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Level of maternal respiratory syncytial virus (RSV) F antibodies in hospitalized children and correlates of protection

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ABSTRACT

Background: Respiratory syncytial virus (RSV) is a major cause of lower respiratory infection among children and no vaccine is available. The stabilized form of the fusion (F) protein – pre-F – is a leading vaccine candidate to target different populations, including pregnant women. This study aimed to determine the magnitude and nature of RSV-directed maternal antibodies (matAbs) in hospitalized children with RSV infection.

Methods: Sixty-five paired blood samples were collected from RSV-infected children aged <6 months and their corresponding mothers. All pairs were screened for levels of pre-F and post-F antibodies using ELISA. The neutralizing antibodies (NAbs) in both groups were measured *in vitro* against mKate RSV-A2 using H28 cells.

Results: It was found that 14% of matAbs ($\log_2$ 12.8) were present in infants at hospitalization, with an average $\log_2$ EP titer of 10.2 directed to both F-protein conformations. Additionally, 61.4% of maternal NAbs ($\log_2$ EC_{50} = 9.4) were detected in infants ($\log_2$ EC_{50} = 8.7), which were mostly pre-F exclusive (81%). Pre-F antibodies in children showed a positive correlation with matAbs titers and negative correlations with age and bronchiolitis score.

Conclusions: The maintenance of neutralizing activity in infants relative to maternal titers was greater than the maintenance of antibody binding based on ELISA, suggesting that higher-potency antibodies may have a longer half-life than weakly neutralizing antibodies.

Introduction

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection and hospitalization of pediatric patients in their first 5 years of life (Nair et al., 2010; Graham, 2017). The virus accounts for up to 60% of pneumonia and bronchiolitis (Piedimonte, 2015), and about 7.0% of deaths in infants between 1 month and 1 year of age (Nair et al., 2010). RSV reinfection is frequent in adults, since exposure to the virus does not provide long-term immunity (Varga and Braciale, 2013), and may result in serious complications. Palivizumab is administered to reduce RSV burden and complications in premature infants at high risk (C.O.I. Diseases, 2009). This FDA-approved prophylactic monoclonal antibody (mAb) is delivered monthly during winter seasons but is costly and limited in its efficacy. Therefore, it has restricted usage in certain developed countries for infants <29 weeks' gestation (Brady et al., 2014; Banerji et al., 2014). For these reasons, to reduce the significant public health burden of RSV infection in young children, there is still a need to develop effective vaccines and more potent mAbs (Modjarrad et al., 2016; Simões et al., 2015).

RSV (*Pneumoviridae* family) is an enveloped virus with a non-segmented 15.2 kbp ssRNA genome, coding for 11 viral proteins (Afonso et al., 2016; Collins, 1991; Kingsbury, 2012). The two main surface glycoproteins are the main target for NAbs and vaccine development. The attachment protein (G) facilitates viral attachment to target cells (Teng et al., 2001) and helps the virus to evade

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the immune system (Taleb et al., 2018). It is also used to define RSV subgroups according to the sequence of the hypervariable regions (HVR) (Johnson et al., 1987). The fusion protein (F) facilitates viral entry to host cells by fusion with the host membrane. It also triggers the production of potent NAbs (McLellan et al., 2013a). F is genetically conserved inter-strain and intra-strain, but is structurally unstable as it irreversibly changes from pre-fusion (pre-F) to post-fusion (post-F) conformations (Mousa et al., 2017). Both conformations share antigenic sites such as sites I and II, which are the palivizumab targets. However, most of the NAbs target the pre-F rather than post-F epitopes (McLellan et al., 2013b). Therefore, stabilized pre-F has been considered a promising vaccine candidate for different populations, including young infants, the elderly and pregnant women (Graham, 2017; Anderson et al., 2013; Krarup et al., 2015). It is therefore crucial to understand the correlation between pre-F-specific antibody responses and protection from RSV disease in infants. This study estimated the magnitude and analyzed the nature of RSV-directed maternal antibodies (matAbs) in hospitalized children with RSV infection.

Methods

Study population and design

This cohort study was designed to enroll children admitted with RSV infection to the Pediatric Emergency Center (PEC) of Hamad Medical Corporation (HMC), Qatar. Because of the nature of RSV seasonality, 65 RSV-positive children and their corresponding mothers were enrolled between 2017–2018. Paired blood samples (from infants and their mothers) and nasal swabs/aspirations (from RSV-infected infants only) were collected at hospitalization. RSV-infected infants are hospitalized in the PEC unless the treating physician determines that they: (1) do not need supplementary oxygen; (2) are feeding adequately without intravenous fluids; (3) have minimal or absent wheezing, crackles and chest retractions; and (4) have an oxygen saturation $\geq$94% and a bronchiolitis severity score <4 on discharge. Bronchiolitis scores were assigned by PEC healthcare providers according to the observer agreement for respiratory signs and oximetry in infants aged <2 years and hospitalized with lower respiratory infections (Wang et al., 1992). Enrollment criteria consisted of infants being: aged <6 months; positive for primary RSV infection; hospitalized at the PEC; and enrolled in this study before receiving any treatment. Collected blood samples from hospitalized infants and their mothers were adsorbed then used to measure antibody binding levels by ELISA and neutralizing capacities by standard and competitive neutralization assays. Nasal aspirates were used to detect RSV load by PCR.

Serum adsorption and ELISA

Sera adsorption from patient samples was performed using 0.5 mg/ml pre-F or post-F. The binding endpoint titer against both conformations was measured by ELISA. Briefly, all sera types (unadsorbed or adsorbed with pre-F/post-F) were serially diluted and incubated with pre-F and post-F pre-coated 96-well plates for 1 h at room temperature. Anti-human IgG-HRP was added to all wells followed by the substrate, and the reaction was then stopped by H_2SO_4. Readings of endpoint (EP) titers were taken at 450 nm. The detailed procedures for adsorption and ELISA have previously been described (Ngwuta et al., 2015).

Neutralization assay and competition neutralization assay

The NAbs titers (effective concentration: EC_{50}) were measured at the Viral Pathogenesis Laboratory (VRC, NIH, USA) as previously stated (Crank et al., 2019). Briefly, original sera were serially diluted and incubated with mKate RSV-A2 (1:16; MOI = 3) with and without a competitor (post-F) for 1 h at 37 °C. The mixture was then added to 80% NCI-H28 cells (ATCC® CRL-5820) and incubated for 24 h at 37 °C. Fluorescence readings were captured at excitation 588 nm and emission 635 nm.

Viral load quantification

The viral load from nasal swabs/aspirations was determined using RT-qPCR, as previously described (Ngwuta et al., 2015). Briefly, extracted viral RNA by QIAamp Viral-RNA Extraction (QIAGEN, Germany) was detected using One-Step reverse-transcriptase quantitative Real-Time PCR kit (Luna® One-Step RT-qPCR Kit, NEB, US), as per the manufacturer's recommendations. The reaction was carried out with the QuantStudio™ 6 Flex Real-Time PCR instrument (Applied Biosystems, USA) using primers and cycle conditions as previously described (Aamir et al., 2013).

Data and statistical analysis

The collected clinical data included patients' age, gestational age, sex, breastfeeding, exposure to smoking, symptoms, and bronchiolitis scores at admission and discharge. Statistical analysis was performed using GraphPad V8 to investigate the potential correlation between matAbs in RSV-infected infants and disease manifestation. Correlations between continuous variables were assessed using Pearson r and Spearman r correlation. In order to account for "age in weeks" in correlations, linear regressions were run between RSV EP titers (infants) and age in weeks to generate residuals that were then correlated with traits of interest using SPSS statistics V27. Significance was applied at $p < 0.05$ in all tests. The name of the statistical test used in each experiment is indicated in the figure legends.

Results

Patients characteristics

Sixty-five pairs were enrolled in one year (February 2017 to February 2018). Infants were: aged 0–6 months (mean: 2; median: 2), previously healthy (90.1%), mostly mature (96.8%), breastfed (87.1%), and not exposed to smoking (89.1%). Regarding sex, there were slightly more males matching the criteria than females (55.3%; 1:1.24 F:M ratio). The bronchiolitis (severity) scores given by nurses/physicians ranged 3–11 (average $\pm$ SD: 5 ± 1.4) at admission, indicating mild infection in 32.3%, moderate in 35.3% and severe in 32.3% of them (Table 1).

Low RSV F-antibody endpoint titers in mothers and hospitalized infants

The endpoint (EP) titers to both pre-F and post-F conformations were calculated using ELISA. In mothers, unadsorbed sera demonstrated similar EP titers to pre-F and post-F, with an average $\pm$ SD of 7370 ± 5669 and 7259 ± 5436 ($\log_2$ 12.8) ($p > 0.05$), respectively. Similarly, unadsorbed sera from infants had equivalent EP titers to pre-F and post-F with an average $\pm$ SD of 1140 ± 929 and 1206 ± 988 ($\log_2$ 10.2) ($p > 0.05$), respectively, representing ~14% of matAbs levels. The adsorption of original sera with pre-F decreased EP titers to pre-F by ~83–90% [EP declined to 191 ± 315 (7.5 $\log_2$) ($p > 0.05$) in infants and to 749 ± 661 (9.5 $\log_2$) ($p < 0.0001$) in mothers] and decreased EP titers to post-F by ~76–87% [EP declined to 283 ± 379 (8.1 $\log_2$) ($p > 0.05$) in infants and to 1014 ± 730 (9.9 $\log_2$) ($p < 0.0001$) in mothers]. The decrease of EP titers post-adsorption was only significant in mothers, since EP titers in infants were originally low. Meanwhile, the adsorption

Table 1
Demographic characteristics of enrolled RSV-positive infants.

Variables	N = 65
Age at admission	
Average ± SD (weeks)	10 ± 5.2
Average ± SD (months)	2 ± 1.2
0–3 months	57 (87.6%)
4–6 months	8 (12.3%)
Gestational age	
Premature (<37 weeks)	2 (3.1%)
Mature (≥37 weeks)	61 (96.8%)
Gender	
Male	36 (55.3%)
Female	29 (44.6%)
Birth weight (kg)	
Average ± SD	3.08 ± 0.5
Underlying illness	
Bronchiolitis	2 (3.2%)
Laryngitis	1 (1.6%)
Propionic acidemia	1 (1.6%)
Hip dysplasia	1 (1.6%)
SLE in mother	1 (1.6%)
None	55 (90.1%)
Symptoms	
Coughing	60 (95.2%)
Runny nose	55 (87.3%)
Sneezing	50 (79.3%)
Wheezing	45 (71.4%)
Fever (>37 °C)	40 (63.5%)
Runny eyes	9 (14.2%)
Pain	2 (3.1%)
Bronchiolitis score	
Average ± SD (at admission)	5 ± 1.4
Average ± SD (at discharge)	3 ± 0.4
Disease severity	
Mild	21 (32.3%)
Moderate	23 (35.3%)
Severe	21 (32.3%)
Breastfeeding	54 (87.1%)
Exposure to smoking	7 (10.9%)

with post-F lowered EP titers by ~57–59% to pre-F and by ~72–77% to post-F. These findings indicate a higher occurrence of pre-F than post-F antibodies in infants' and mothers' sera (Figure 1).

Average to low F-NAbs titers in mothers and infants

To determine the F NT capacity in patients' sera, standard and competitive neutralization assays were performed using mKate RSV-A2. Unadsorbed sera from mothers blocked the viral infection of H28 cells at EC_{50} of 716 ± 612 (log_2 9.4). Meanwhile, infants' sera neutralized the virus at EC_{50} of 440 ± 583 (log_2 8.7), suggesting that significant maternal NAbs (61.4%; $p < 0.05$) were detected in infants. Acknowledging that most antibodies' neutralizing activity is pre-F exclusive (Ngwuta et al., 2015), post-F protein was used as a competitor to estimate the magnitude of pre-F neutralization. The Post-F competition removed 65% of the neutralizing activity in mothers' sera [EC_{50} declined to 250 ± 380 (log_2 7.9); $p < 0.0001$], but 19% of the neutralizing activity in infants' sera [EC_{50} declined to 356 ± 570 (log_2 8.4); $p > 0.05$]. These results suggest that NAbs are mostly directed to post-F in mothers and to pre-F in infants (81%) (Figure 2).

Correlation analysis of the antibody levels with demographics and clinical manifestations

Antibody levels in newborns at birth are utmost maternal and affected by several factors, including age, health and nutritional status of the infant (van den Berg et al., 2011). MatAbs will eventually wane during the first months before naturally-occurring Ab develop later in life (Niewiesk, 2014). Therefore, various correlation analyses were performed to investigate the potential factors affecting the binding and neutralizing activities of the infants' antibodies.

Although ~14% of matAbs remained in infants, significant positive correlations were found between maternal and infant EP titers ($p < 0.0001$; $r = 0.5$) (Figure 3(a)) and between pre-F EP titers and age in weeks ($p < 0.01$; $r = -0.4$) (Figure 3(b)). Interestingly, a positive correlation was found between pre-F EP titers and viral loads ($p < 0.05$; $r = 0.3$) but not with post-F (Figure 3(c)). These correlations were also significant after correcting for age in weeks, including the correlation between pre-F EP titers and viral load ($p < 0.05$; $r = 0.3$) (Supplementary Figure S4). Further, there was a slight correlation between gestational age (in weeks) and matAbs levels in hospitalized children (Supplementary Figure S5). No significant correlation was seen between maternal and infant NAbs levels in both unadsorbed and post-F competed sera ($p > 0.05$; $r = 0.2$ and $r < 0.1$, respectively) (Figure 4). As expected, a significant negative correlation was seen between NAbs titers and infection severity (bronchiolitis score), meaning that F NAbs titers were lower in patients with severe infection ($p < 0.05$; $r = -0.3$) (Figure 5(a)). Finally, pre-F NAbs showed no significance with age in weeks and sex ($p > 0.05$) (Figure 5(b) and (c)). More details about these correlation analyses are shown in the Supplementary figures.

Discussion

RSV is a leading cause of lower respiratory tract infection (LRTI) in the pediatric population, for which a vaccine is not available. RSV surface proteins, F and G, are the primary targets for NAbs, thus, considered for vaccine development. The F-protein has two conformations, one of which (pre-F) possesses more potent epitopes than the palivizumab's site. Vaccination during pregnancy has been an efficient approach to protect young children against pathogens such as influenza, pertussis, diphtheria, and tetanus (Lindsey et al., 2013). Therefore, pre-F-protein is currently under clinical trials as a promising vaccine candidate in pregnant women (NCT04032093).

This cross-sectional study found that all hospitalized infants and most mothers carried low levels of binding antibodies to pre-F and post-F, with average log_2 EP titers of 10.2 and 12.8, respectively. Naturally, matAbs reach up to 120% in infants at birth, then rapidly decay after birth (van den Berg et al., 2011). The half-life of RSV matAbs ranges 36–42 days (Chu et al., 2014). This study found 14% (range: 3–84%) of matAbs present in infants at hospitalization, which is consistent with the waning of passively-transferred antibodies during the early months of life (Chu et al., 2014, 2017). This percentage does not reflect the actual matAb transfer, since blood samples were not collected at birth, rather 1–24 weeks later. Considering the low matAb titer that was found in infants, future vaccines should aim to boost antibody titers to high levels in pregnant women. Hence, vaccine developers should think of novel approaches to achieve this goal using adjuvanted-vaccine or new platforms such as vector-based, DNA or RNA delivery systems (Niewiesk, 2014).

Unadsorbed sera from mothers and infants showed similar bindings to both F conformations, undoubtedly due to shared sites (I, II, IV), representing half the surface of both proteins (McLellan et al., 2013b). The current adsorption assays indicated that most binding antibodies (89.8%) in mothers were directed to pre-F epitopes, lining up with a previous study in adults (Ngwuta et al., 2015). In infants, pre-F antibodies positively correlated with matAbs and negatively with their age ($p < 0.05$). Unfortunately, this analysis was performed on a single time point, thus it was unable to provide better insight into the maintenance of matAbs in infants over time. Multiple factors could influence transfer and decay rates of matAbs, including gestational age and sex (Palmeira

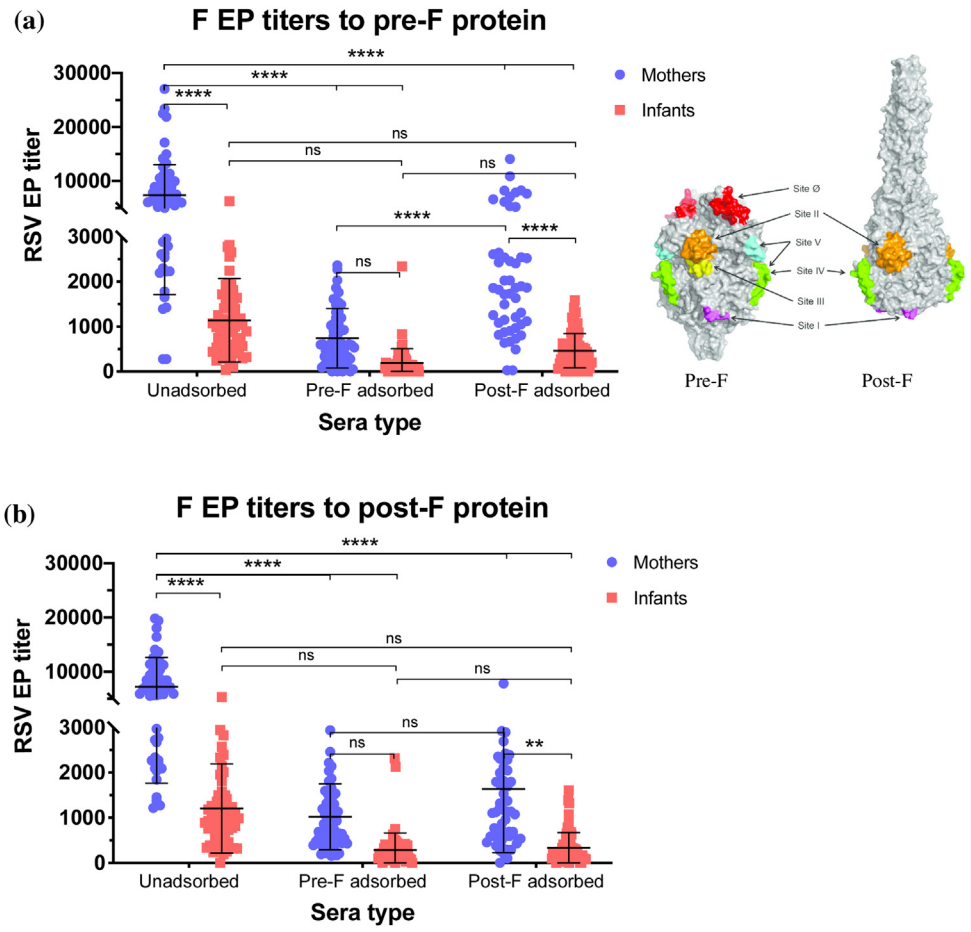

Figure 1. Levels of pre-F and post-F antibodies in infants and corresponding mothers. Sera samples were adsorbed with pre-F or post-F proteins before testing for the EP titer using ELISA f in serial dilutions. (a) EP titer to Pre-F and (b) to post-F protein. EP titers were calculated from non-linear curve fitting of data from individual patients, corresponding to the O.D. reading at highest dilution four times above the background. Statistical analyses were performed in pairs (mothers and infants) in respect to sera types (original; pre-F adsorbed; post-F adsorbed) using two-way ANOVA (multiple comparisons). Significance was applied at *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Illustration of pre-F and post-F protein conformations is adopted from [Flynn JA, 2016].

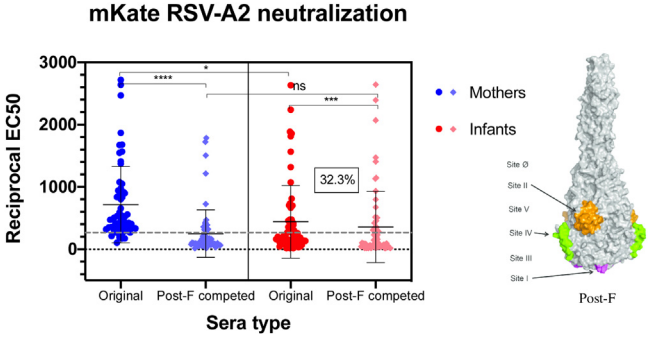

Figure 2. Neutralizing activity of mothers' and infants' sera. Serially-diluted sera (original or post-F competed were incubated with mKate RSV-A2 (diluted 1:16; MOI = 1) for 1 h at 37 °C, then added to 80% confluent H28 cells (ATCC® CRL-5820™) and incubated for 24 h at 37 °C. Plates were then read at excitation 588 nm and emission 635 nm and data were analyzed using nonlinear regression (curve fit); dose-response — inhibition; log (inhibitor) vs. response; variable slope (four parameters) to determine EC_{50} values. The level of protective NAbs titer is demonstrated in a dashed grey line (8 log_2; 256). In the box is the percentage of infants with NAbs above the protective titer calculated from post-F competed sera. Significance was applied by paired t-test at *p-value <0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Illustration of post-F protein conformation is adopted from [Flynn JA, 2016].

et al., 2011). In the current study group, two infants (3.1%) were premature, hence, no statistical analysis could be applied, and no difference in matAb titers was observed between sexes.

Since levels of binding antibodies might not necessarily correlate with less disease severity, hospitalization and delayed primary infection, the neutralizing activity of mothers' and infants' sera was tested against RSV-A2. The correlation of NAbs titers with age, sex, severity score, and viral load was then measured. Mothers had log_2 EC_{50} of 9.4 compared with 8.7 (61.4%) in their infants at hospitalization. No correlation was found between the binding antibody levels and their neutralizing capacity. Following the post-F competition, NAbs significantly decreased to log_2 7.9 and 8.4 in mothers and infants, respectively. The competitive neutralization assay (with post-F) indicated that a substantial fraction of NAbs in mothers were directed to post-F conformation (shared sites). However, 81% of detected matAbs in infants were directed to pre-F conformation. Such observation might propose a phenomenon of selective RSV antibody transfer from mothers to infants, where only potent anti-F-antibodies are passing placentally to infants. According to literature, antibody transfer is affected by certain factors, including IgG subclass (IgG1, followed by IgG4, IgG3, then IgG2), antigen specificity, antibody avidity, and affinity to FcRN (Marchant et al., 2017), whereas antibodies of specific antigens are transferred at different ratios, and those of high avidities to

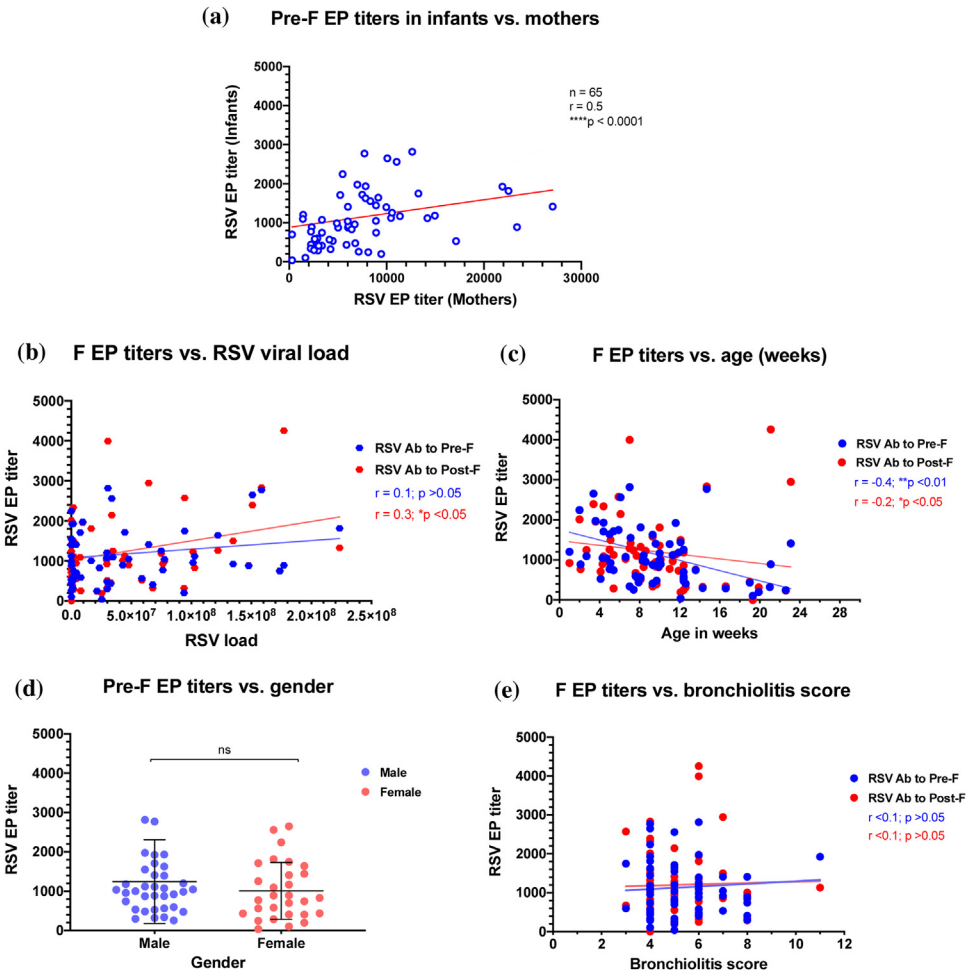

Figure 3. Correlation analysis of RSV pre-F Abs in infants with (a) maternal RSV pre-F-antibodies, (b) infants' age (in weeks), (c) RSV viral load, (d) infants' sex and (e) infants' severity (bronchiolitis) score. To determine viral load, a standard curve was created using extracted RNA from the generated RSV stock (10_9 PFU/ml) in serial dilutions to quantify viral RNA load in all tested samples (copies/reaction equivalent to PFU/ml). Levels of infants' RSV Abs binding to pre-F from original sera were analyzed for significant correlations using Pearson r in (a; b; c; e) and Mann–Whitney test in (d). Significance was applied at $^*p < 0.05$.

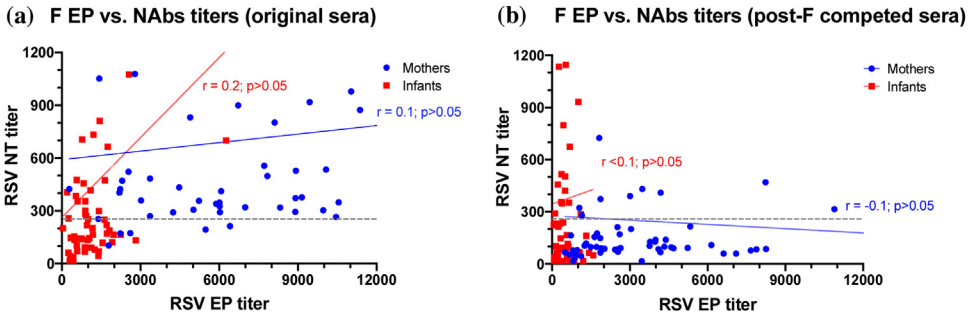

Figure 4. Correlation analysis between RSV-F EP and NAbs titers in infants and mothers: (a) original sera and (b) post-F competed sera. The level of protective NT titer is demonstrated in a dashed grey line (8 $\log_2$; 256). Levels of RSV-F-NAbs in infants were tested for significant correlation using Pearson r in (a; b). Significance was applied at $^*p < 0.05$.

antigens and high affinity to FcRN are favorably transferred. Likewise, matAb decay in infants is dependent on IgG subclass, antigen specificity, antibody glycosylation (directly, longer half-life for highly glycosylated antibodies), total IgG concentration (inversely, shorter half-life in case of high total IgG concentration), and ethnic groups (Marchant et al., 2017). That said, it was anticipated that pre-F exclusive NAbs were preferentially crossing the placenta among maternal RSV Abs, and that possibly passively transferred post-F Abs had shorter half-life in infants than pre-F Abs. Pre-F directed antibodies represent the majority of the RSV Ab repertoire in adults, with high neutralizing potency and high binding affinity to pre-F specific sites compared to poorly neutralizing antibodies (Gilman et al., 2016).

It is believed that the protective RSV titer is differently reported among species and subtypes, and according to the detection method. Recently, Buchwald et al. showed another threshold at 80% inhibitory concentration (IC_{80}) of 240 among infants during their first 3 months of life in case of RSV-A

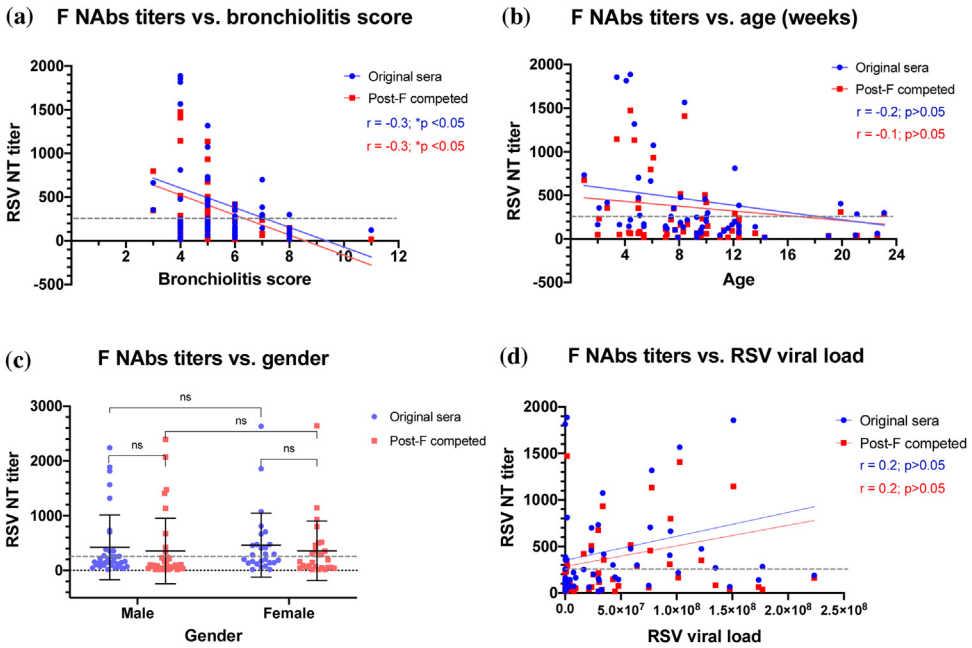

Figure 5. Correlation analysis of demographics and clinical outcomes with neutralization titer in infants (a) severity (bronchiolitis) score, (b) age (in weeks), (c) sex, and (d) RSV viral load. To determine viral load, a standard curve was created using extracted RNA from the generated RSV stock (10^9 PFU/ml) in serial dilutions to quantify viral RNA load in all tested samples (copies/reaction equivalent to PFU/ml). The level of protective NT titer is demonstrated in a dashed grey line (8 $\log_2$; 256). Levels of RSV-F-NAbs in infants were tested for significant correlation using Pearson r in (a; b; d) and paired t-test in (c). Significance was applied at $^*p < 0.05$.

infection (Buchwald et al., 2020). Accordingly, this study extrapolated EC_{80} values for the samples, to compare them with the latter threshold, and found discrete results (67.7% of hospitalized infants had higher levels) possibly from the differences in the assay method and and/or the reading format. Thereafter, 1:265 ($\log_2$ EC_{50} of 8) was considered as a convenient protective titer reference (Piedra et al., 2003) for hospitalized infants at hospitalization, as used in many other studies. One-third of the hospitalized infants (32.3%) had NAbs above 1:265. Despite having protective NAbs levels according to the reported thresholds, early-life immunity is too immature (and Th2-driven) to fully clear RSV or other viral infections (Ruckwardt et al., 2016). Hence, the protection from viral infections is not necessarily antibody-dependent, and requires effective adaptive immunity, which hospitalized infants lack in early life.

This study had certain limitations. It enrolled infants of different ages (0–6 months) at the time of hospitalization, and prospective follow-up would have enabled a better analysis of correlates of protection. Also, it only investigated antibody binding and neutralizing levels, while measurements like antibody avidity, total IgG concentration, and other immune factors such as T-cell phenotypes, ADCC, cytokines, and complements would widen understanding of RSV pathogenicity in infants, their corresponding immune response and maternal correlates of protection. Further, the neutralization assay was performed with one virus (RSV-A2) and one competitor (subtype A post-F), while a more comprehensive analysis is needed to avoid bias results. Additionally, this study lacked the molecular analysis of the infectious viruses in admitted infants, where some might carry different RSV subtypes or important escape mutations. Such research is critical in follow-up studies, as identifying variants in the F-epitopes might dictate the design of future RSV vaccines.

This study found that 14% of matAbs remained in infants at hospitalization, and 61.4% were neutralizing, of which 81% were pre-F exclusive NAbs. These pre-F NAbs in infants inversely correlated with age (in days) and severity scores ($p < 0.05$). These findings indicate that the maintenance of neutralizing activity in infants relative to maternal titers was greater than the maintenance of antibody binding based on ELISA, suggesting that higher potency antibodies may have a longer half-life than weakly neutralizing antibodies. Accordingly, vaccinating pregnant women with pre-F in the third trimester might be a successful strategy to protect their infants during their first 6 months of life, but only if the vaccine induces high antibody levels. Finally, the observed positive correlation between post-F antibody levels and viral load in hospitalized infants is worth considering while adopting a future vaccine. That is: the next RSV vaccine shall only contain a stable pre-F construct that does not adopt the post-F conformation, but will induce a substantial antibody to the surfaces that are shared with the post-F molecule that does retain some neutralizing epitopes.

Conflict of interest

The authors declare no conflict of interest.

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Ethics approval

Ethical approval was obtained from Hamad Medical Corporation (# 16196/16) and Qatar University (# QU-IRB 890-E/18).

Author's contribution

HY designed the work; ST performed experiments; KA recruited patients; AA and GN provided supervision; ADC and TR ran neutralization experiments; BG provided reagents; all authors revised the manuscript and accepted its final form.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2021.06.015.

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