Epidemiol. Infect. (2016), **144**, 1622–1633. © Cambridge University Press 2016
doi:10.1017/S0950268815003143

Prospective clinical and serological follow-up in early childhood reveals a high rate of subclinical RSV infection and a relatively high reinfection rate within the first 3 years of life

A. KUTSAYA[1]*, T. TEROS-JAAKKOLA[2], L. KAKKOLA[1], L. TOIVONEN[2], V. PELTOLA[2], M. WARIS[1] AND I. JULKUNEN[1,3]

[1] *Department of Virology, University of Turku, Turku, Finland*
[2] *Department of Pediatrics, Turku University Hospital, Turku, Finland, and Turku Institute for Child and Youth Research, University of Turku, Turku, Finland*
[3] *Viral Infections Unit, National Institute for Health and Welfare, Helsinki, Finland*

Received 24 March 2015; Final revision 22 October 2015; Accepted 24 November 2015; first published online 6 January 2016

SUMMARY

Children encounter repeated respiratory tract infections during their early life. We conducted a prospective clinical and serological follow-up study to estimate the respiratory syncytial virus (RSV) primary infection and reinfection rates in early childhood. Sera were collected from 291 healthy children at the ages of 13, 24 and 36 months and antibody levels against RSV antigens were determined by enzyme immunoassay. The RT–PCR method was also used for identifying the possible presence of RSV in symptomatic patients. At ages 1, 2 and 3 years, 37%, 68% and 86%, respectively, of studied children were seropositive for RSV. In children seropositive at age 1 year, RSV reinfection rate was at least 37%. Only one of reinfected children showed evidence for a third reinfection by age 3 years. Of children who turned RSV seropositive between ages 1 and 2 years, the reinfection rate was 32% during the third year of life. The mean antibody levels at primary infection were very similar in all age groups. The average decrease of antibody levels was 25–30% within a year. In 66 cases RSV infection was identified by RT–PCR. RSV infection rate in early childhood is 86% and reinfection rate is around 35%. This prospective serological follow-up study also provided evidence for the presence of RSV infections in children that did not show clinical signs warranting RSV RNA detection.

Key words: Antibodies, infants, reinfection, RSV, seroprevalence.

INTRODUCTION

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection in infants and most children are infected by age 3 years [1, 2]. Although the severity of the disease is of the greatest during the first year of life, RSV infection also leads to a substantial disease burden in older children [3]. The infection is frequently complicated by wheezing, bronchiolitis, pneumonia and acute otitis media. RSV infection-related mortality in healthy children is low, but hospitalization due to severe lower respiratory tract symptoms is relatively common. However, in prematurely born infants and immunocompromised individuals, RSV infection can be life-threatening [4].

Typically RSV causes annual epidemics leading to a high rate of primary infections in young children and secondary infections throughout the life [5, 6]. RSV isolates have been divided into two major genetic and antigenic subgroups, A and B. Usually, both group A and B viruses co-circulate during epidemics,

* Author for correspondence: Miss A. Kutsaya, Department of Virology, University of Turku, Kiinamyllynkatu 13, 20520 Turku, Finland.
(Email: anna.kutsaya@utu.fi)

but the predominant RSV genotype varies from year to year [7]. In Finland, an alternating prevalence of groups A and B has been observed in 2-year cycles [8, 9]. RSV infection induces normal virus-specific antibody responses, thus the presence of RSV-specific IgG in serum can be used to estimate the cumulative incidence of RSV infection in the population [10, 11]. Previously, it was shown that children who had suffered from one genotype RSV infection were relatively more resistant to reinfection by homologous than heterologous groups of RSV [8, 9]. However, it remains unclear to what extent primary infection confers immunity and protection against RSV reinfection and what is the kinetics of antibody decay after primary and secondary infections.

Despite of significant efforts, there is still no licensed vaccine, nor specific antiviral treatment for RSV infection. The only option is the immunoprophylaxis of high-risk individuals with monoclonal antibodies. Currently, the treatment of RSV infection is generally limited to supportive care, although ribavirin can be considered in severe disease forms [12, 13]. Better understanding of the characteristics of host humoral immune responses against RSV infection and the effects of age on immunity and protection against RSV infection have a great importance for the further development of an effective vaccine.

In this prospectively conducted, clinical and serological study we have used serial serum specimens from a birth cohort of children during a 3-year follow-up. This material allowed us to carry out a seroprevalence-based immunological study to estimate the mean RSV-specific antibody levels, seropositivity rates at different ages, reinfection frequency, and the decrease of antibody level after the primary infection.

MATERIALS

Patients and serum specimens

Samples were obtained from children, who participated in an interdisciplinary, prospective observational birth-cohort study, i.e. the STEPS Study [14]. The STEPS study was approved by the ethical committee of the Hospital District of Southwest Finland (decision no. 16/180/2008). An informed consent was signed by the parents of the children. The cohort group consists of 1827 children born in the Hospital District of Southwest Finland between 2008 to 2010. Children were enrolled into the study before or soon after birth with no exclusion criteria. A subset of 923 children was followed intensively for respiratory infections during the first 2 years of life. Whenever children experienced respiratory tract infections nasal swabs were obtained during the study clinic visits, or alternatively, at home by the parents. The parents were trained and well-versed with the sampling technique. Serum samples were collected during study clinic visits scheduled at specified ages of the children.

A full series of three serum samples, collected at ages 13, 24, and 36 months from a total of 291 children was available for this serological survey. All sera were stored at −70 °C until tested.

Enzyme immunoassays (EIAs)

Serum samples were tested for the presence of anti-RSV IgG by EIA using RSV group A Randall strain whole virus antigen cross-reacting with antibodies formed after a primary infection with both group A and B strains [15]. The antigen purification and EIA were performed essentially as described previously [15]. Assay conditions were optimized for the measurement of relatively low concentrations of IgG in a single serum dilution. The viral antigen was dissolved in phosphate-buffered saline (PBS), pH 7·2 (2·5 μg/ml, 100 μl/well) and adsorbed onto the wells of polystyrene microtitre plates (Combiplate, 96-well format, Thermo Scientific, USA) at room temperature for 24 h followed by washing the plates twice with PBS containing 0·5% Tween 20 and stored at +4 °C. The serum samples were diluted 1:300 in a dilution buffer (5% normal swine serum in PBS containing 0·5% Tween 20) and 100 μl was incubated in duplicates at 37 °C for 2 h. Bound IgG was detected with polyclonal rabbit anti-human IgG conjugated to horseradish peroxidase (1:2000 in dilution buffer, Dako A/S, Denmark; 100 μl/well). The enzymatic reaction was developed by adding a fresh substrate solution (0·3% 1,2-phenylenediamine and 0·02% hydrogen peroxide in citrate-phosphate buffer, pH 5·5) and terminated by adding 1 M hydrochloric acid. The result was read by a multilabel plate reader (Victor 3 V – 1420 Multilabel Counter, PerkinElmer, USA) at 490 nm.

Reverse transcription–polymerase chain reaction (RT–PCR) for RSV detection

Nasal swabs were vortexed with 500 μl PBS and 200 μl of the suspension was used for nucleic acid extraction using MagnaPure 96 (Roche, Germany) or NucliSense easyMag (bioMérieux, The Netherlands) automated

extractor with 50 µl elution volume. RSV was detected in a 3-plex RT–qPCR assay for rhinoviruses, enteroviruses and RSVs as described previously [16, 17]. Reverse transcription included RSV F gene-specific forward primer and 5 µl of extracted RNA in a total volume of 20 µl. Amplification step included RSV F gene-specific primers, an internal dual-label LNA probe, and 5 µl cDNA in a total volume of 20 µl. Relative RNA copy numbers were calculated using pre-determined amplification efficiency (85%) and a RSV cDNA standard (10^5 copies). No-template controls (water) of extraction, cDNA reaction and PCR were included in each run.

RSV-N A/B group typing PCR

Reverse-transcription reactions were carried out with 5 µl extracted RNA and random hexamer primers using QuantiTect Reverse Trasncription kit (Qiagen, The Netherlands). PCR mix contained 200 nM RSV N gene-specific primers (forward type A: GGCTC TTAGCAAAGTCAAGTTGAA; type B: AAAGA TGGCTCTTAGCAAAGTCA; reverse type A: CC TGTGCTCCGTTGGATG; type B: TGCACATC ATAATTGGGAGTG) and 100 nm probes (A: FAM-ACACTCAACAAAGATCAACTTCTGTCA-TCCAG-BHQ1; B: Cy5-ATACATTAAATAAGGA TCAGCTGCTGTCATCCA-BHQ2), 2× Maxima Probe qPCR Master Mix (Fermentas, Germany) and 2 µl cDNA in a total volume of 20 µl. Amplifications were performed using a Rotor Gene 6000 cycler (Corbett Research, Australia) with initial denaturation at 95 °C for 10 min and 45 cycles of 95 °C for 10 s and 55 °C for 60 s. Primers and probes were modified from those described previously [18] and obtained from Oligomer (Finland) and Eurogentec (Belgium), respectively. No-template controls (water) of extraction, cDNA reaction and PCR were included in each run.

Surveillance data for RSV infections in Finland

Nationwide and Hospital District of Southwest Finland surveillance data of RSV infections in children aged <5 years were obtained from the public database of the National Infectious Disease Registry (NIDR) in Finland maintained by the National Institute for Health and Welfare (THL) (http://www.thl.fi/ttr/gen/rpt/tilastot.html). Surveillance samples have been collected mostly from hospitalized children.

Statistical analysis

Each EIA assay included a high (100 EIA units), an intermediate (70 EIA units) anti-RSV positive and four negative calibrator sera (0 EIA units). All specimens were tested in duplicate. The cut-off level was calculated as the mean absorbance value of the negative calibrator sera +3 standard deviation (S.D.) units. The antibody concentration of the samples was expressed as EIA units calculated from the linear plot of the calibrator. The data were analysed using Microsoft Excel version 2010 (Microsoft Corp., USA). All serum specimens of the same individual were tested simultaneously. Data depicted on box plots were analysed and graphed using Origin, v. 8 (OriginLab, USA). Statistical significance of differences between different groups were analysed by independent-samples *t* test using IBM SPSS Statistics v. 22 software (IBM Corp., USA). A *P* value of <0·05 was considered significant.

RESULTS

Epidemiology of RSV infections in 2009–2013 in Southwest Finland and the whole country

During the study period RSV epidemics in the Hospital District of Southwest Finland followed the same pattern as RSV infections in the whole country (Fig. 1). Moreover, RSV findings in our study population by direct methods (RSV-specific RT–PCR) at the acute phase of respiratory infections, which were mostly mild and not necessitating hospitalization, coincided with the NIDR surveillance data (data not shown). The collection of serum samples took place during 2009–2013 covering two 2-year cycles of RSV occurrence. The collection periods for 1-, 2- and 3-year samples (arrows in Fig. 1) overlapped with yearly RSV outbreaks, indicating that most of the enrolled children were probably exposed to RSV infection every year.

RSV IgG seropositivity and reinfection

To estimate the RSV seropositivity rate, antibody levels and possible reinfection rates, serial serum specimens collected at ages 13 months (1 year), 24 months (2 years) and 36 months (3 years) from 291 children were analysed for the presence of anti-RSV IgG antibodies by EIA. IgG antibodies against RSV were detected in 37% ($n = 109$) of 1-year-old children indicating a relatively high primary RSV infection rate during the first year of life (Fig. 2). Further analysis

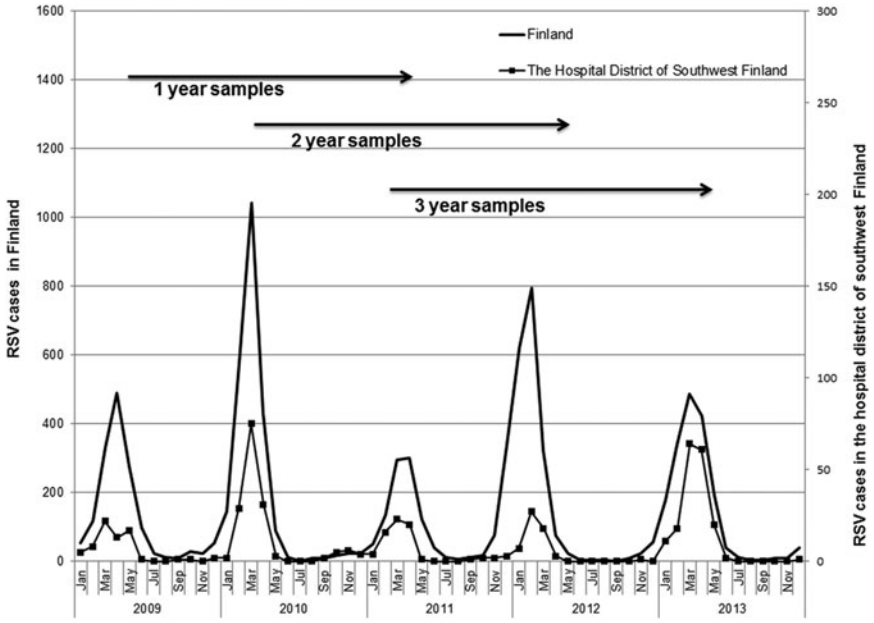

Fig. 1. Epidemiology of respiratory syncytial virus (RSV) infections during the study period. Diagnosed cases of RSV infections (number of cases per month) in children aged 0–4 years in Finland (——), and in the Hospital District of Southwest Finland (■–■) in 2009–2013. Arrows indicate the time line when the serum samples used in this study were collected. The data are based on National Infections Disease Registry, National Institute for Health and Welfare, Finland.

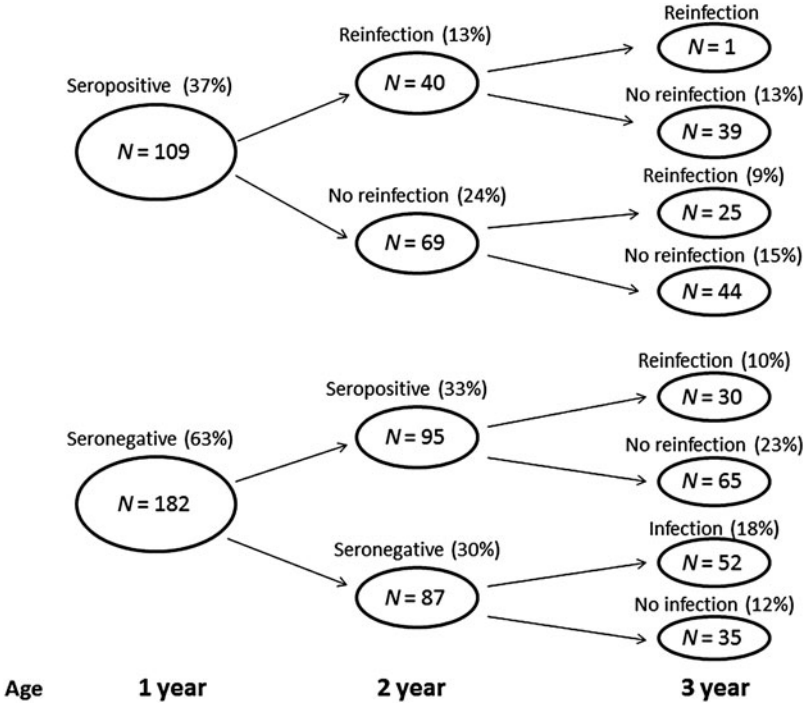

Fig. 2. General description of respiratory syncytial virus (RSV) infections in the child cohort during the follow-up. The study included 291 children from whom serum specimens were collected at aged 1, 2 and 3 years. Seropositivity is based on positive anti-RSV IgG antibody test results in whole RSV antigen enzyme immunoassay (EIA). Reinfection is determined as a significant (>25 EIA units) rise in serum anti-RSV IgG antibody levels between the yearly drawn serum specimens. The numbers in circles indicate the number of children and the percentages in parentheses indicate the percentage of children from the whole cohort ($n = 291$).

of seronegative children at 1 year ($n = 182$, 63% of all children) or 2 years ($n = 87$, 30%) showed that 50% and 60% of children, respectively, turned seropositive during the follow-up (Fig. 2). Altogether only 12% of children did not acquire anti-RSV antibodies during the 3-year follow-up.

In order to estimate the possible RSV reinfection rate in seropositive children aged between 1–2 years and 2–3 years we compared anti-RSV antibody levels between the serial samples. A rise in antibody level of $\geqslant 25$ EIA units was considered to indicate a reinfection. This value corresponded to ~3 S.D. of the values for the inter-assay variation of control sera. Of the children who acquired primary RSV infection by age 1 year, ~60% acquired an RSV reinfection within the two subsequent years ($n = 40 + 25/109$; Fig. 2). The children who became RSV seropositive by age 2 years (seronegative at 1 year) showed a reinfection rate of ~32% ($n = 30/95$) between the second and third years of life. Only one child showed evidence for two reinfections (Fig. 2).

RSV IgG seropositivity and reinfection rates by RSV-specific RT–PCR

From the studied group ($n = 291$), 186 children were followed more intensively for respiratory tract infections during the first 2 years of life. Nasal swab specimens taken during symptoms of infection were analysed by RT–PCR for the presence of RSV RNA. Of the 186 children, 66 had at least one RT–PCR-confirmed RSV infection. Of these RSV RT–PCR-positive individuals, 92% turned IgG seropositive. The five (8%) serological non-responders were very young children (aged <9 months). Thereby, RSV positivity rates detected by RT–PCR correlate with seropositivity based on positive anti-RSV IgG antibody tests, which indicates that our serological assay is sufficiently sensitive to represent the overall rate of seropositivity. Moreover, according to RT–PCR results, 10 children experienced RSV reinfection and one individual two reinfections. Reinfected individuals showed a further rise in IgG antibody levels of $\geqslant 10$ EIA units (Table 1). Exercising caution in our serological assay, a higher rise in antibody levels ($\geqslant 25$ EIA units) was considered to indicate a confirmed reinfection. This suggests that reinfection rates detected by a rise in antibody levels tend to be underestimated rather than overestimated.

RSV strains from 11 children who had repeated infections were identified as either group A or group B by RSV-N A/B group typing PCR. During the first infection, six children had group B strains and three had group A strains. In two cases, the determination failed due to initially low amount of viral RNA in the samples. Of the six children with group B strains during their first infection, four had a group B and two a group A strain during the second infection. Of the three children with group A strains during their first infection, two had a group A and one had a group B strain during their second infection.

RSV seroprevalence and mean RSV IgG levels increase with age

Figure 3 summarizes the RSV seropositivity rates and mean RSV IgG antibody levels at different ages. Of the serial serum specimens collected from 291 children, 256 (88%) became RSV seropositive (specific IgG detected in at least one serum specimen). The RSV IgG seropositivity rate increased with age and achieved 86% by age 3 years (Fig. 3*a*). The 2% discrepancy between the 3-year seropositivity rate and overall infection rate (88%) is explained by six children that were initially seropositive, but turned seronegative by age 3 years. In addition to increased seroprevalence, the mean IgG antibody levels rose significantly between 1 and 2 years (EIA unit values: 36 *vs.* 50, $P < 0{\cdot}001$) while the rise between 2 and 3 years remained more modest (EIA unit values: 50 *vs.* 54, $P = 0{\cdot}103$) (Fig. 3*b*). The rise in mean anti-RSV IgG levels is likely due to reinfections and antibody levels are higher if the primary infection is contracted at an older age (between 1 and 2 years *vs.* <1 year, $P < 0{\cdot}001$).

RSV IgG antibody levels after primary infection

To further analyse whether the child's age contributes to disease susceptibility and/or whether the antibody level after primary infection protects from a reinfection, seropositive individuals were divided into groups according to the age at the time of seropositivity (Fig. 4). Children who acquired RSV infection (were seropositive) by age 1 year ($n = 109$), showed increased mean antibody levels (36 EIA units *vs.* 52, $P < 0{\cdot}001$) at age 2 years suggesting a high rate of RSV reinfection. The same children showed rather similar antibody levels at age 3 years (52 EIA units at 2 years *vs.* 54 at 3 years, $P = 0{\cdot}784$) suggesting the lack of a second reinfection and relatively slow decay of anti-RSV IgG antibodies. Similarly, children turning seropositive between ages 1 and 2 years ($n = 95$) showed

Table 1. *Relationship between detection of RSV RNA in nasal swabs during respiratory infections before age 24 months and RSV IgG antibody levels in sera at ages 13 and 24 months*

Case number(s)	Mean age (range) in months at RSV RNA detection (s) (<13 months)	RSV IgG at 13 months		Mean age (range) in months at RSV RNA detection(s) (13–24 months)	RSV IgG at 24 months		Change in mean EIA units
		Mean EIA units (range)	Seropositivity rate or status		Mean EIA units (range)	Seropositivity rate or status	
Cases with a single RT–PCR confirmed RSV infection episode							
1–31	7·7 (0·8–13·5)	32 (3–82)	87%	n.d.	49 (4–127)	97%	+17
32–55	Not detected	0 (0–6)	4%	17·3 (13·1–24·7)	52 (6–162)	100%	+52
Cases with two RT–PCR confirmed RSV infection episodes							
56	1·9 (group B)	35	Positive	25·4 (group B)	82	Positive	+47
57	2·6 (group B)	5	Positive	18·3 (group B)	15	Positive	+10
58	2·8 (group B)	1·3	Negative	23·9 (group B)	54	Positive	+53
59	4·2 (group A)	43	Positive	16·0 (group A)	64	Positive	+21
60	6·1 (group B)	25	Positive	17·1 (group A)	41	Positive	+16
61	9·7 (group B)	24	Positive	23·1 (group B)	86	Positive	+62
62	11·2 (group A)	43	Positive	21·2 (group B)	89	Positive	+46
63	2·3, 12·3 (group B, A)	30	Positive	n.d.	46	Positive	+16
64	2·9, 9·7 (group A, A)	10	Positive	n.d.	7	Positive	−3
65	4·1, 10·4 (group n.d., B)	41	Positive	n.d.	91	Positive	+50
Cases with three RT–PCR confirmed RSV infection episodes							
66	5·8, 7·4 (group n.d., A)	21	Positive	18·3 (group A)	49	Positive	+28

RSV, Respiratory syncytial virus; EIA, enzyme immunoassay; n.d., not detectable.

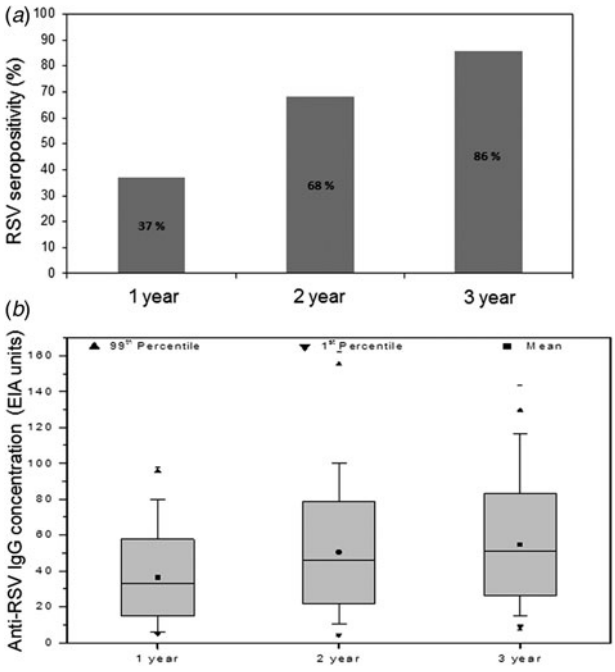

Fig. 3. Respiratory syncytial virus (RSV) IgG seroprevalence in relation to the children's age. (*a*) RSV IgG seropositivity rate in different age groups. (*b*) The mean RSV IgG antibody levels in different age groups. The boxes represent the interquartile range (25–75%); the whiskers denote 5th and 95th percentiles; the horizontal lines inside boxes show a median; the dashes show minimum and maximum antibody levels. The values are shown as EIA units that have been calculated in relation to the values for negative control specimens (EIA unit value 0) and highly positive serum specimen (EIA unit value 100).

increased mean antibody levels at age 3 years (42 EIA units at 2 years *vs.* 58 at 3 years, $P < 0{\cdot}001$). The antibody levels after the primary infection were slightly higher if the infection was acquired at the age of 2–3 years, or 1–2 years, compared to <1 year (42 and 41 *vs.* 36 EIA units at 1 year, $P < 0{\cdot}05$). Overall, primary infection led to an average antibody level of 35–45 EIA units, and reinfection increased the mean antibody levels by nearly 40%.

High RSV IgG antibody levels develop after reinfection

To define the mean RSV IgG antibody level after reinfection and to determine how a high post-infection antibody level likely protects from reinfection, the children were divided into groups according to the age at the time of primary infection and reinfection. Of the 109 children who acquired RSV infection by age 1 year, 40 were reinfected by age 2 years (Fig. 5*a*) and 25 by age 3 years (Fig. 5*c*) while 44 showed no serological evidence for a reinfection (Fig. 5*b*). As expected the children in both reinfection groups showed a significant increase in mean antibody levels after reinfection ($P < 0{\cdot}001$). The result strongly suggests that antibody level after the second infection is on an average at a protective level, since only one child showed evidence for a third infection. However, of those reinfected by age 2 years, IgG levels declined by 25% at age 3 years ($P < 0{\cdot}01$). Interestingly, in 25 children who acquired primary infection during the first year of life and who did not contract a reinfection during the second year, the antibody levels declined by nearly 28% by age 2 years ($P = 0{\cdot}082$). It is likely that in these children the anti-RSV IgG levels declined below the protective level and thus these children became susceptible to a new RSV infection between years 2 and 3 of follow-up. The group of 44 children, who did not become reinfected, showed ~40% higher mean antibody levels after primary infection than the other two groups (Fig. 5*b*). Moreover, their RSV IgG antibody levels remained constant without significant decline at age 3 years ($P = 0{\cdot}878$ and $P = 0{\cdot}230$). We can, however, not rule out the possibility that some exposure to RSV was present leading to weak booster responses (<25 EIA units) that remained under the diagnostic reinfection criteria ($\geqslant$25 EIA units).

Of the 95 children who acquired their first RSV infection by age 2 years (seronegative at 1 year; Fig. 2), 30 contracted a second RSV infection and showed a significant increase in IgG antibody levels ($P < 0{\cdot}001$) by age 3 years (Fig. 6*a*). The remaining 65 of those who acquired an initial infection by age 2 years, were not reinfected although their post-infection antibody levels at age 2 years were at a similar level compared to those who became reinfected. It is noteworthy that of these children the mean anti-RSV IgG levels did not decrease ($P = 0{\cdot}863$) at age 3 years (Fig. 6*b*).

Rate of RSV IgG antibody decline after primary infection

To determine the rate of decline in anti-RSV IgG antibody levels, we divided children who likely experienced only one RSV infection into two age groups and calculated the geometric mean antibody levels at different time points separately for groups of children who were initially low, moderate or high responders. The antibody levels remain fairly stable with time if RSV IgG antibodies were initially at a low or a

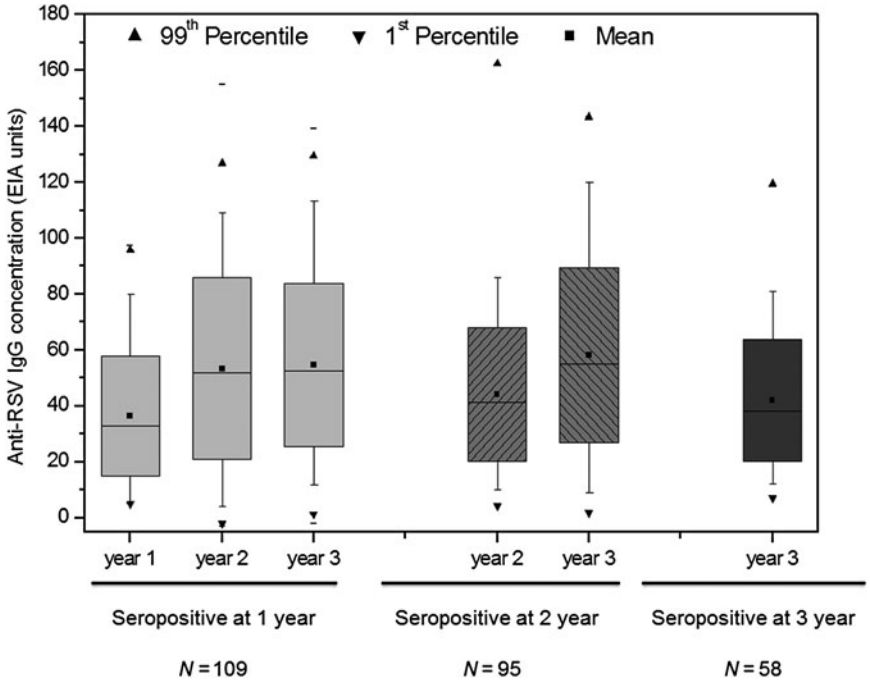

Fig. 4. Follow-up of mean respiratory syncytial virus (RSV) IgG levels post-infection and in subsequent years in different age groups. The boxes represent the interquartile range (25–75%); the whiskers denote 5th and 95th percentiles; the horizontal lines inside boxes show a median; the dashes show minimum and maximum. Child serum specimens were separated into three different groups based on their seropositivity at a given time point. The mean RSV IgG antibody levels are shown for the samples of the same children in each part of the figure as indicated.

moderate level (<50 EIA units). However, in the high responder group (>50 EIA units), the mean anti-RSV antibody levels fell by 15% within the first year and by 45% 1 year later (Fig. 7*a*). Similarly, anti-RSV IgG antibodies declined more in high responder children who acquired the infection by age 2 years (Fig. 7*b*). Overall, the higher the initial anti-RSV IgG antibody level was after the primary infection the faster the decline was (Fig. 7).

DISCUSSION

In the present study, we estimated RSV primary and reinfection rates in early childhood by examining serially collected serum specimens in a prospectively followed birth cohort comprising of 291 children. Based on anti-RSV IgG antibody seroprevalence analysis at least 37% of young children were infected with RSV during the first year of life, 68% by age 2 years and 86% by age 3 years. At least one third of the children that had undergone an RSV infection were susceptible to RSV reinfection during the subsequent years, suggesting that the primary RSV infection gives only partial protection against reinfection in early childhood. In the present study we used a highly sensitive, whole virus-specific EIA method to detect antibodies against RSV. The assay was well controlled and all test series included negative control specimens from children of the same age. The criteria for seropositivity and diagnostic antibody rise were relatively stringent which rather leads to underestimation than overestimation of the rate of RSV infections.

Antibodies induced by a natural RSV infection are important mediators of protection against subsequent infections [11]. However, there is no general concept of anti-RSV antibody levels or their specificity that would provide protection against reinfection. Maternal antibody levels correlate with protection against RSV infection in very young infants and administration of anti-RSV immunoglobulins prevent infections in premature infants [19]. Maternal RSV-specific antibodies decline rapidly after birth and they are largely absent at age 6 months [20, 21]. The immune system of children aged <1 year has not yet fully developed. However, the present study showed that in primary RSV infection the induced antibody responses were not necessarily so strongly age-dependent. Only modestly, although not significantly, higher mean

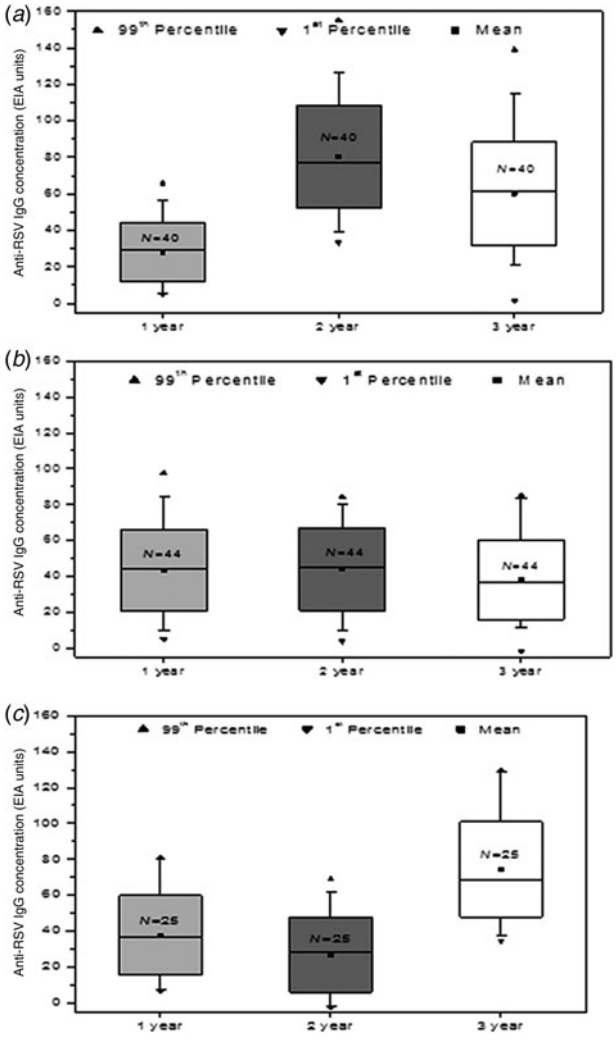

Fig. 5. Respiratory syncytial virus (RSV) IgG levels in children who were RSV IgG seropositive at 1 year (n = 109). Children were separated into three groups based on their likely RSV reinfection. (*a*) Mean RSV IgG levels in children showing significantly increased IgG levels at age 2 years compared to the 1-year sample (likely reinfection between 1 and 2 years, $P < 0{\cdot}001$). (*b*) Mean RSV IgG levels in children showing no increase in IgG levels during follow-up (no evidence for reinfection, $P = 0{\cdot}878$ and $P = 0{\cdot}230$). (*c*) Mean RSV IgG levels in children showing increased IgG levels at 3 years compared to 2-year samples (likely reinfection between ages 2 and 3 years, $P < 0{\cdot}001$). The boxes represent the interquartile range (25–75%); the whiskers denote 5th and 95th percentiles; the horizontal lines inside boxes show a median; the dashes show minimum and maximum; N is the number of children in different subgroups.

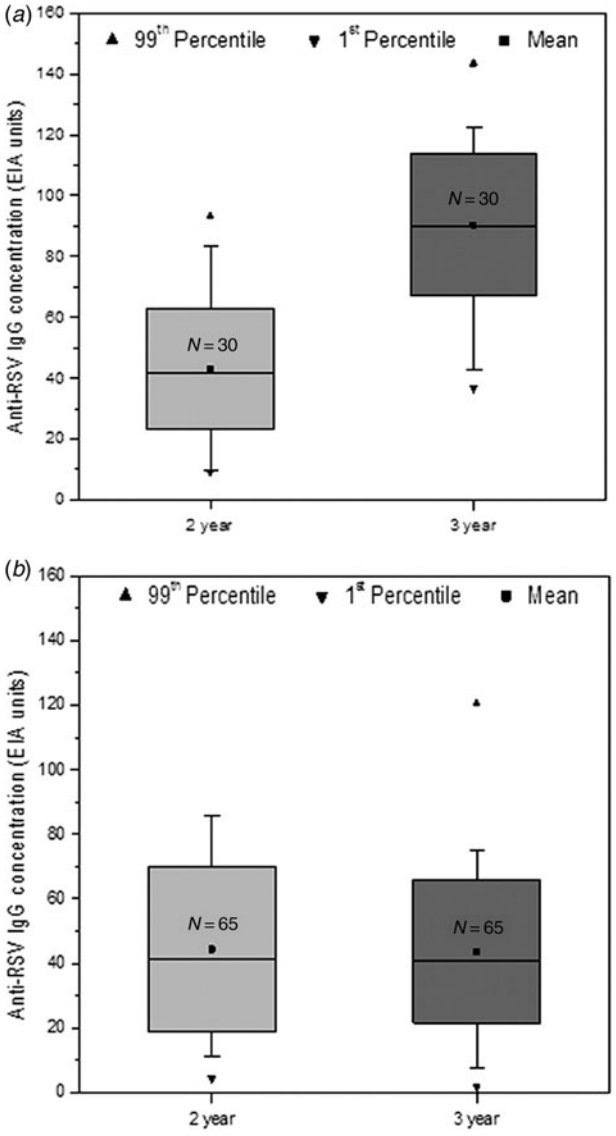

Fig. 6. Respiratory syncytial virus (RSV) IgG levels in children ($n = 95$) who acquired initial infection between ages 1 and 2 years. Children were separated in two groups based on their likely RSV reinfection between the second and third year of follow-up. (*a*) Mean RSV IgG levels in children ($n = 30$) showing significantly increased IgG levels ($P < 0{\cdot}001$) between ages 2 and 3 years (likely reinfection between 2 and 3 years). (*b*) Mean RSV IgG levels in children ($n = 65$) showing no increase in IgG levels ($P = 0{\cdot}863$) during the follow-up (no evidence for reinfection). The boxes represent the interquartile range (25–75%); the whiskers denote 5th and 95th percentiles; the horizontal lines inside boxes show a median; the dashes show minimum and maximum; N is the number of children.

anti-RSV antibody levels were observed in the group of older children (2 and 3 years *vs.* 1 year) after primary RSV infection.

Our data show that at least one third of children experienced a RSV reinfection during the second or third years of follow-up. It was of interest that the reinfection rate was approximately the same in children who had the primary infection within the first or the second year of life. This may indicate that the primary

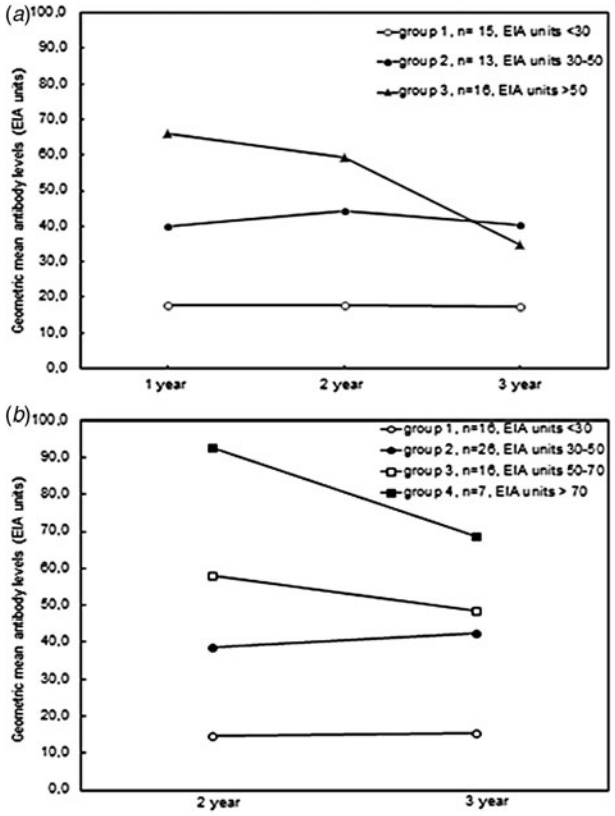

Fig. 7. Kinetics of anti-respiratory syncytial virus (RSV) IgG antibody decay after the primary infection. (*a*) Mean RSV IgG antibody decline in children who acquired the infection by age 1 year (seropositive at 1 year) and who failed to show evidence for reinfection. Seropositive children ($n = 44$) were separated into three groups based on their RSV IgG antibody level at age 1 year and their mean antibody levels were followed up at ages 2 and 3 years. The children were grouped as high [>50 enzyme immunoassay (EIA) units], moderate (30–50 units) and low (positive to <30 units) responders. (*b*) Mean RSV IgG antibody decline in children ($n = 65$) who acquired the infection by age 2 years (seronegative at 1 year). The children were grouped as very high (>70 EIA units), high (50–70 units), moderate (30–50 units) and low (positive to <30 units) responders.

infection may provide equally weak (in reinfection-prone children) or stronger protective responses (in reinfection-free children) in very young (<1 year) and young (1 to <2 years) children. Moreover, it was interesting to note that in children who had undergone two RSV infection episodes, only one child had evidence of two reinfections. Overall, immune response to the primary infection was relatively weak but, after a reinfection, a significant booster response in RSV IgG antibodies was observed resulting in 50–60% increase in the mean anti-RSV IgG antibody levels. These results strongly suggest that reinfection or repeated exposure to the virus leads to the development of high antibody levels and protection against additional RSV infections that is consistent with a previous study [22].

Some evidence for the protective role of initially higher anti-RSV antibody levels comes from a comparison of groups of children who became reinfected or remained free of reinfection. The children who turned seropositive within the first year of life and who were reinfected by age 2 years showed lower anti-RSV antibody levels at 1 year compared to children who did not become reinfected. This observation was supported by RT–PCR analysis (Table 1), which showed that children undergoing a virologically confirmed RSV reinfection mostly had low anti-RSV IgG antibody levels prior to reinfection. In addition, many children whose humoral immunity waned within the second year after primary infection (antibody levels declined by 28%) were reinfected by age 3 years. In adults, low serum antibody levels are a risk factor for reinfection [23]. However, in children who received their primary infection by age 2 years, anti-RSV antibody levels were not different from those of reinfection-prone and reinfection-free children. Our findings indicate that higher antibody levels give only relative protection against reinfection. It is, however, likely that after two episodes of RSV infection, the antibody levels, together with cellular immunity, rise to a level that confers good protection against RSV infection at least for some time. Even when the production of RSV-specific antibodies following second infection is robust, humoral immunity is relatively short-lived and wanes over time. Even if anti-RSV antibody levels declined by 25% within 1 year after reinfection they still remained at a higher level compared to the levels seen in children, who had likely experienced only one infection episode. It is interesting to note that if children did not experience a reinfection, their antibody levels following primary infection remained relatively stable. These findings agree with the rate of antibody decline seen in adults after RSV infection [24].

One-year-old seropositive children, who did not suffer a reinfection in the next year showed an average decline of 25% in their anti-RSV antibody levels. This observation is consistent with previous studies where RSV neutralizing antibodies induced by primary infection in infants declined to pre-infection levels rapidly (~3 months post-infection) [25]. Another study showed that RSV-specific antibodies were absent or present only at low levels 1 year after the

primary infection [22]. Short duration of primary RSV infection-induced antibody response can be the reason for the susceptibility of young children to RSV re-infection(s). On the other hand, it has been suggested that the ability of RSV to cause recurrent infections may be due to antigenic variation [26]. It is plausible that the reason for repeated RSV infections in early childhood is the sum of (1) the inability of very young children to develop efficient protective immunity against RSV infection, (2) a rapid decline of antibody levels, and (3) due to antigenic variation of RSV strains in subsequent epidemic seasons. It is also possible that there are certain differences in the virulence between the two groups of RSV. Some studies have suggested that subgroup A RSV isolates are associated with a more severe form of the disease while some other studies fail to support this concept (reviewed in [27]). However, it has been reported that the group A viruses replicate to higher titres than the group B viruses [28] and patients show higher avidity and antibody titres against subgroup A than against subgroup B RSV antigens [15]. By mathematical modelling of epidemics in Finland and Wales over a 20-year period, the reinfection rate was estimated to be reduced by 64% with the homologous group and 16% with the heterologous group during a 2-year period of temporary immunity [9]. It was also predicted that the transmission rate of group A is 8% higher than that of group B. From the limited data of this study, there is no clear pattern of occurrence of RSV groups in children with two infections. Of the nine children, six had second infections with the homologous group and three had second infections with the heterologous group.

In the present prospective clinical and serological study we have demonstrated that young children are highly susceptible to RSV infections and the frequency of RSV reinfections is relatively high. However, two (or more) RSV infection episodes within the first 2 years of life apparently provide a good immunological response and a good protection against a third infection episode within the third year. We and others [25] observed that anti-RSV antibody levels decrease relatively rapidly, which has to be taken into account in infant RSV vaccine development. The data presented show that if the future vaccines induce antibody responses of comparable duration to that induced by a natural infection, there will be a need for the administration of booster doses of the vaccine in order to maintain antibodies at protective levels. Since RSV infections are extremely common and contribute to significant morbidity in early childhood the availability of efficient and safe RSV vaccines would be very desirable.

ACKNOWLEDGEMENTS

Ritva Kajander is acknowledged for excellent technical support. We thank all the children and their parents for participation in the STEPS study.

This work was supported by Erasmus Mundus Triple I mobility programme (TRIPLEI2011B1595), Academy of Finland (grant nos. 123 571 and 140 251), The Pediatric Research Foundation (TYKS-Sapa, Finland) and research funding from the Ministry of Social Affairs and Health to TYKS ERVA.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Nair H, *et al.*** Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet* 2010; **375**: 1545–1555.
2. **Berkley JA, *et al.*** Viral etiology of severe pneumonia among Kenyan infants and children. *Journal of the American Medical Association* 2010; **303**: 2051–2057.
3. **Hall CB, *et al.*** The burden of respiratory syncytial virus infection in young children. *New England Journal of Medicine* 2009; **360**: 588–598.
4. **Ohuma EO, *et al.*** The natural history of respiratory syncytial virus in a birth cohort: the influence of age and previous infection on reinfection and disease. *American Journal of Epidemiology* 2012; **176**: 794–802.
5. **Glezen WP, *et al.*** Risk of primary infection and reinfection with respiratory syncytial virus. *American Journal of Diseases of Children* 1986; **140**: 543–546.
6. **Henderson FW, *et al.*** Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. *New England Journal of Medicine* 1979; **300**: 530–534.
7. **Hall CB, *et al.*** Occurrence of groups A and B of respiratory syncytial virus over 15 years: associated epidemiologic and clinical characteristics in hospitalized and ambulatory children. *Journal of Infectious Diseases* 1990; **162**: 1283–1290.
8. **Waris M.** Pattern of respiratory syncytial virus epidemics in Finland: two-year cycles with alternating prevalence of groups A and B. *Journal of Infectious Diseases* 1991; **163**: 464–469.
9. **White LJ, *et al.*** The transmission dynamics of groups A and B human respiratory syncytial virus (hRSV) in England & Wales and Finland: seasonality and cross-protection. *Epidemiology and Infection* 2005; **133**: 279–289.

10. **Domachowske JB, Rosenberg HF.** Respiratory syncytial virus infection: immune response, immunopathogenesis, and treatment. *Clinical Microbiology Reviews* 1999; **12**: 298–309.
11. **Roca A, *et al.*** Prevalence of respiratory syncytial virus IgG antibodies in infants living in a rural area of Mozambique. *Journal of Medical Virology* 2002; **67**: 616–623.
12. **Englund JA, Piedra PA, Whimbey E.** Prevention and treatment of respiratory syncytial virus and parainfluenza viruses in immunocompromised patients. *American Journal of Medicine* 1997; **102**: 61–70; discussion 75–6.
13. **Zambon M.** Active and passive immunisation against respiratory syncytial virus. *Reviews in Medical Virology* 1999; **9**: 227–236.
14. **Lagstrom H, *et al.*** Cohort profile: Steps to the healthy development and well-being of children (the STEPS Study). *International Journal of Epidemiology* 2013; **42**: 1273–1284.
15. **Meurman O, Waris M, Hedman K.** Immunoglobulin G antibody avidity in patients with respiratory syncytial virus infection. *Journal of Clinical Microbiology* 1992; **30**: 1479–1484.
16. **Osterback R, *et al.*** Simultaneous detection and differentiation of human rhino- and enteroviruses in clinical specimens by real-time PCR with locked nucleic acid probes. *Journal of Clinical Microbiology* 2013; **51**: 3960–3967.
17. **Toivonen L, *et al.*** Blood MxA protein as a marker for respiratory virus infections in young children. *Journal of Clinical Virology* 2015; **62**: 8–13.
18. **Hu A, *et al.*** Simultaneous detection, subgrouping, and quantitation of respiratory syncytial virus A and B by real-time PCR. *Journal of Clinical Microbiology* 2003; **41**: 149–154.
19. **Groothuis JR, *et al.*** Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. The Respiratory Syncytial Virus Immune Globulin Study Group. *New England Journal of Medicine* 1993; **329**: 1524–1530.
20. **Ochola R, *et al.*** The level and duration of RSV-specific maternal IgG in infants in Kilifi Kenya. *PLoS ONE* 2009; **4**: e8088.
21. **Brandenburg AH, *et al.*** Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. *Journal of Medical Virology* 1997; **52**: 97–104.
22. **Welliver RC, *et al.*** The antibody response to primary and secondary infection with respiratory syncytial virus: kinetics of class-specific responses. *Journal of Pediatrics* 1980; **96**: 808–813.
23. **Walsh EE, Falsey AR.** Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. *Journal of Infectious Diseases* 2004; **190**: 373–378.
24. **Falsey AR, Singh HK, Walsh EE.** Serum antibody decay in adults following natural respiratory syncytial virus infection. *Journal of Medical Virology* 2006; **78**: 1493–1497.
25. **Sande CJ, *et al.*** Kinetics of the neutralizing antibody response to respiratory syncytial virus infections in a birth cohort. *Journal of Medical Virology* 2013; **85**: 2020–2025.
26. **Pothier P, *et al.*** Antigenic variations of respiratory syncytial virus in recurrent infections. *European Journal of Clinical Microbiology* 1987; **6**: 212.
27. **Walsh EE, *et al.*** Severity of respiratory syncytial virus infection is related to virus strain. *Journal of Infectious Diseases* 1997; **175**: 814–820.
28. **Hierholzer JC, *et al.*** Subgrouping of respiratory syncytial virus strains from Australia and Papua New Guinea by biological and antigenic characteristics. *Archives of Virology* 1994; **136**: 133–147.