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RESEARCH PAPER

Antibody response to respiratory syncytial virus infection in children <18 months old

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ABSTRACT
The development of a safe and effective respiratory syncytial virus (RSV) vaccine might be facilitated by knowledge of the natural immune response to this virus. The aims of this study were to evaluate the neutralizing antibody response of a cohort of healthy children <18 months old to RSV infection. During the RSV season, 89 healthy children <18 months old were enrolled and followed up weekly for 12 weeks. At each visit, a nasopharyngeal swab was obtained for RSV detection by real-time polymerase chain reaction (PCR). During the study period, 2 blood samples were drawn and they were used to determine RSV geometric mean neutralizing antibody titres (GMT) against RSV. A total of 35 (39.3%) children had RSV detected during the study period. Among RSV-positive patients, children ≥7 months showed a significantly higher increase in antibody response ($p<0.001$). A significantly higher number of patients with a ≥4 -fold increase in GMT were ≥7 months old ($p = 0.02$) and presented lower respiratory tract infections (LRTIs) during the study period ($p = 0.01$). Viral shedding was longer among children aged ≥7 months ($p = 0.06$), those with viral load $\geq 10^6$ copies/mL ($p = 0.03$), and those with LRTIs during the study period ($p = 0.03$), but it was not associated with the immune response ($p = 0.41$). In conclusion, natural RSV infection seems to evoke a low immune response in younger children. To be effective in this infant population, which is at highest risk of developing severe LRTIs, vaccines must be able to induce in the first months of life a stronger immune response than that produced by the natural infection.

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Introduction

Respiratory syncytial virus (RSV) is the major cause of severe respiratory tract infection in infants and young children, particularly in those who were born prematurely or who suffer from congenital heart disease or bronchopulmonary dysplasia.[1] Moreover, RSV infection is recognized as a predisposing factor to the development of severe lower respiratory tract infection (LRTI) in the short term[2] and of wheeze and asthma later in life.[3] Previous studies have shown that the neutralizing antibody concentration plays a fundamental role in conditioning susceptibility to RSV disease and its severity.[4] High titres of maternally derived neutralizing antibodies to RSV correlate well with the protection of younger infants,[4] and the administration of polyclonal or humanized monoclonal antibodies against RSV has been associated with a significant reduction in the number of severe cases and hospitalizations from RSV infection in high-risk infants.[5]

Active immunization with RSV vaccines could reduce most of the clinical, economic and societal problems strictly related to RSV infection. Unfortunately, the development of safe and effective RSV vaccines remains elusive. Presently, no RSV vaccine has been licensed for use in humans. In the 1960s, a formalin-inactivated vaccine was used, but it was found to be associated with a high risk of severe RSV disease upon re-exposure due to the presence of high levels of non-neutralizing antibodies.[6] In the 50 y since then, multiple vaccine strategies have been investigated in preclinical and limited clinical settings.[7] These vaccines generally have not progressed to clinical evaluation or have been met with limited success in human trials. Progress has been hampered by limited immunogenicity, the induction of Th2-biased immunity, or unacceptable levels of adverse events. Overcoming these problems is essential to prepare a safe and affective RSV vaccine. Moreover, the development of a safe and effective RSV vaccine might be greatly facilitated by the knowledge of the natural immune response of infants and young children to RSV infection.[8]

The evaluation of the immune response generated by natural infection in early infancy could offer useful information to evaluate the best vaccination strategy and to understand the factors that might modify the immune response. However, though several studies of this type have been performed in recent years, the immunological outcome of primary RSV infection has not been completely defined. The aims of this study were to evaluate the neutralizing antibody response of a cohort of healthy children <18 months old to RSV infection and to analyze which factors could influence antibody production. The prospective study of a

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cohort of healthy young children, followed weekly for 4 months, was considered the ideal design for the evaluation of the immune response to RSV in presence of asymptomatic infections and acute diseases with different severity.

Results

Among the 89 enrolled children, 35 (39.3%) had RSV detected during the study period (Table 1). None of them was hospitalized and received corticosteroids. Co-infection with other viruses, mainly rhinovirus, was found in 11 (31.4%) cases. Because a previous analysis of antibody response in children with only RSV infection and in those with co-infection did not show any difference between groups, all of the infected children were considered together. In comparison with subjects without RSV, those RSV-positive were significantly more often ≥5 months old (children 5–8 months old and 9–16 months old vs children 0–4 months old: p=0.03 and p=0,003, respectively). Moreover, RSV-positive children attended significantly more frequently day-care (p=0.001), showed significantly more often a previous history of LRTI (p=0.0002) and other infections from birth (p=0.007), and received antibiotics for respiratory tract infection significantly more frequently (p=0.0004) than subjects without RSV. These results were initially observed with an unadjusted model and were later confirmed in the adjusted model that included birth date and gender as predictors. No other significant difference was observed when comparing RSV-positive and RSV-negative children.

Geometric mean titres (GMT) of RSV-neutralizing antibodies decreased from V1 to V2 in RSV-negative children (GMT

Table 1. Characteristics of the cohort of 89 children.

				Unadjusted model[a]			Adjusted model[b]		
Predictor variable	Overall (n=89)n (%)	RSV- (n=54)n (%)	RSV+ (n=35)n (%)	OR	95% CI	p-value	OR	95% CI	p-value
Gender									
Females	39 (44)	28 (52)	11 (31)	Ref.					
Males	50 (56)	26 (48)	24 (69)	2.3	(1.0, 5.7)	0.06	2.1	(0.9, 5.4)	0.10
Age at enrolment									
0–4 mo	29 (33)	24 (44)	5 (14)	Ref.					
5–8 mo	30 (34)	17 (31)	13 (37)	3.7	(1.1, 12.2)	0.03	7.7	(1.5, 38.9)	0.01
9–16 mo	30 (34)	13 (24)	17 (49)	6.3	(1.9, 20.9)	0.003	16.3	(2.1, 126.9)	0.008
Birth date									
<7 months	41 (46)	30 (55)	11 (31)	Ref.					
≥7 months	48 (54)	24 (44)	24 (69)	2.7	(1.1, 6.7)	0.03	2.5	(1.0, 6.3)	0.04
Gestational age (weeks)	39.0 (1.3)	39.0 (1.2)	39.1 (1.3)	1.1	(0.8, 1.6)	0.47	1.1	(0.8, 1.6)	0.47
Exposure to passive smoke*									
No	63 (72)	39 (74)	24 (69)	Ref.					
Yes	25 (28)	14 (26)	11 (31)	1.3	(0.5, 3.3)	0.61	1.2	(0.5, 3.3)	0.69
Siblings									
No	54 (61)	37 (69)	17 (49)	Ref.					
Yes	35 (39)	17 (31)	18 (51)	2.3	(1.0, 5.5)	0.06	2.3	(0.9, 5.8)	0.08
Breast-feeding									
No	22 (25)	13 (24)	9 (26)	Ref.					
Yes	67 (75)	41 (76)	26 (74)	0.9	(0.3, 2.4)	0.86	1.3	(0.4, 3.8)	0.62
Day-care attendance									
No	68 (76)	48 (89)	20 (57)	Ref.					
Yes	21 (24)	6 (11)	15 (43)	6.0	(2.0, 17.7)	0.001	5.5	(1.7, 18.5)	0.005
Allergies									
No	83 (93)	50 (93)	33 (94)	Ref.					
Yes	6 (7)	4 (7)	2 (6)	0.8	(0.1, 4.4)	0.76	1.1	(0.2, 7.1)	0.94
URTIs from birth									
No	34 (38)	24 (44)	10 (29)	Ref.					
Yes	55 (62)	31 (56)	25 (71)	2.0	(0.8, 5.0)	0.13	1.7	(0.7, 4.5)	0.27
LRTIs from birth									
No	76 (85)	52 (96)	24 (69)	Ref.					
Yes	13 (15)	2 (4)	11 (31)	11.9	(2.4, 58.0)	0.002	15.9	(2.8, 88.8)	0.002
Infections from birth									
No	28 (31)	23 (43)	5 (14)	Ref.					
Yes	61 (69)	31 (57)	30 (86)	4.5	(1.5, 13.2)	0.007	4.1	(1.3, 12.8)	0.002
Hospitalizations									
No	82 (92)	50 (93)	32 (91)	Ref.					
Yes	7 (8)	4 (7)	3 (9)	1.2	(0.2, 5.6)	0.84	1.0	(0.2, 5.0)	0.99
Antibiotics for respiratory infections*									
No	69 (78)	49 (92)	20 (57)	Ref.	(2.7, 31.1)	0.000	8.4	(2.4, 30.1)	0.001
Yes	19 (22)	4 (8)	15 (43)	9.2		4			
Antibiotics for non-respiratory infections*									
No	81 (92)	49 (92)	32 (91)	Ref.					
Yes	7 (8)	4 (8)	3 (9)	1.1	(0.2, 5.5)	0.86	1.0	(0.2, 4.9)	0.96

95% CI, 95% confidence interval; LRTIs, lower respiratory tract infections; OR, odds ratio; RSV, respiratory syncytial virus; URTIs, upper respiratory tract infections.
*Data on 88 children.
[a]Logistic regression model.
[b]Multiple logistic regression model including birth date and gender as predictors.

Table 2. Antibody response to RSV during the study period among 35 RSV-positive patients.

	GMT at V1 (SD), log_2 units	GMT at V2 (SD), log_2 units	GMT fold change, log_2 units	95% CI of fold change, log_2 units	p-value
Birth date					
< 7 months	7.1 (1.5)	7.9 (1.1)	0.8	(−0.2, 1.9)	Ref.
≥ 7 months	5.3 (1.4)	9.2 (1.0)	3.9	(3.1, 4.6)	<0.001
Virus type					
RSV A	5.7 (1.6)	8.8 (1.2)	3.1	(2.3, 4.0)	Ref.
RSV B	6.9 (1.5)	8.5 (1.1)	1.7	(−0.1, 3.4)	0.15
Viral load					
< 1^6 (copies/mL)	6.5 (1.8)	8.8 (1.3)	2.2	(1.2, 3.3)	Ref.
≥ 1^6(copies/mL)	5.3 (1.3)	8.8 (1.1)	3.5	(2.5, 4.6)	0.11
Viral shedding					
<15 days	5.6 (1.8)	8.6 (1.2)	3.0	(1.9, 4.1)	Ref.
≥15 days	6.2 (1.5)	8.9 (1.2)	2.7	(1.5, 3.8)	0.70
Symptoms					
URTIs	6.3 (1.7)	9.1 (1.0)	2.7	(1.6, 3.9)	Ref.
LRTIs	5.5 (1.5)	8.5 (1.3)	3.0	(1.9, 4.1)	0.76

95% CI, 95% confidence interval; GMT, geometric mean titres; LRTIs, lower respiratory tract infections; RSV, respiratory syncytial virus; URTIs, upper respiratory tract infections.

log_2 units ± standard deviation [SD], 6.5 ± 1.7 at V1 *vs* 5.5 ± 1.0 at V2; −0.9 log_2 unit GMT fold change, 95% confidence interval [CI] −1.4 – −0.4). On the contrary, in RSV-positive children, a 2.9 log_2 unit fold change (95% CI 2.1 – 3.7) was observed (GMT log_2 units ± SD, 5.8 ± 1.7 at V1 *vs* 8.8 ± 1.2 at V2). Table 2 summarizes the neutralizing antibody response to RSV during the study period among RSV-positive children. To evaluate immune response according to age, the cut-off level of 7 months was chosen according to previous studies showing that the greatest immune response to RSV infection occurs after this age.[9-15] Those <7 months old had higher baseline levels than those ≥7 months old. However, older children showed a significantly higher increase in the antibody response from V1 to V2 in comparison to those aged <7 months (p<0.001). Virus type, viral load, duration of shedding and respiratory infections during the study period did not appear to significantly influence the antibody response.

Similar findings were observed when RSV-positive children were analyzed according to the degree of increase in neutralizing antibody titres after infection (Table 3). A 4-fold increase was chosen to differentiate higher and lower antibody response according to the definition of effective response usually considered to establish seroconversion after vaccination.[16] In comparison to children with less than a 4-fold increase in antibody titres, a significantly higher number of patients with a ≥4 -fold increase in the neutralizing antibody GMT from V1 to V2 were ≥7 months old (p=0.02). Virus type and viral load did not influence the amount of antibody production. Also in RSV-positive children, virus type and viral load did not influence the levels of antibody production. However, in comparison to children with less than a 4-fold increase in antibody titres, a significantly higher number of children with a ≥4 -fold increase presented LRTIs during the study period (p=0.01).

Table 4 describes the duration of RSV shedding. Shedding was longer among children aged ≥7 months (median days, 28 vs 14; p=0.06), among those with a viral load ≥10^6 copies/mL (median days, 27 vs 14; p=0.06 in the simple Cox regression model and p=0.03 in a multiple Cox regression model

Table 3. Analysis of fold change (FC) of neutralizing antibody titer to RSV from baseline among 35 RSV-positive patients.

Variable	FC < 4 (n=12)n (%)	FC ≥ 4 (n=22)n (%)	OR[a]	95% CI[a]	p-value[a]	Adjusted OR[b]	95% CI[b]	p-value[b]
Birth date								
< 7 months	7 (58)	4 (18)	Ref.					
≥ 7 months	5 (42)	18 (82)	6.3	(1.4, 33.9)	**0.02**			
Virus type								
RSV A	9 (75)	19 (86)	Ref.			Ref.		
RSV B	3 (25)	3 (14)	0.5	(0.1, 3.0)	0.41	0.3	(0.0, 1.9)	0.19
Viral load								
< 1^6 (copies/mL)	6 (50)	11 (52)	Ref.			Ref.		
≥ 1^6(copies/mL)	6 (50)	10 (48)	0.9	(0.2, 3.8)	0.90	0.6	(0.1, 3.1)	0.56
Viral shedding								
<15 days	6 (50)	10 (46)	Ref.			Ref.		
≥15 days	6 (50)	12 (54)	1.2	(0.3. 4.9)	0.80	1.7	(0.3, 8.7)	0.50
Symptoms								
URTIs	10 (83)	7 (32)	Ref.			Ref.		
LRTIs	2 (17)	15 (68)	10.7	(2.1, 83.2)	0.01	11.1	(1.9, 107.8)	0.01

95% CI, 95% confidence interval; FC, fold change; LRTIs, lower respiratory tract infections; OR, odds ratio; RSV, respiratory syncytial virus; URTIs, upper respiratory tract infections.

[a]In columns 4, 5, and 6, the odds ratios of the 4r-fold change between the 2 modalities of the variable of interest and the corresponding 95% CI and p-value are obtained by a simple logistic regression model.

[b]In columns 7, 8, and 9, the adjusted OR of the 4-fold change between the 2 modalities of the variable of interest and the corresponding 95% CI and p-value are obtained by a multiple logistic regression model including birth date as an adjusting factor.

Table 4. Duration of shedding among 35 RSV-positive patients.

Variable	Median shedding duration in days (95% CI)	HR[a]	95% CI[a]	p-value[a]	Adjusted HR[b]	95% CI[b]	p-value[b]
Birth date							
< 7 months	14 (13, 21)	Ref.					
≥ 7 months	28 (15, 50)	2.3	(1.0, 5.3)	**0.06**			
Virus type							
RSV A	15 (14, 28)	Ref.			Ref.		
RSV B	19 (7, 42)	1.1	(0.4, 2.8)	0.82	0.8	(0.3, 2.1)	0.66
Viral load							
< 1^6 (copies/mL)	14 (9, 21)	Ref.			Ref.		
≥ 1^6(copies/mL)	27 (14, 42)	0.5	(0.2, 1.0)	**0.05**	0.4	(0.2, 0.9)	**0.03**
Symptoms							
URTI	14 (9, 28)	Ref.			Ref.		
LRTI	21 (14,35)	0.7	(0.3, 1.3)	0.23	0.4	(0.2, 0.9)	**0.03**
Antibody titer increase between V1 and V2							
<4-fold	15 (9, 35)	Ref.			Ref.		
≥4 -fold	16 (14, 28)	1.1	(0.5, 2.3)	0.84	0.7	(0.3, 1.6)	0.41

95% CI, 95% confidence interval; GMT, geometric mean titres; LRTIs, lower respiratory tract infections; RSV, respiratory syncytial virus; URTIs, upper respiratory tract infections.
[a]In columns 3, 4, and 5, the HRs of the shedding duration between the 2 modalities of the variable of interest and the corresponding 95% CI and p-value are obtained by a simple Cox regression model.
[b]In columns 6, 7, and 8, the adjusted HRs of the shedding duration between the 2 modalities of the variable of interest and the corresponding 95% CI and p-value are obtained by a multiple Cox regression model including birth date as an adjusting factor.

including birth date as an adjusting factor), and among those with LRTI (median days, 21 vs 14; p=0.23 in the simple Cox regression model and p=0.03 in a multiple Cox regression model including birth date as an adjusting factor). Viral type and antibody response did not seem to influence shedding duration.

Discussion

Considering that no child with gestational age <36 weeks was included in the study, none received corticosteroids during the study period, follow-up was carried out weekly, and RSV neutralizing antibodies were determined after at least 4 and no later than 6 weeks from infection onset, it can be concluded that the study has measured the RSV-neutralizing antibody titres in the period during which they had reached their highest level after RSV infection. This study shows that, although all subjects with RSV detection in the nasopharynx had a demonstrable specific immune response when infected by this virus, younger infants had a significantly lower antibody production in comparison to older infants and children. This finding is in contrast with the report by Shinoff et al.,[17] who found that the neutralizing antibody titer in the acute-stage serum sample (presumably acquired through the passive transfer of maternal antibodies) and not the age was the most important determinant of the response. However, these authors studied antibody production after RSV infection in the Navajo and White Mountain Apache American Indian children, a group of subjects who frequently suffer from immune deficiencies that could *per se* explain the different results. On the other hand, all the other studies regarding specific antibody production in children infected by RSV showed results quite similar to those reported in our study.[10-16] Moreover, similar results were recently confirmed by Sande et al.,[18] who reported that in comparison to the mean acute phase antibody titer, the mean convalescent titer was lower in the 0–1.9 month age class, no different in the 2–3.9 month age class and greater in all age classes ≥4 months. The relative immaturity of the immune system, the pressure of passively acquired maternal antibodies interfering with the development of a more solid immune response or both these factors could be the cause of the lower immune response of younger infants to RSV. Furthermore, particularly in older children, previous RSV infection might have led to the development of an immune memory able to induce a significant antibody production in case of a new infection.

Unfortunately, in this study we did not collect information on maternal antibodies and we did not test at different time points neonates' and infants' antibodies in order to understand the decay of maternal antibodies. However, this cohort study, with a careful weekly follow-up, stresses the role of age in RSV-specific antibody response, although it does not solve the problem of the factor(s) that could have influenced the final results. The origin of the antibody concentrations evidenced at baseline is not known. However, independently of the reason for the low antibody response, in the first months of life, it is unlikely that infants could mount strong neutralizing antibody responses to live RSV vaccines, and other strategies to protect them have to be explored.[19]

In this study, viral type, viral load, and duration of shedding did not influence the antibody response. The finding of a lack of a correlation between viral type and antibody response is in disagreement with the data reported by Roca et al.[15] These authors found that although an immune response after RSV infection was evidenced in all children regardless of the viral type, different RSV strains could evoke different immune responses in patients infected by RSV A, who showed the highest increments in antibody titres. In our study population, no statistically significant difference in GMT was found between children infected by RSV type A and those affected by RSV type B, although GMT fold changes were higher in RSV-A cases. However, the number of RSV-B-infected children was too small to permit definitive conclusions to be drawn.

The lack of any association of the viral load and the duration of shedding with the antibody response could be explained with

possible differences in the moment of sampling in relation to the moment of infection. Because sampling was performed every week, it is possible that in some cases nasopharyngeal secretions were collected at the beginning of the infection and in other children after some days when the viral load was higher. This could have influenced the evaluation of shedding duration. However, the data collected with this study are in agreement with the data published by Wright et al..[20] These authors prospectively evaluated 77 infants with RSV bronchiolitis and found that the severity of the illness was influenced by age and host risk factors but not by RSV-neutralizing antibody titres, the amount of virus nasal secretion, or the duration of viral shedding.

Most children with the highest antibody response had an LRTI during the study period, suggesting a potential association between immune response and the development of disease more severe than common URTIs. However, in this study the mean number of URTIs and LRTIs from which children <7 months old and those ≥7 months old suffered was similar and children with LRTI had a relatively mild disease because none of them was hospitalized. Consequently, it is possible that the difference in severity between groups was too small to be associated with relevant differences in immune response. Previous studies have evidenced a positive correlation between the RSV load, the duration of virus elimination, and the clinical course of the disease.[21-23] Another study highlighted the poor role of antibody concentration in conditioning the severity of RSV disease because it showed that children with high levels of maternally derived neutralizing antibodies, despite having a reduced risk of infection, have disease with the same severity of children with a low antibody concentration when RSV infection occurs.[4] In this study, it was expected that the lack of an association between viral factors and immune response and the severity of the disease does not influence immune response. However, the small number of children with LRTI involved in the study, the moment of viral load determination and the methods used to identify the virus could be the reason for this result.

In conclusion, this study shows that natural RSV infection seems to evoke a low immune response in younger children, those with the highest risk of severe disease. To be effective in this infant population, which is at highest risk of developing severe LRTIs, vaccines must be able to induce in the first months of life a stronger immune response than that produced by the natural infection. Otherwise, to protect younger infants, the immunization of women during pregnancy could be a realistic strategy given the recent progress in the development of well-tolerated and immunogenic recombinant RSV vaccines.[24] Through the mother, younger infants could receive adequate neutralizing antibody titres that are able to delay the onset or reduce the severity of RSV disease in most of these very vulnerable subjects. However, further studies involving a greater number of patients are needed to solve the problem of the relationships between viral characteristics and immune response and to define the best RSV vaccine.

Methods

Study population

This prospective study was conducted by the Pediatric Highly Intensive Care Unit of the University of Milan's Department of Pathophysiology and Transplantation and approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy. The study involved a cohort of healthy children aged <18 months enrolled between 15 and 30 October 2013 and followed up with by means of weekly household visits from 1 November 2013 to 28 February 2014. The study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy.

A letter explaining the nature of the study was sent to 150 families with children born in our institution during the previous 2 y The letter specified that only healthy, full-term infants could be enrolled and that any child born prematurely or with a severe chronic underlying disease diagnosed after birth would be excluded. It also stated that the family would need to remain in the Milan area throughout the study period and accept weekly household visits. The 89 families expressing interest were invited to attend a first hospital appointment with their child, during which demographic, socio-economic and medical information was collected, and the final selection was made after written informed consent of the parents or legal guardians was obtained.

The 89 enrolled children were followed up with by means of weekly household visits by fieldworkers, where the parents/guardians were interviewed, and a record was made of the date of the onset, duration and specific symptoms of respiratory or other acute infections occurring during the preceding week. Children with respiratory tract infections were classified into different disease groups (i.e., acute otitis media, rhinosinusitis, pharyngitis, croup, infectious wheezing, acute bronchitis, radiographically-confirmed pneumonia) on the basis of signs and/or symptoms using well-established criteria[25] and were finally subdivided into 2 subgroups: URTIs (that included acute otitis media, rhinosinusitis, pharyngitis, and croup) and LRTIs (that included infectious wheezing, acute bronchitis, and radiographically-confirmed pneumonia). At each visit, nasopharyngeal (NP) secretions were collected using a flexible pernasal flocked swab (Eswab 490CE.A, Copan Italia, Brescia, Italy), which was immediately placed in a mini-tube containing 1 mL of transport medium (Enat medium, Copan Italia), taken to the research laboratory, and stored at −70°C until being analyzed for the presence of RSV that occurred every week. All of the fieldworkers were trained in collecting data and samples and in recognizing respiratory signs and symptoms by means of workshops and reviews of educational material prepared for the World Health Organization's Integrated Management of Childhood Illnesses (IMCI) protocol.[26] In addition, a first blood sample of all participants was drawn at enrolment (V1), and a second sample was collected either when an RSV-positive swab was identified or in negative cases at the end of the study period (V2). In positive cases, blood collection was carried out after 25–32 d of the execution of the nasopharyngeal swab.

RSV identification

Viral nucleic acids were extracted from the NP samples by means of a NucliSENS® EasyMAG automated extraction system (BioMéerieux, Craponne, France). The extract was tested for respiratory viruses using the respiratory virus panel xTAG RVP

FAST v2 (Luminex Molecular Diagnostics, Inc.., Toronto, Canada), which simultaneously detects influenza A virus (subtypes H1 or H3), influenza B virus, RSV, parainfluenza virus-1, parainfluenza virus-2, parainfluenza virus-3 and parainfluenza virus-4, adenovirus, human metapneumovirus, coronaviruses 229E, NL63, OC43 and HKU1, enterovirus/rhinovirus and human bocavirus. The RSV-positive viral nucleic acid extracts were re-tested for confirmation using a single-tube real-time polymerase chain reaction (PCR) kit (TaqMan One-Step RT-PCR Master Mix Reagents Kit, Applied Biosystems, New Jersey, USA) and a 7,900 HT real-time PCR system (Applied Biosystems, New Jersey, USA). The N genes of RSV-A and RSV-B were targeted for the confirmation with primers and probes with minor modifications, in accordance with Van Elden et al..[27] The viral load was determined as previously described.[28]

RSV-neutralizing antibody assay

Serum RSV-neutralizing antibody assays were performed at Viroclinics Biosciences, Rotterdam, The Netherlands. Briefly, approximately 100 plaque forming units (PFU) of RSV-A2 (ATCC VR-1540) per well were mixed with 8 serial 2-fold dilutions of heat-inactivated sera, in triplicate, and incubated at 37°C for 1 hour prior to the inoculation of HEp-2 cell monolayers in 96-well plates. Following further incubation at 37°C, infection was scored by RSV-specific immunostaining and the automated counting of PFU per well. Neutralization titres represented the reciprocal dilution causing 50% plaque reduction and were calculated by non-linear regression fitting 4-parameters curves with a variable slope using GraphPad 5.03.

Statistical methods

For each categorical variable, the numbers of occurrences and percentages were reported, whereas continuous variables were described by mean and standard deviation due to the approximately symmetric distribution. The association between binary responses and other demographic and clinical variables was assessed by odds ratios (ORs) estimated from logistic regression models. Linear regression was used with a log-transformed antibody titer fold change between V2 and V1 and the antibody titer at V2 as responses. The effect of covariates on time to recovery, defined as the time interval between the first positive swab and the subsequent first negative swab, was evaluated by hazard ratios (HRs) from the Cox model. In all regression models, point estimates, 95% confidence intervals and Wald test p-values were obtained. Median times to recovery were estimated by Kaplan-Meier curves. All analyses were performed with R,[29] version 3.1.1.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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