05$). Likewise, Post-F adsorption of mothers' sera removed 46% and 78% of binding to Pre-F and Post-F, respectively ($p<0.05$) [Figure 7]. These numbers indicate that approximately 80% of total RSV Abs are directed to Pre-F protein in both mothers and corresponding infant. It is worth reminding that both F- forms share four antigenic sites, such as site II and site IV, while some other antigenic sites like site Ø and site V are strictly found on Pre-F structure and are responsible for eliciting high potent Abs (Graham, 2017).

4.4 RSV viral load and subgroups circulating among young infected infants

Quantification of RSV viral load in infected children was done using qRT-PCR on the RNA extracted from nasal aspirates. Although all recruited patients in this study were initially screened for RSV infection at PEC-HMC using PCR, yet, viral RNA load was reperformed to confirm RNA detection and to quantify RSV in all patients. The viral load for RSV-positive infants (100%) ranged from 1.73×10^2 to 2.23×10^8, with a mean of 4.48×10^7 copies/reaction.

In parallel, PCR-typing revealed dominance of RSV-A (34%) over RSV-B (19%) throughout the study period. Interestingly, 47% of enrolled infants had mixed infections (both RSV-A and RSV-B) [Figure 8].

4.5 Correlation of F antibody titers with demographics and circulating RSV subgroups

The correlation between infants' anti-F Abs titers with maternal RSV Abs titers, patients' demographic characteristics, RSV load and subgroups were all investigated to understand the infection manifestation among young infants. The inspected correlations were analyzed by Pearson's and Spearman's correlation tests in case of parametric and non-parametric samples, respectively. All investigated correlations between unadsorbed, Pre-F and Post-F adsorbed sera were evaluated by two-way ANOVA and multiple t-tests. Significance was considered at $p < 0.05$ in all performed statistical analysis.

4.5.1 Association between infants' and maternal Ab titers

We first ran the analysis to study the correlation between infants' and mothers' Abs titers as indicated above. Our results indicated positive correlation between the aforementioned variables (Pearson r= 0.48; $p<0.05$), demonstrating that the higher RSV antibody titers a mother had, the higher they were in her infant and vice-versa [Figure 9].

4.5.2 Association of RSV Ab titers (Pre-F and Post-F) with age

Maternal Abs are known to wean out within 6 months after birth and hence, we aimed at analyzing the association between infants' Pre-F (0.03×10^3 2.81×10^3) and Post-F ranged (0.01×10^3 to 3.99×10^3), and age in weeks. Unlike Post-F Abs, Pre-F Abs were negatively associated with infants' age (Pearson r= -0.45; $p<0.05$). These outcomes showed that maternal Pre-F Abs in infants were lower in older infants, concluding that Pre-F Abs decrease in titer over time as expected [Figure 10].

4.5.3 Association of RSV Ab titers (Pre-F and Post-F) with gender

Males are known to be more prone to RSV infection than females (Watkiss, 2012). To study the correlation of F-specific maternal Abs and gender, Man-Whitney test was used. We found no significant correlation between gender and maternal Abs to F-proteins, suggesting that the levels of RSV Abs do not significantly vary depending on gender [figure 11].

4.5.4 Association of RSV Ab titers (Pre-F and Post-F) with bronchiolitis score

Some Ab responses have been shown to elicit enhanced disease illness, like the ones seen in FI-RSV vaccine trails. Some of these Abs have been shown to be directed to the Post-F form of the protein that is expressed on the FI-virus. Accordingly, we ran an

analysis to study the correlation between F-specific antibody titer (Pre- and Post-F) and bronchiolitis score which is an indication of infection severity. Surprisingly, no significant relationships were established in regards of bronchiolitis score with Pre-F, nor with Post-F antibody binding titers (rs = 0.07 and 0.1, respectively; $p>0.05$) [Figure 12].

4.5.5 Association of RSV Ab titers (Pre-F and Post-F) with viral load and genotypes

Infection severity and illness are known to increase with the increase of virus replication in a particular organ. Accordingly, we estimated viral titers in infected patient and ran correlation analysis with sera level of F-specific Abs. Results revealed slightly significant inverse correlation between RSV Pre-F antibody binding titers and the viral load (Pearson r = -0.28, $p<0.05$), indicating that as Pre-F Abs decrease, RSV replicates more [Figure 13]. Further, we found no correlation between RSV subgroups and Ab titers [Figure 14].

4.6 RSV F gene sequencing analysis

All enrolled RSV-infected children were included in F gene sequencing method to determine potential mutations in most important antigenic sites of F protein. For that, F1 subunit was selected for sequencing as it includes the most imported Pre-F specific site and the most common shared site, site Ø (partially) and site II, respectively. Sequenced F1 subunits were translated, aligned and compared to Protein Data Bank # PDB: 4JHW as a reference protein. At site Ø (aa196 – 210), amino acids alignment showed a very conserved antigenic site among most infants (93.7%), expect in 4 (6.5%) who showed a

bulk of amino acid substitutions at positions: 196, 197, 198, 199, 202, 203, 204, 209. Meanwhile, at site II (aa255 – 276), amino acids alignment showed variations at different positions with different frequencies among infants, of which positions: 258, 262, 263, 268 and 275 were 100%, 4.7%, 46.8%, 100% and 6.2% substituted, respectively [Table 3] [Figure 15].

Table1: RSV detection and subtyping primers used in this study

No.	Primer Name	Sequence (5'–3')	Reference
I		qReal-time RT-PCR for RSV	
1	RSV Forward	GGCAAATATGGAAACATACGTGAA	(Aamir et al., 2013)
2	RSV Reverse	TCTTTTTCTAGGACATTGTAYTGAACAG	
3	RSV probe	FAM-CTGTGTATGTGGAGCCTTCGTGAAGCT-BHQ-1	
II		Nested RT-PCR for RSV subtyping	
1	RSV-AB forward	GTCTTACAGCCGTGATTAGG	(Abels et al., 2001).
2	RSV-AB Reverse	GGGCTTTCTTTGGTTACTTC	
3	RSV-A Forward	GATGTTACGGTGGGGAGTCT	
4	RSV-A Reverse	GTACACTGTAGTTAATCACA	
5	RSV-B Forward	AATGCTAAGATGGGGAGTTC	
6	RSV-B Reverse	GAAATTGAGTTAATGACAGC	
III		F gene sequencing	
1	RSV-A F forward	GGC AAA TAA CAA TGG AGT TG	(Tapia et al., 2014)
2	RSV-A F reverse	AAG AAA GAT ACT GAT CCT G	

Table 2: Bronchiolitis score given to enrolled infected patients at admission (n=62) and at discharge (n=51)

Bronchiolitis score at admission		No. of patients	Percentage (%)
mild	3	2	3.225806452
	4	19	30.64516129
moderate	5	14	22.58064516
	6	15	24.19354839
	7	5	8.064516129
severe	8	6	9.677419355
	11	1	1.612903226
Bronchiolitis score at discharge		**No. of patients**	**Percentage (%)**
1		1	1.960784314
2		5	9.803921569
3		45	88.23529412

Table 3: F1 subunit of fusion protein sequencing results among enrolled RSV-infected infants for site and site II

Antigenic site	Amino acid position	Mutations		
		Name (%)	Reported or not	Effect/importance
Site Ø (partial)	196-210	L196D (6.5%)	-	-
		N197T (6.5%)	-	-
		Y198A (6.5%)	-	-
		I199M (6.5%)	-	-
		G202L (6.5%)	-	-
		L204P (6.5%)	-	-
Site II	255-276	L258N*/C/T (*95%)	-	-
		N262C*/K (*3%)	reported	Resistance to Palivizumab neutralization in vivo and vitro
		D263K*/N (*44%)	-	
		N268L*/F (*98%)	reported	
		S275L*/I/M (*3%)	reported	Syncytium formation impairment

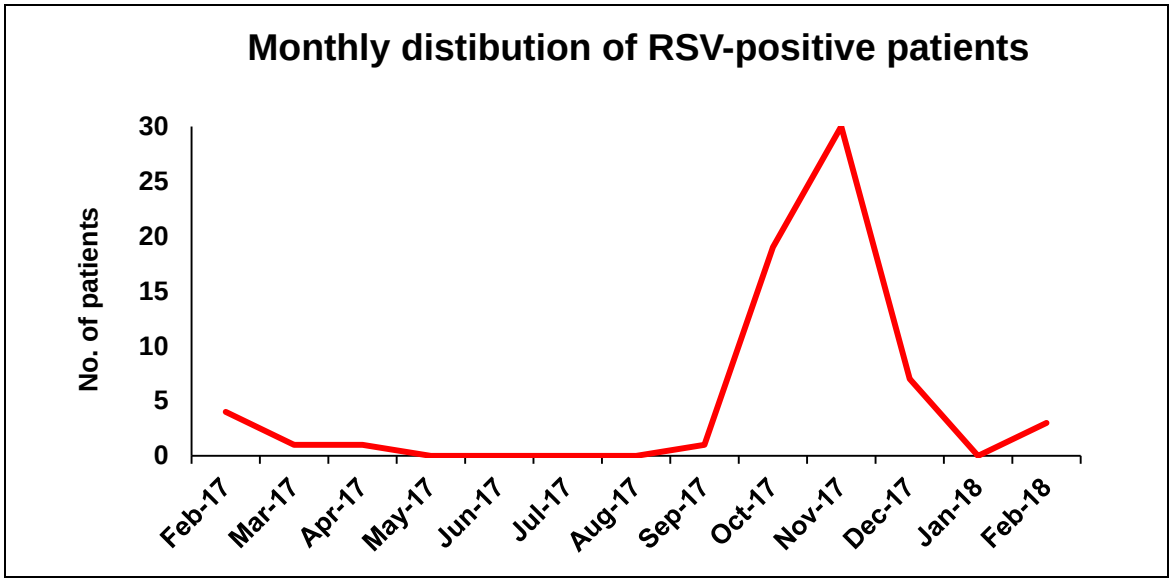

Figure 5: Monthly distribution of enrolled RSV-infected patients in this study from February 2017 to February 2018.

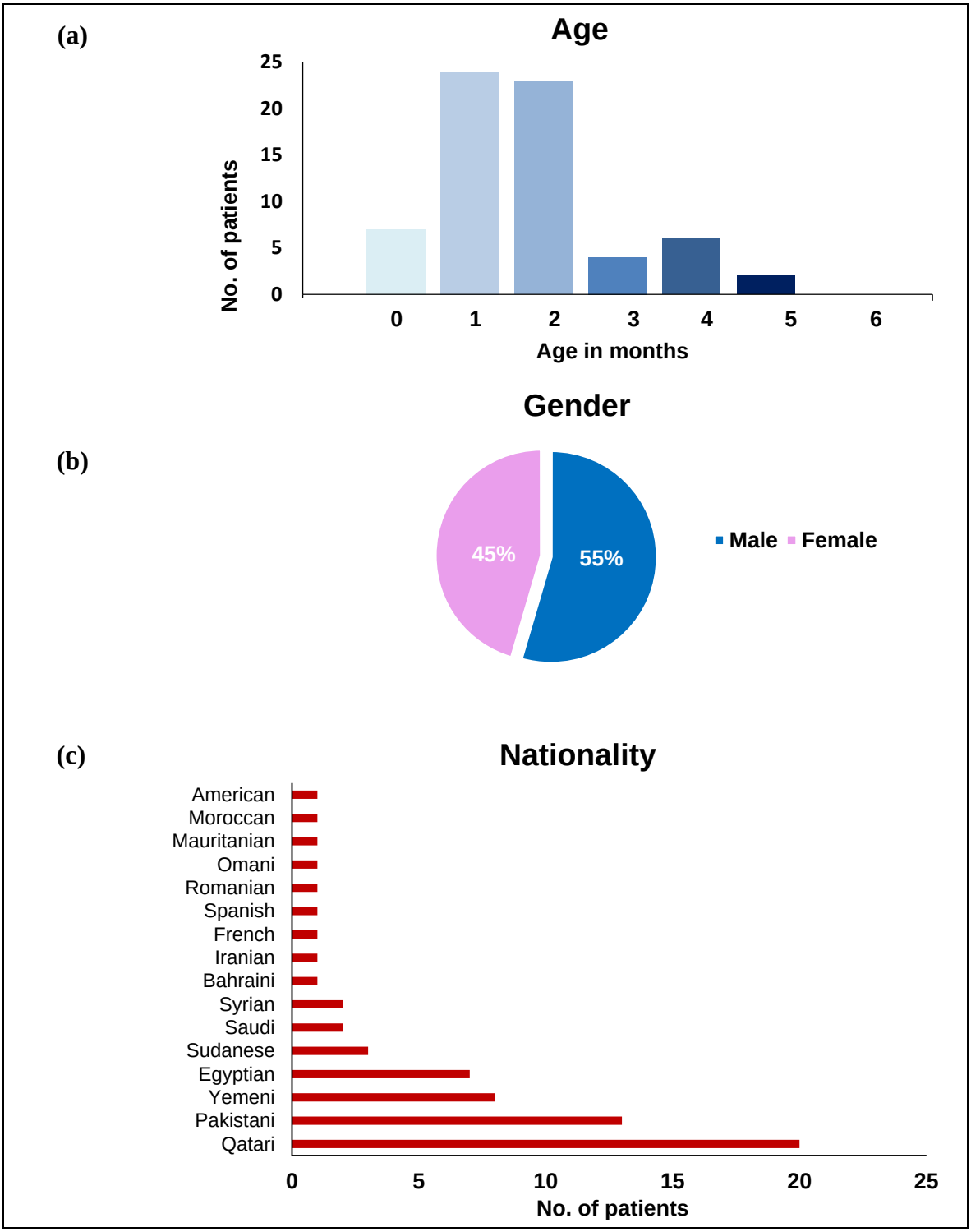

Figure 6: Demographic characteristics of all enrolled patients in regards with (a) age, (b) gender and (c) nationality.

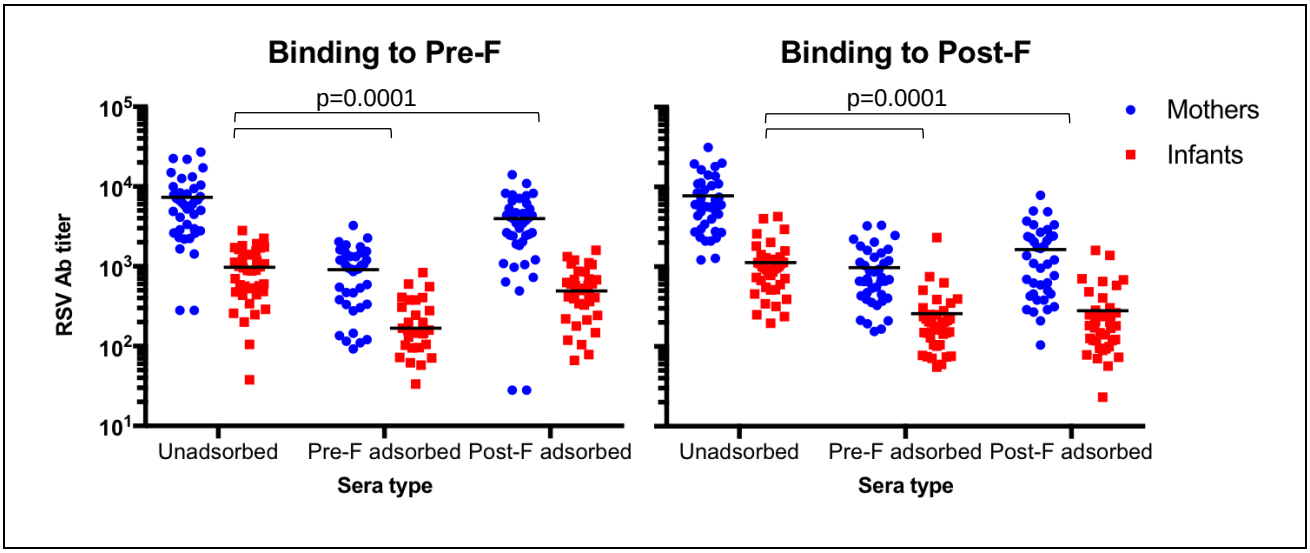

Figure 7: Evaluation of RSV antibody binding activities to both F glycoprotein structures in infants and their corresponding mothers by ELISA. Serially diluted unabsorbed, Pre-F adsorbed, and Post-F adsorbed sera were tested for binding to Pre-F and Post-F proteins and endpoint titers (inverse of log10) were calculated from non-linear curve fitting. EP titers were plotted in dot plot format and statistical analysis was done using Two-way ANOVA tests. Significance was applied when $p < 0.05$.

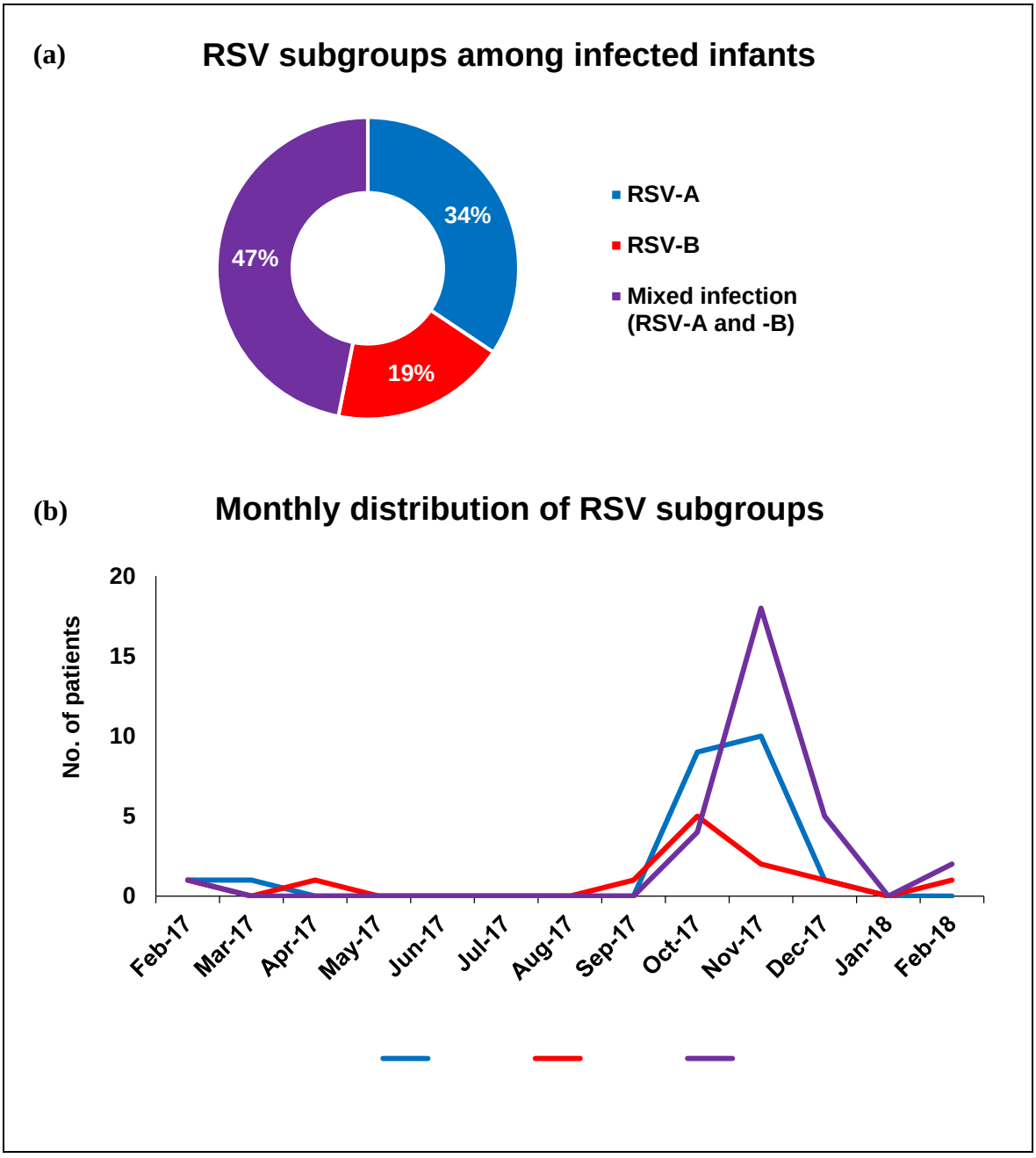

Figure 8: (a) **Detected RSV genotypes** among infected infants and (b) their monthly distribution throughout the study timeline (from February 2017 to February 2018).

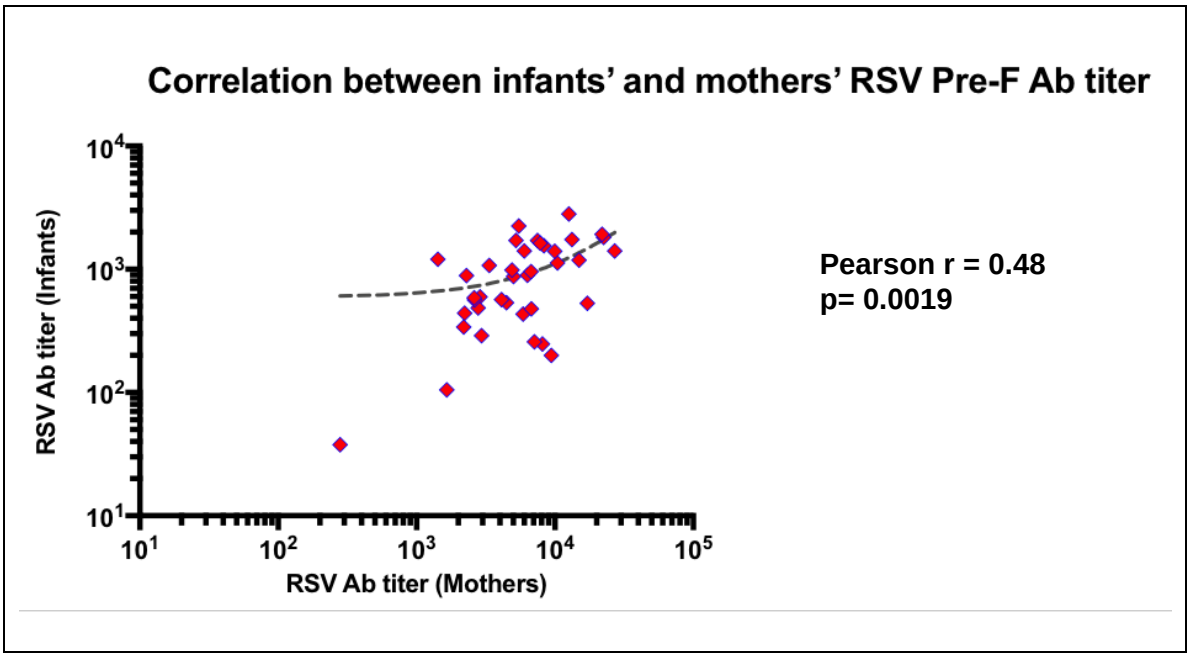

Figure 9: Association between infants' and mothers' Ab titers to Pre-F-protein. Correlation was tested using Pearson's correlation test r. Significance was applied at $p < 0.05$.

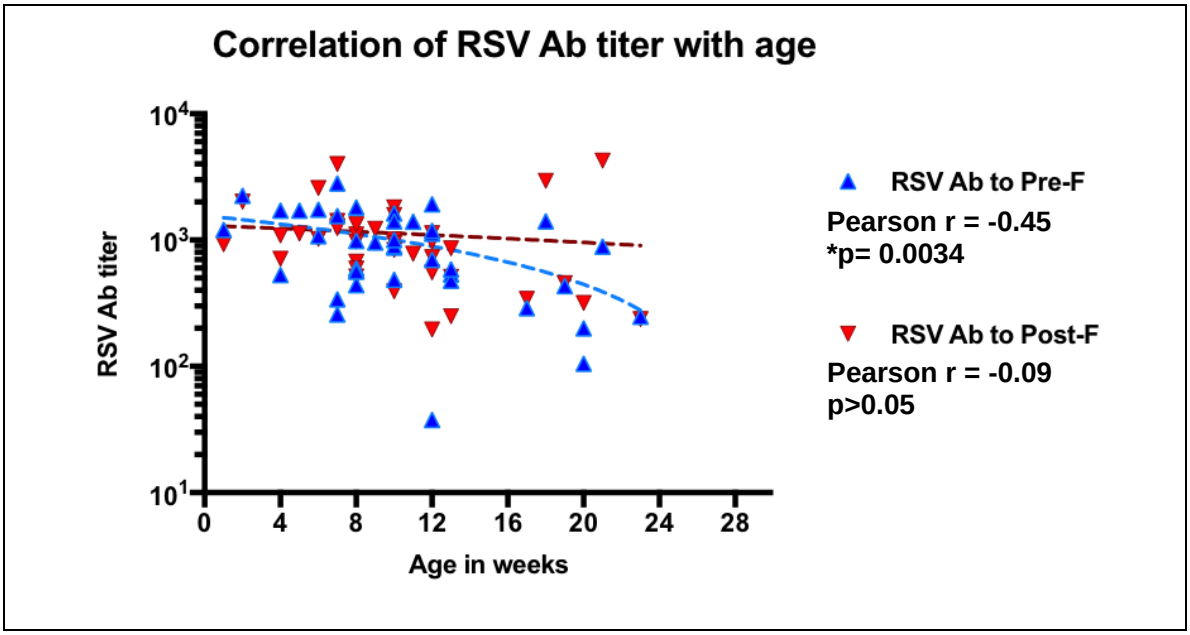

Figure 10: Association between infants' F-specific Ab titers and age expressed in weeks. Analysis was done for both Pre-F (blue) and Post-F (red) Ab titers. This correlation was tested using Pearson's correlation test r. Significance was applied at $p < 0.05$.

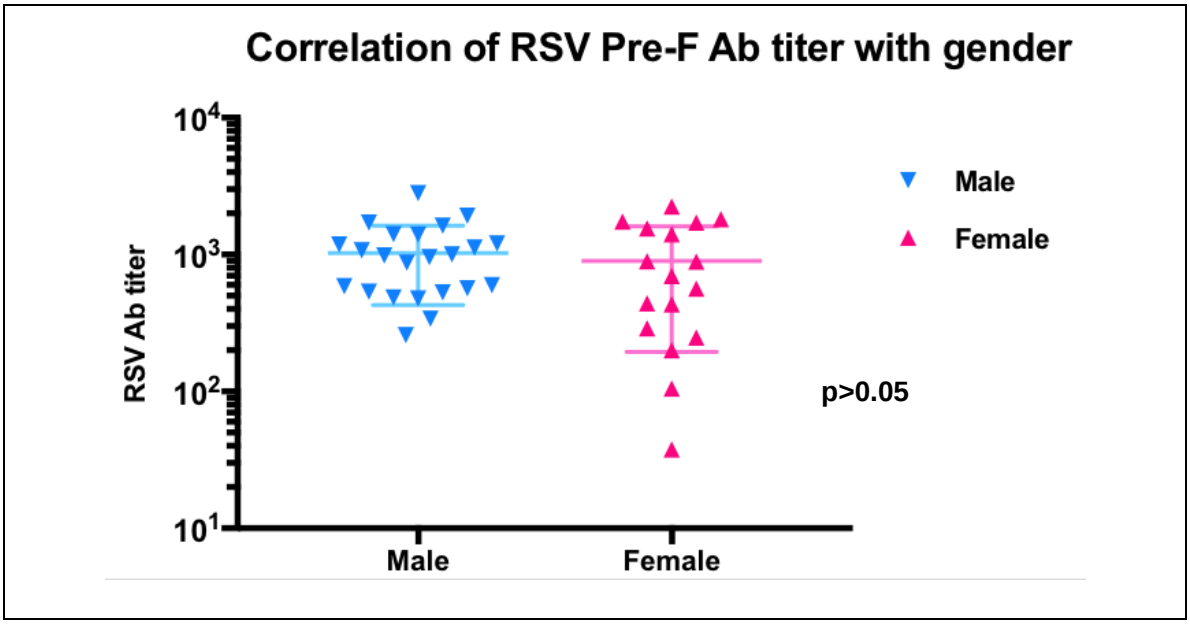

Figure 11: Association between infants' Pre-F specific Ab titer and gender. This correlation was tested using Man-Whitney test. Significance was applied at $p < 0.05$.

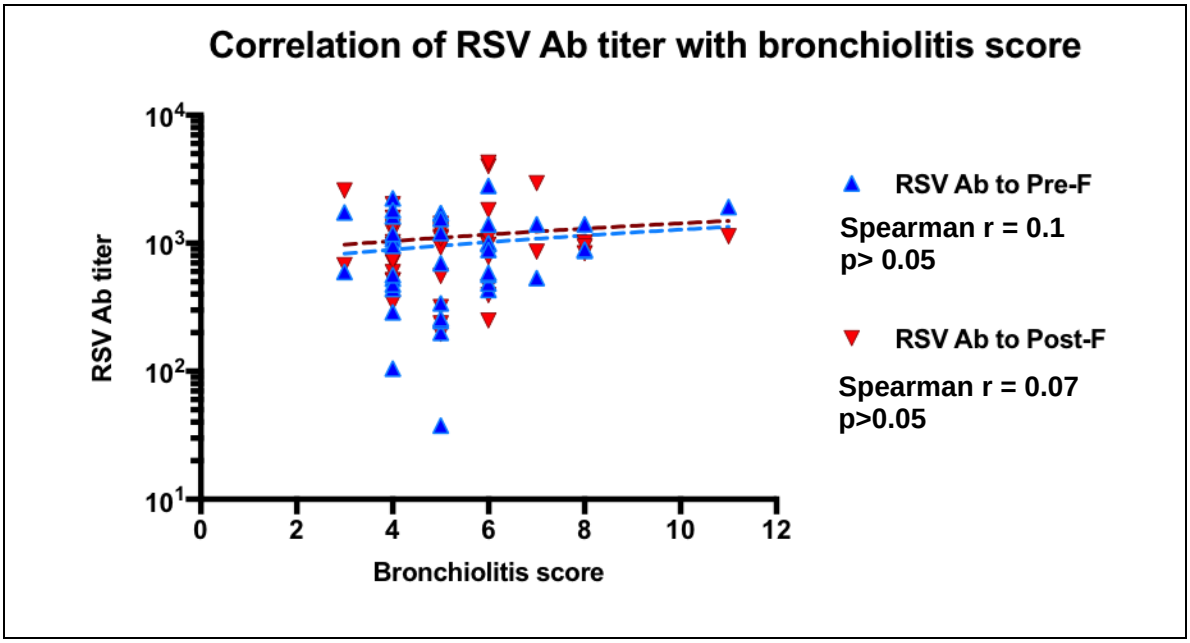

Figure 12: Association between infants' Pre-F specific Ab titer and bronchiolitis score. This correlation was tested using Spearman's correlation test rs. Significance was applied at $p < 0.05$.

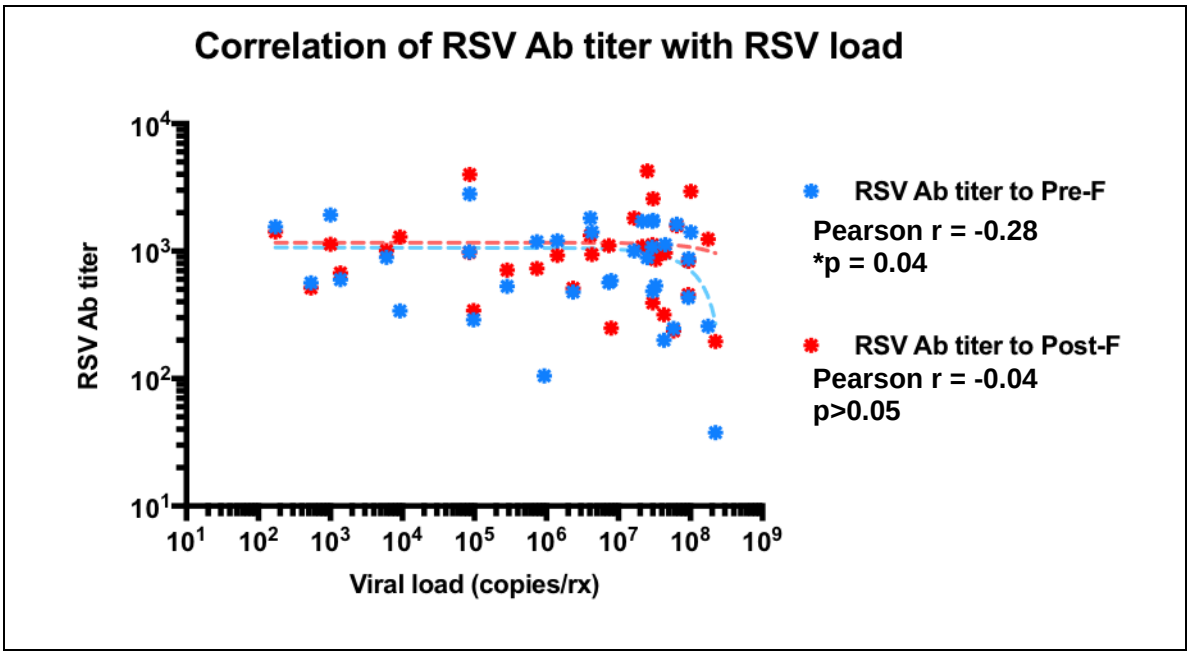

Figure 13: Association between infants' Pre-F specific Ab binding titer and RSV load. This correlation was tested using Pearson's correlation test r. Significance was applied at $p < 0.05$.

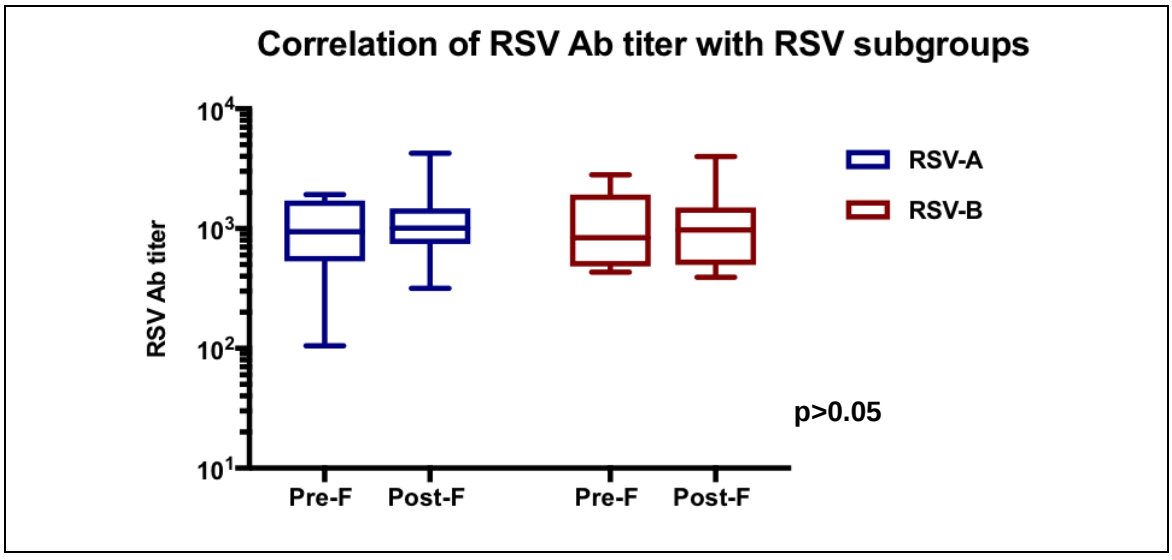

Figure 14: Association between infants' F-specific Ab titers and RSV subgroups. Correlation matrix and Two-way ANOVA were used. Significance was applied at $p < 0.05$.

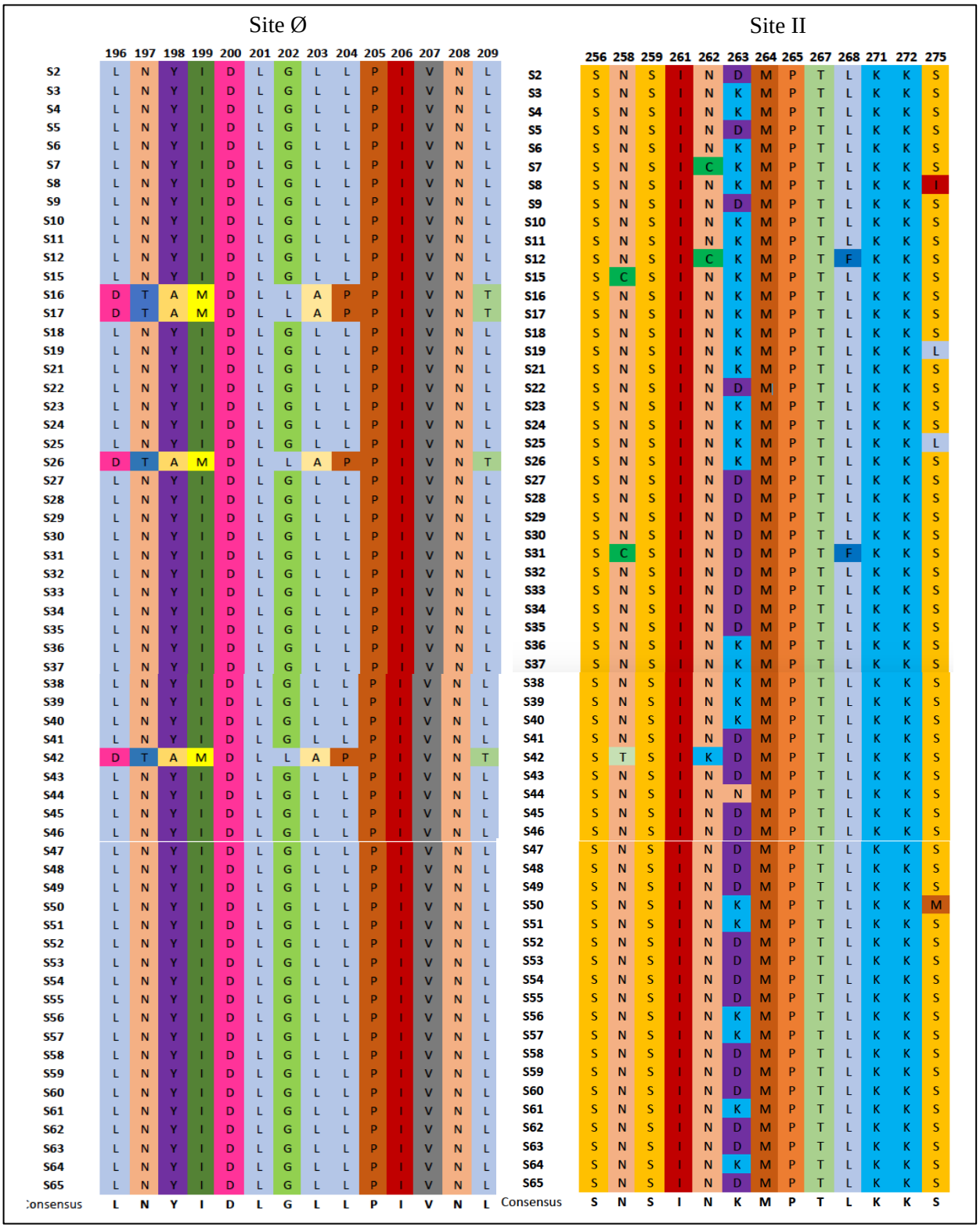

Figure 15: Amino acid alignment of site Ø and site II of F1 subunit of fusion protein among enrolled RSV-infected infants

V- DISCUSSION

RSV expresses two surface glycoproteins, of which, F protein is metastable and changes conformation to a more stable structure spontaneously (Gilman et al., 2016). In fact, recent studies showed that an RSV virion loses its Pre-F confirmation structure within few hours. Furthermore, formalin inactivation of RSV results in significant changes in the F-structure within 72 hours of treatment, which partially explains the weakness in immunogenicity and the lack of protection following FI-RSV vaccine trials (Killikelly et al., 2016). Following Pre-F stabilization with cavity-filling-mutations, new epitopes were identified on Pre-F which seem to induce more potent Abs than Post-F. These Pre-F specific epitopes include site Ø, site V and site VIII (Mousa et al., 2017). Consequently, stabilized Pre-F has been selected as a potential vaccine candidate. Considering failures of previous RSV clinical trials in children, where most vaccines induced enhanced disease illness rather than protection, alternative vaccine strategies have been considered (Graham et al., 2015). Knowing that infants below 3 months of age are the most vulnerable group to RSV infection, it is thought that RSV vaccination during pregnancy could boost pre-existing neutralizing antibody titers, providing passive protection to newborns (McLellan, Chen, Leung, et al., 2013) (Graham, 2017).

Previous studies on maternal Abs role in RSV protection were focused on Post-F Abs, which were found to be non-protective regardless of their titer levels (Swanson et al., 2011). With the availability of Pre-F protein that was generously provided to us by our NIH colleagues, we sought for determining the role of maternally derived Pre-F Abs

in RSV protection in infants. Accordingly, magnitude of maternally derived Pre-F Abs were determined in 65 RSV-hospitalized infants using binding and neutralizing procedures.

Expectedly, our recruited patients were all below the age of six months in which, 89.2% of them were below three months old. Gender-wise, 55.3% of them were male. And nationality-wise, a huge variability was observed among our samples as Qatar in a multi-national county. That said, Qataris, Pakistanis then Yemenis represented 30.7%, 20%, and 12.3% of patients' cohort, respectively. To better understand the role of anti-F Abs in protection or enhanced diseases illness in hospitalized patients, we recorded data about disease manifestation, such as severity and bronchiolitis scores, to include in the analysis. Most infants had a score between 4 and 6 (77.4%) at admission, and a score of 3 (88.2%) at discharge.

Evaluation of binding activities of maternally transferred RSV Abs revealed low presence of Ab levels against Pre-F- and Post-F-directed Abs with an average $0.97x10^3$ and $1.12x10^3$ end-point titer, respectively. These binding titers are considered remarkably low to provide enough neutralizing titers according to previous studies which found that a minimum end-point titer of 1:256 (8 log2) is required to provide protection from RSV infection (Piedra, Jewell, Cron, Atmar, & Glezen, 2003) (Jounai et al., 2017). To further investigate the levels of Abs directed to each form of the F-protein, we ran adsorption assay followed by ELISA binding with both, Pre-F and Post-F proteins. Unlike Post-F, adsorption of sera samples with Pre-F removed most binding activity to both fusion conformations (approximately 80%) in both infants and mothers.

These results are similar to what have been reported recently by Ngwuta et al., where anti-F Abs accounted for more than 90% of the binding Abs in apparently healthy individuals aged between 7 and 93 years old (Ngwuta et al., 2015). Accordingly, a Pre-F based vaccine would be ideal to stimulate and boost pre-existing memory B cell repertoire in pregnant women to induce high levels of potent maternal Abs. However, our study is missing healthy control group, age-matched RSV-negative children, to compare RSV antibody levels between affected and non-affected populations to drive better conclusions. In fact, it would be more ideal to run a longitudinal study that follow up the levels of Abs of infants from birth until six months and relate that to RSV infection and mode of illness. Nonetheless, these studies are not easy to do, considering all required ethical approvals and commitment of participants.

Abs could be beneficial or harmful according to their type and epitopes they target. Antibody-mediated enhanced disease illness has been reported with many viral infections including RSV. In fact, enhanced disease illness in FI-RSV vaccine trials was partially attributed to the presence of post-F antibody response (Acosta, Caballero, & Polack, 2016). Accordingly, we ran several correlation analyses to study the link between Abs level to both F forms, demographics and clinical manifestations. Specifically, correlations of maternally transferred RSV Pre-F binding Abs with their age, gender, bronchiolitis score, maternal RSV Pre-F antibody titer, and RSV load and subgroups were examined. In average, around 14% of maternal pre-F Abs were transferred according to our analysis. We found significant correlation of Pre-F Abs in infants with mothers' Pre-F Abs, age (in weeks), and viral load, but not with gender nor

with bronchiolitis score. In healthy conditions, neonatal Abs correlate significantly with maternal Abs. That is, the higher antibody titer a mother has, the higher level of Abs are transferred to her infant (Palmeira, Quinello, Silveira-Lessa, Zago, & Carneiro-Sampaio, 2011). Consequently, maternally transferred Abs would decrease over time and naturally induced Abs would increase following infection. Therefore, it would be interesting to follow up with these patients to see whether they elicited Pre-F specific response after their primary infection, and whether such response has been affected negatively or positively by the levels of maternal Abs. On the other hand, the low levels of infant's Pre-F specific Abs (average 0.97×10^3) are attributed to the low level of corresponding mothers [average end-point titers of Pre-F- and Post-F-binding antibody were 7.33×10^3 and 7.75×10^3, respectively]. Finding 14% of maternally transferred Abs, and knowing that a titer of 1:256 (8 log2) is needed for protection, an effective vaccine should elicit a minimum titer of 50 log2 in pregnant women to ensure good transfer of protective antibody titer in infants. Pre-fusion RSV has been shown to strongly boost Pre-F specific neutralizing responses in animal models, reaching 14-fold increase in pre-existing titers would result in 4-5-months of neutralizing antibody titers above the protective titer of 1:180 in babies (Steff et al., 2017). A clinical trial to test dose, safety, tolerability and immunogenicity of a stabilized RSV Pre-F subunit protein vaccine in healthy adults is on-going (ClinicalTrials.gov identifier NCT number: NCT03049488). Combined results from this trial and our study will provide better insight on the potential success rate of the vaccine in pregnant women.

No correlation was found between Pre-F antibody levels with gender and bronchiolitis score, similarly to few recent studies (Jans et al., 2017). Unlike with gender, bronchiolitis score was expected to reflect disease severity and correlate negatively with RSV Pre-F Abs or/and positively with Post-F Abs. However, no association was observed with Pre-F Abs, nor with Post-F Abs. This could be partially attributed to the low level of Pre-F Abs in all enrolled cohorts and to the absence of healthy control groups. Further, different clinicians would rate the disease severity in patients differently. Finally, a higher number of samples might be needed to generate better statistical values for our analysis.

Molecular analysis revealed high virus titers in the nasal aspirates, with an average of 4.48×10^7 copies/reaction (PFU/ml) in all recruited infants. This high level of virus replication was expected considering the low antibody titer to the F-forms. Since virus replication is an indication of illness severity, correlations between Pre-F Abs titers and viral titers were tested. We expected a negative relationship between RSV antibody titers, mainly Pre-F-directed, and the viral load. Indeed, inverse correlation was seen between Pre-F Abs and viral load ($p < 0.05$). Subgrouping revealed more circulation of RSV-A than RSV-B with incidence of 84% vs. 66%, respectively, in which 47% of patients had mixed infections. It has been reported that RSV subgroups can coinfect individuals either at the same period of throughout a lifetime (Tan et al., 2013). However, our mixed infections scores were surprisingly high compared to adjacent regions and worldwide. For instance, in Saudi study done in 2014, 77% of RSV-positive infants belonged to hRSV-A, and 23% belonged to hRSV-B. And a co-infection with

both strains represented 5.7% (Ahmed et al., 2016). While in Morocco, it was documented that among 18.2% of RSV-positive cases, a mixed infection of RSV-A and RSV-B was witnessed in 37.9% (Jroundi et al., 2016). Meanwhile in China, a 3-year study revealed that 51.2% of patients were RSV-A positive compared to 48.8% RSV-B positive. No co-infection with both viruses was reported (W. Liu et al., 2016). Both RSV subgroups can be recognized through cross-reactive antibodies indicating presence of cross-immunity to both subgroups, then through the high variable region of G gene (Tan et al., 2013). Unless our co-infected children go to a common nursery, or acquired the second viral infection from the PEC itself, our findings require further analysis and confirmation to determine an accurate RSV subgrouping prevalence.

No correlation was found between antibody titers to Pre-F/Post-F and RSV subgroups A and B, where subgrouping is determined based on G-protein rather than F-protein. Interestingly, we could not detect amplified fragment by gel electrophoresis following by nested RT-PCR in one out of 65 patients, although all samples tested positive at PEC – HMC and at BRC - QU using PCR and displayed RSV-like illness. These discrepancies might be due to low RNA quality and quantity, or due to presence of G gene mutations in this patient.

F gene sequencing was performed among all enrolled RSV-infected children to investigate the presence of any potential mutations in the most imported Pre-F specific antigenic site and the most common shared site targeted by the only approved prophylactic drug, site Ø (partially) and site II, respectively. Amino acids alignment in site Ø (aa196-210) conserved among 93.7% of isolated RSV. Yet, 6.5% of them had the

following mutations: L196D, N197T, Y198A, I199M, G202L, L203A, L204P, L209T. These exact mutations were interestingly never reported before it the literature. Instead, D200N, K201N, and K209Q were the most reported ones at site Ø in other studies (Okamoto et al., 2018) (Hause et al., 2017). To eliminate any bias outcomes, reperforming F gene sequencing is highly considered to validate our mutations. Further, once confirmed, and if those mutations happen to at the binding site of monoclonal site Ø Pre-F specific antibodies, this could affect the virus neutralization by maternal antibodies and thus explain infants' hospitalization with RSV. Regarding site II (aa255 – 276), amino acids alignment showed variations of mutation frequencies among infants, such as: L258N, N262C, D263K, N268L and S275L. Compared to literature, N262K, N268I/L and S275L mutations were reported in other studies (Chen et al., 2018) (Hashimoto & Hosoya, 2017; Ogunsemowo, Olaleye, & Odaibo, 2018) and showed resistance of mutant RSV isolates to Palivizumab neutralization, knowing that its binding site is aa262 to aa276 (Hause et al., 2017). It would be interesting to follow up with our patients to investigate whether isolated mutant viruses acquired resistance to Palivizumab or/and to Pre-F Abs. These findings could be in favor of introducing stabilized Pre-F antigen as a vaccine in pregnant woman since circulating RSV started acquiring mutations at the binding site of the only prophylactic drug, resulting in resistance to its neutralizing activity.

Further analysis like measuring RSV neutralization should be tested in future. Nonetheless, we expect very low titers of neutralizing Abs considering all the above findings. Additionally, it would also be important to run similar analysis on age-

matched RSV-negative controls to validate most of our conclusions. However, recruitment of more patients for the sake of this study was a huge obstacle due to the virus seasonality, and hesitation of guardians to enroll their kids in the study.

VII-CONCLUSION

This is the first study that aims at providing an evidence-based overview on the contribution of Pre-F specific maternal Abs in RSV protection. Data from this study shall guide the development of strategies to evaluate Pre-F RSV vaccine in pregnant women. Our results indicated that only 14% of maternal Abs to RSF F-protein are transferred to their infants. Having in mind that at titer of at least 1:256 (8 log2) is required for protection in infants below the age of 6 months, a vaccine shall induce an antibody titer of 50 log2 in pregnant women to insure sufficient transfer of protective Abs. According to other studies, approximately "every 2-fold rise in cord blood neutralizing antibody titers reduces the risk of RSV-associated hospitalization in the first 6 months of life by 26–30%" (Steff et al., 2017). This approach seems very applicable considering our findings, where Pre-F Abs represented more than 80% of the response in infants' and mothers' samples. That is, a Pre-F vaccine would stimulate and boost pre-existing Pre-F specific memory B cell repertoire in pregnant women to induce high levels of potent Abs.

A major drawback in our study is that we analyzed F-forms RSV Abs only at one time point which is not a good indicative of the maternal antibody transfer in this regard. Accordingly, an ideal analysis would involve a serial sampling of infants in their first six months of life, while RSV monitoring infection status. This kind of study is hard to manage due to the needed ethical approvals, commitment of enrolled families, and continuous follow-up.

Interestingly, we could not find any correlation between Pre-F antibody titers and clinical manifestations such as gender, bronchiolitis score. This could be attributed to several factors including: low number of samples, inconsistency of reporting severity score between different practitioners, and absence of healthy control. Further, our analysis was focused on Pre-F Abs considering their protective potential, while a deeper analysis on Post-F might be required considering their recognized effect on enhanced disease illness.

Few groups have recently started phase 1 to 3 clinical trials implementing different RSV vaccines in adults and elderly people (Falloon et al., 2016). Importantly for us, a study is currently running in the USA in collaboration between NIH Vaccine Research Center and Novavax company to test RSV Pre-F+Alum in pregnant women in their last trimester. The study, which is anticipated to be completed on May 2020, aims to determine the safety, immunogenicity, and efficacy of this vaccine in protecting young infants from RSV infection after 90 days from delivery through maternal immunization (Thomas, 2020). It would be ideal to contribute in making such clinical trials successful by providing more conclusive finding in regards to support the importance of introducing RSV Pre-F antigen vaccine to pregnant mothers.

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Appendix A. Ethical Approvals

مؤسسة حمد الطبية
Hamad Medical Corporation
مركز البحوث الطبية
Medical Research Center

Hamad Medical Corporation

Institutional Review Board

Email: irb@hamad.qa Tel: 00974-44390614
HMC-IRB Registration: SCH-HMC-020-2015
IRB-MoPH Assurance: MOPH-A-HMC-020

Approval Notice:
Protocol Title: Magnitude of respiratory syncytial virus (RSV) Fusion Protein-Specific Antibodies in Infants and Corresponding Mothers in Qatar
Study Number: 16196/16
HMC Principal Investigator: Khalid Al Ansari
Date of HMC-IRB Approval: 05 December 2016
Date of Letter Issued: 08 December 2016
Review Type: Expedited
Decision: Approved
Approved HMC Enrollment: 100 Pairs (Mother & Child)

The IRB has reviewed the submitted documents of the above titled research and approval for the study has been granted. The list of approved document(s) is attached.

IRB oversight expires 12 months from the date of approval indicated above.

It is the responsibility of the Investigator to ensure timely renewal of study oversight. Progress reports for continuing review must be approved prior to expiration date; therefore submissions must be received by the IRB 60 to 90 days prior to the expiration date.

The IRB notes the extensive delegation log for this study and in keeping with the previous discussions about such extensive logs in the PEC, it is of great importance that the plan of education and communication that was presented to the IRB is applied for the staff involved in this study.

Requested Resolutions: **None**

Any resolutions submitted must include a letter indicating that the submission is a follow up request by the IRB; this will ensure that resolutions are processed appropriately and in a timely manner.

Please note; this approval only covers HMC, you may also need approvals from other institutions involved in your study. You should not start your study until all of these have been obtained

If you have any questions or need additional information, Please contact IRB at the above mentioned email address or telephone number. Your responsibilities as Principal Investigator are listed in the "Important Note" at the end of this letter.

Sincerely,

Prof. David Barlow
Chairman Institutional Review Board
Hamad Medical Corporation

جامعة قطر
QATAR UNIVERSITY

Qatar University Institutional Review Board
QU-IRB

March 7, 2018

Ms. Sara Taleb
Graduate Student Project
Qatar University
Email: st1000813@student.qu.edu.qa

Dear Ms. Sara Taleb,

Sub.: **Research Ethics Review Exemption / Graduate Student Project**
Ref.: **Project titled, "Magnitude of RSV Fusion Protein-Specific Antibodies in Infants and Corresponding Mothers in Qatar"**

We would like to inform you that your application along with the supporting documents provided for the above proposal, is reviewed and having met all the requirements, has been exempted from the full ethics review.

Please note that any changes/modification or additions to the original submitted protocol should be reported to the committee to seek approval prior to continuation.

Your Research Ethics Approval No. is: **QU-IRB 890-E/18**

Kindly refer to this number in all your future correspondence pertaining to this project.

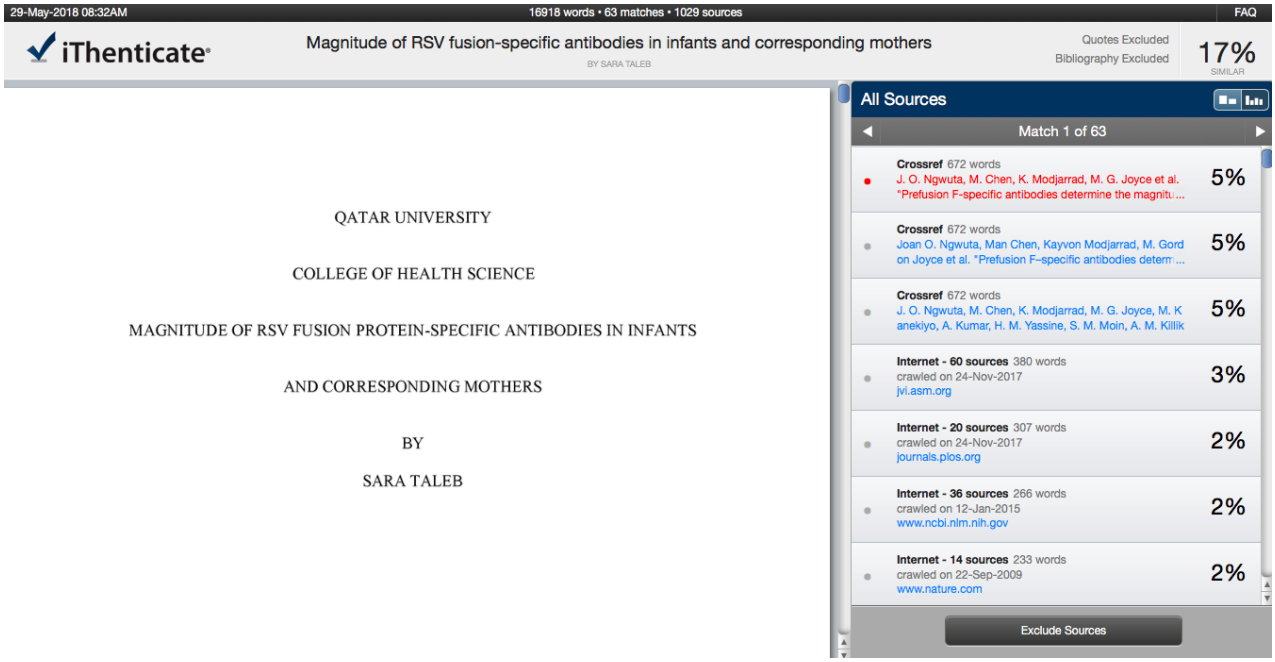
Best wishes,

K. Alali

Dr. Khalid Al-Ali
Chairperson, QU-IRB

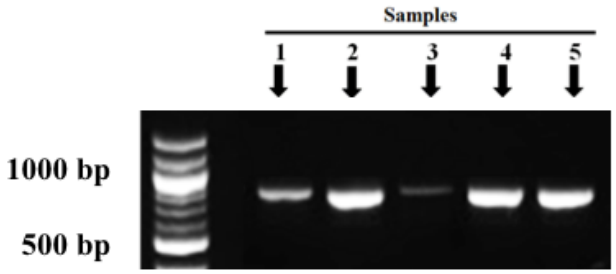
جامعة قطر
QATAR UNIVERSITY
Institutional Review Board
(IRB)
Office Of Academic Research

Appendix B. Plagiarism report

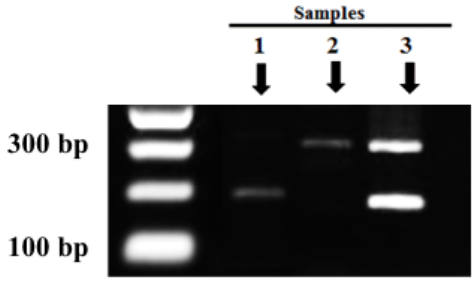


Appendix C. Gel electrophoresis for G gene and F gene

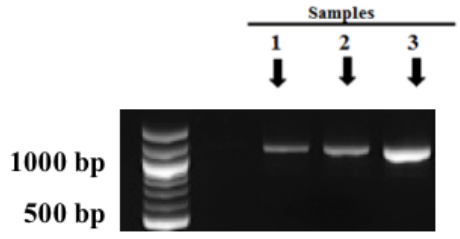
1. G gene of either RSV-A or RSV-B
Product: 836bp



2. G gene of either RSV-A and RSV-B
Product: 334bp and 183 bp, respectively



3. F gene of either RSV-A or RSV-B
Product: 1046bp or 1017bp, respectively



Appendix D. 3D Crystal Structure of Respiratory Syncytial Virus Fusion Glycoprotein Stabilized in the Prefusion Conformation (4JHW)