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Temporal development of the humoral immune response to surface antigens of *Moraxella catarrhalis* in young infants

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ABSTRACT

The primary *Moraxella catarrhalis*-specific humoral immune response, and its association with nasopharyngeal colonization, was studied in a cohort of infants from birth to 2 years of age.

Results indicated that the levels of antigen-specific IgG, IgA and IgM showed extensive inter-individual variability over time, with IgM and IgA levels to all 9 recombinant domains, from 7 different OMPs, being relatively low throughout the study period. In contrast, the level of antigen-specific IgG was significantly higher for the recombinant domains $Hag^{385-863}$, $MID^{764-913}$, $MID^{962-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ in cord blood compared to 6 months of age ($P \leq 0.001$). This was a most likely a consequence of maternal transmission of antigen-specific IgG to newborn babies, possibly indicating a future role for these 3 surface antigens in the development of an effective humoral immune response to *M. catarrhalis*. Finally, at 2 years of age, the levels of antigen-specific IgG still remained far below that obtained from cord blood samples, indicating that the immune response to *M. catarrhalis* has not matured at 2 years of age.

We provide evidence that a humoral antibody response to OMPs UspA1, UspA2 and Hag/MID may play a role in the immune response to community acquired *M. catarrhalis* colonization events.

Keywords: *Moraxella catarrhalis*; colonization; immune response; surface antigens; vaccine; children

INTRODUCTION

Moraxella catarrhalis is an aerobic, Gram-negative diplococcal commensal of the respiratory tract. Although healthy children are frequently colonized with this bacterium, it is also able to cause disease, being especially associated with otitis media (OM), as well as exacerbations of chronic obstructive pulmonary disease (COPD) in adults.

Studies have shown that the bacterium colonizes the nasopharynx soon after birth and that the carriage rate of *M. catarrhalis* in healthy children may differ per geographical region, season and year of isolation [1]. For example, in a German study (November 1991 to April 1992), 9% of children attending day-care centers ranging in age from 4 months to 3 years old were colonized with *M. catarrhalis* [2]. In a Japanese study conducted in 1999, children aged 1 month to 5 years attending day-care centers, 35% were found to be colonized [3]. In The Netherlands, a comparative study of 1.5 to 14 month old children born between February 2003 and August 2005, indicated a carriage rate for children ranging from 11.8% at the age of 1.5 months to 29.9% at the age of 6 months and 29.7% at the age of 14 months [4]. In general, despite local geographical variation, infants tend to become colonized with *M. catarrhalis* at a very early age, resulting in a nasopharyngeal colonization peak for *M. catarrhalis* at 2 years of age [5].

Bacterial adherence to the respiratory mucosa is an essential step towards colonization of the human respiratory tract epithelium, and research has indicated that the most important adhesins responsible for the attachment of *M. catarrhalis* to host cells include the outer membrane proteins (OMPs) UspA1, UspA2 and Hag/MID, though several other surface-exposed outer membrane proteins have been described that may also play a role in the process. Further, with respect to *M. catarrhalis*, it has been shown that colonization of the human respiratory tract epithelium results in an increased risk of disease, specifically OM disease (both chronic and acute) in children [6-7]. Further, two distinct genetic lineages

related to 3 different 16S rRNA types have been identified for *M. catarrhalis*, which differ phenotypically in their ability to resist the killing effect of human serum (sero-resistant versus sero-sensitive), and in their ability to adhere to human epithelial cells [8-9]. Therefore, it is reasonable to expect that an effective immune response raised against UspA1, UspA2 and Hag/MID, for example via vaccination, will have a significant effect on colonization and disease.

OM is one of the major childhood diseases that necessitate visits to general practitioners [10]. In 2004, the American Academy of Pediatrics (AAP) published new guidelines that addressed the diagnosis and treatment of acute otitis media (AOM), largely because the treatment of AOM is not always appropriate, and the long-term overuse of antibiotics increases the risk of the development of antimicrobial resistance. The AAP guidelines recommended the use of observation as a potential strategy for the treatment of AOM, although global rates of antibiotic prescription for AOM still vary greatly [11-13].

An alternative strategy to the use of antibiotics in the treatment of OM disease is vaccination [14]. However, there is currently no licensed vaccine available against *M. catarrhalis*, and none of the antigens so far described (which may serve as potential vaccine candidates) have progressed to clinical trials. The challenge in identifying potential vaccine candidates for *M. catarrhalis* lies in identifying antigens that are able to generate an appropriate immune response that prevents the process leading from colonization to infection, and are conserved among global strains [15]. It is known that healthy adults possess naturally acquired serum antibodies directed against several *M. catarrhalis* OMPs, apparently via the acquisition and elimination of many different *M. catarrhalis* strains [16]. Further, changes in antibody response are observed in adults suffering from *M. catarrhalis*-mediated COPD disease [17].

The introduction of a vaccination strategy against *M. catarrhalis* (either in children and/or in adults) is still a topic for debate, though the continuing high prevalence of OM disease in children and the rising prevalence of COPD in adults means that *M. catarrhalis*-associated disease continues to increase in global significance. Further, the introduction of successful vaccines against respiratory bacterial pathogens that occupy the same niche as *M. catarrhalis* e.g. *Streptococcus pneumoniae* and *Haemophilus influenzae* could facilitate a concomitant increase in *M. catarrhalis* colonization and infection.

Several new *M. catarrhalis* OMP vaccine candidates have been described in the literature, and previous studies have suggested that a multivalent vaccine comprising a combination of epitopes of these *M. catarrhalis* OMP vaccine candidates should form the basis of a vaccine to prevent *M. catarrhalis*-mediated colonization and disease [16, 18-19].

However, relatively little is known about the humoral immune response to these vaccine candidates, especially within the first few years of life. The present study was performed to determine the humoral immune response to potential *M. catarrhalis* vaccine candidates in healthy Dutch children from birth to 2 years of age. The previously described *M. catarrhalis* recombinant domains UspA1$^{557\text{-}704}$, UspA2$^{165\text{-}318}$, MID$^{764\text{-}913}$, MID$^{962\text{-}1200}$, Hag$^{385\text{-}863}$, MhaC, McaP$^{51\text{-}333}$, orf238 and orf296 were used in this study [16, 20-24]. These 9 recombinant proteins (from 7 different OMPs) represented the majority of published *M. catarrhalis* immunogenic proteins discovered at the time that the study was initiated. Further, the relationship between *M. catarrhalis* colonization and humoral immune response was also investigated.

MATERIALS AND METHODS

Study cohort

This study was embedded in the Generation R Study, a population-based prospective cohort study, designed to identify early environmental and genetic causes of normal and abnormal growth, development and health from fetal life until young adulthood [25]. This study was performed in a randomly selected subgroup of Dutch children whose parents are ethnically homogeneous (two parents and four grandparents born in The Netherlands), in order to exclude possible confounding factors associated with ethnicity.

In total, 57 infants who were born between February 2003 and August 2005 were included in this study. Three or 4 serial serum samples were collected from each infant for inclusion in the study. The collection totalled 177 samples, comprising 54 (31%) cord blood samples, 32 (18%) samples obtained at 6 months, 46 (26%) samples obtained at 14 months, and 45 (25%) samples obtained at 24 months of age. The bacterial colonization status was determined by taking nasopharyngeal swabs at the ages of 1.5, 6, 14 and 24 months of age, with swabs being taken at the same time as serum samples. Swabs were obtained from 40 (70%), 49 (86%), 50 (88%) and 48 (84%) infants at 1.5, 6, 14 and 24 months of age, respectively. The colonization status was determined using standard *M. catarrhalis* culture and detection techniques [1].

Moraxella catarrhalis antigens

The previously described *M. catarrhalis* recombinant domains UspA1$^{557-704}$ (aa 557–704 of UspA1), UspA2$^{165-318}$, MID$^{764-913}$, MID$^{962-1200}$, Hag$^{385-863}$, MhaC, McaP$^{51-333}$, orf238 and orf296 were used in this study [16, 20-24]. These 9 recombinant proteins (from 7 different OMPs) represented the majority of published *M. catarrhalis* immunogenic proteins

discovered at the time that the study was initiated, and are derived from the reference *M. catarrhalis* strains Bc5 (UspA1$^{557-704}$, UspA2$^{165-318}$, MID$^{764-913}$ and MID$^{962-1200}$) and O35E (MhaC, McaP$^{51-333}$ and Hag$^{385-863}$) [16, 20, 22, 26]. Orf238 and orf296 are hypothetical proteins that share homology with lipoprotein family A proteins and with the *M. osloensis* disulfide isomerase gene, which encodes a virulence factor, respectively.

Antigen coupling

Recombinant proteins were coupled to SeroMAP^{TM} beads, which are carboxylated beads that are developed for serological applications. The coupling procedure was performed as detailed by Verkaik *et al.* (2008) [27]. Briefly, 5.0×10^6 microspheres were resuspended in 100 mmol/L monobasic sodium phosphate (pH 6.2) buffer. For activation of the carboxyl groups on the surface of the beads, 10 µl of 50 mg/ml of *N*-hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was used (Pierce Biotechnology, Rockford, IL). The coupling buffer consisted of 50 mmol/L 2-(*N*-morpholino) ethanesulfonic acid, pH 5.0 (Sigma-Aldrich, Zwijndrecht, The Netherlands) in which 25 µg of protein was added. The final concentration of microspheres was adjusted to 4000 beads/µl with blocking-storage buffer (PBS-BN; PBS, 1% bovine serum albumin, and 0.05% sodium azide [pH 7.4]). The microspheres were protected from light and stored at 4°C until required. All centrifugation steps were performed at 12,000 g for 2 min at room temperature (RT).

Uncoupled beads were used as a negative control, and to determine non-specific binding. If minor non-specific binding was observed, then the median fluorescence intensity (MFI) values obtained from this non-specific binding was subtracted from the antigen-specific results.

Multiplex *M. catarrhalis* antibody assay

The multiplex procedure was performed as described elsewhere [27]. Briefly, after validation of the assay (where human pooled serum (HPS) MFI multiplex assay values were compared to corresponding HPS MFI singleplex assay values), the different antigen-coupled microspheres were mixed to a working concentration of 4000 beads per color per well. Serum samples were diluted 1:100 in PBS-BN for measurement of antigen-specific IgG and IgA and 1:50 for measurement of IgM. Fifty microliters per diluted sample was incubated with the microspheres in a 96-well filter microtiter plate (Millipore) for 35 min at room temperature on a Thermomixer plate shaker (Eppendorf). The plate was washed twice with PBS-BN that was aspirated by vacuum manifold, and the microspheres were resuspended in 50 µl of PBS-BN. In separate wells, 50 µl of a 1:100 dilution of R-phycoerythrin (RPE)-conjugated AffiniPure goat anti-human IgG and IgA and 50 µl of a 1:50 dilution of RPE-conjugated donkey anti-human IgM (Jackson Immuno Research) were added. The plate was incubated for 35 min at room temperature and washed. The microspheres were resuspended in 100 µl of PBS-BN. Measurements were performed on the Luminex 100 instrument (BMD) using Luminex IS software (version 2.2). Tests were performed in duplicate, and the fluorescence intensity values, reflecting quantitative antibody levels, were averaged. The coefficient of variation of these values was then calculated for each serum sample and averaged per protein and antibody isotype.

Vaccine candidate gene carriage

M. catarrhalis isolates were grown from glycerol stocks at 37^{o}C overnight on blood agar plates. DNA was extracted using the MagNA Pure LC System (Roche Applied Science). PCR was performed to detect the major identified *M. catarrhalis* vaccine candidates *uspA1*, *uspA2* and *hag/mid* genes. Primer pairs were used to detect the *uspA1* (RTF1-8 5'-cgttatgcactaaaagagcaggtc and RTB1-8 5'-gcatctgaccagcttagaccaatc) and *uspA2* (RTF2-10 5'-

gcatctgcggataccaagtttg and RTB2-10 5’-ttgagccatagccaccaagtgc) genes according to the protocol of Meier *et al.* (2002) [28]. For the detection of the *hag/mid* gene, the primers McatHag-2 (5’-gtcagcatgtatcatttttttaagg) and McatHagR4 (5’-tgagcggtaaatggtttaagtg) were used [19]. The *uspA2*, *uspA2H* and *uspA1* screening primers are situated at the 3'-end of the respective genes, whilst the *hag/mid* primers amplify a region at the 5'-end of the gene, including a small region of the promotor.

Further, PCR was performed to detect 16S rRNA types as previously described by Verhaegh *et al*. (2008) [19].

To identify the 16S rRNA types of individual *M. catarrhalis* isolates, 16S rRNA PCR products were digested using the enzymes *Fsp*BI (10 U) and *Hha*I (10 U) according to Verhaegh *et al*. (2008) [19].

Isolate genotyping

M. catarrhalis genotyping was performed on 30 isolates (representing all *M. catarrhalis* culture positive swabs obtained during the course of the study), by pulsed-field gel electrophoresis (PFGE) as detailed by Verduin *et al*. (2000) [29]. Briefly, *M. catarrhalis* plug digestions were performed using *Spe*I at 20 U/reaction and an electrophoresis protocol comprising a 1st block with a constant voltage of 6 V cm^{-1}, a pulse time from 3.5 to 25 seconds during the first 12 hours, followed by a 2nd block of 8 hours where the pulse time increased linearly from 1 to 5 seconds. All PFGE patterns were analyzed using BioNumerics (Applied Maths), with gel lanes normalized against a lambda DNA ladder (Bio-Rad) and band tolerance set to 1.5%. PFGE products between 48.5 and 339.5 kb were included in the band matching analysis.

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Statistical analysis

Statistical analyses were performed using SPSS PASW 17.0.2. The Wilcoxon signed-rank test was used to compare the anti-*Moraxella* antibody levels between different age groups. The Mann-Whitney *U*-test was used to compare differences in antibody levels between colonized and non-colonized children. A *P*-value of ≤ 0.05 was considered to be statistically significant.

RESULTS

Isolate genotyping and vaccine candidate gene carriage

A high degree of genotypic heterogeneity in *M. catarrhalis* isolates colonizing children in the focus cohort was maintained over the entire study period, with no association found between genotype and any of the antigen-specific MFI values. In total, 28 different genotypes were observed, with only two children being colonized more than once (Fig. 1).

Ninety-seven percent (29/30) of the *M. catarrhalis* isolates were found to be positive for *uspA1*, with 90% (27/30) positive for *uspA2*, and 87% (26/30) positive for *hag/mid* gene carriage. In total, 87% (26/30) of the *M. catarrhalis* isolates were categorized into 16S type lineage 1 (16S type 1; seroresistant lineage), with the remaining 13% belonging to the 16S type lineage 2 (16S type 2 and 3; serosensitive lineage) (Table 1).

Dynamics of the anti-*Moraxella* antibody response

The changes measured in anti-*M. catarrhalis* IgG, IgA and IgM during the first 2 years of life are shown in Fig. 2. The levels of antigen-specific IgG, IgA and IgM showed extensive inter-individual variability over time. The level of antigen-specific IgG in cord blood (maternal antibody) was significantly higher for $Hag^{385-863}$, $MID^{764-913}$, $MID^{962-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ than at 6 months of age ($P \leq 0.001$), presumably due to passive

immunization by maternally acquired IgG antibodies *in utero*. Such passive immunity typically remains until approximately 6 months after birth [30].

IgG levels against $MID^{764-913}$, $MID^{962-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ rose significantly between 6 months to 2 years of age. IgG levels to *M. catarrhalis* OMPs $Hag^{385-863}$, $McaP^{51-333}$, MhaC, orf238 and orf296 remained relatively low and did not significantly increase over the 6 month to 2 year time period.

IgM and IgA levels to all 9 recombinant domains of 7 different OMPs were relatively low throughout the study period. However, IgM levels to MhaC, $MID^{764-913}$, $MID^{962-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$, and IgA levels to $Hag^{385-863}$, $MID^{764-913}$, $MID^{962-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ increased significantly ($P \leq 0.05$) over the 6 month to 2 year time period. Finally, not every infant developed an antigen-specific IgG, IgA or IgM response to all of the recombinant proteins tested in the first 2 years of life.

Relationship between colonization and anti-*M. catarrhalis* antibody levels

In order to relate colonization status to changes in anti-*M. catarrhalis* antibody levels (which would provide an estimation of the efficacy of the immune response in preventing *M. catarrhalis* colonization), results were utilized from sera and nasopharyngeal colonization data of children at 6, 14 and 24 months of age where concurrent sera and nasopharyngeal swab data was available. Children were divided into colonized or non-colonized at each time period and their IgG levels to $Hag^{385-863}$, $MID^{764-913}$, $MID^{962-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ plotted.

In total, 9 (33%), 10 (24%) and 11 (29%) of the children were found to be colonized with *M. catarrhalis* at the time of sampling at 6, 14 and 24 months, respectively. There was no significant difference in IgG levels for all antigens between colonized and non-colonized children, except for $MID^{962-1200}$ at 24 months of age (P=0.04) (Fig. 3). Further, the increase in

IgG antibody response did not result in a decrease in the percentage of infants nasopharyngeal colonized by *M. catarrhalis*, although antigen-specific IgG levels significantly increased for $MID^{764\text{-}913}$, $MID^{962\text{-}1200}$, $UspA1^{557\text{-}704}$ and $UspA2^{165\text{-}318}$ between 6 months and 2 years of age (Fig. 4).

DISCUSSION

The research performed in this publication represents the most extensive study of the infant immune response to potential vaccine candidates of *M. catarrhalis* performed to date, utilizing 9 recombinant domains representing 7 different *M. catarrhalis* OMPs in a cohort of 57 healthy children followed from birth until 2 years of age. Further, the study was performed using multiplexed Luminex's xMAP technology that proved to be a rapid method for research into humoral immune response changes during *M. catarrhalis* colonization.

In our study, the level of antigen-specific IgG to *M. catarrhalis* antigens in cord blood was significantly higher compared to the anti-*M. catarrhalis* IgG level at 6 months, most likely due to the presence of maternally derived IgG antibodies that were transferred to the fetus through the placenta. The passage of antibodies between mother and baby, via the umbilical cord, gives rise to "passive immunity", which generally tends to confer humoral protection against infection until approximately 6 months after birth [30]. During this 6 month period, passively acquired antibodies disappear and are replaced by antibodies generated by the infants' own "actively acquired" humoral immune response. This actively acquired immune response may be generated by successive rounds of colonization and/or infection by pathogens, leading to the development of a host-specific immune response and eventual pathogen clearance. In this respect, Ejlertsen *et al.* (1994) showed a significant fall in antibody concentration during the first 3 months of life, and a steady low level was maintained in the age group from 3 to 10 months, similar to the results obtained in this study

[31]. Further, from the age of 1 year, the immune response of the children in the study of Ejlertsen *et al.* (1994) and Tan *et al.* (2006) increased slowly to reach maternal levels at the age of 10 years and in healthy adults, and though only sampling children up to 2 years of age, our study also showed increases in IgG antibody response for the antigens $MID^{764-913}$, $MID^{962-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ between years 1 and 2. In contrast, MhaC, MhaP, orf238 and orf296 did not induce major humoral immune responses in this cohort of children. Although *M. catarrhalis* is considered to be a major mucosal pathogen of the human respiratory tract, the IgA response towards OMPs of *M. catarrhalis* remained relatively low throughout the study period. Two forms of IgA can be distinguished based upon their location (serum IgA and secretory IgA). In its secretory form, IgA is the main immunoglobulin found in mucous secretions, including respiratory epithelium. Studies have shown that human salivary IgA response is directed consistently against a small number of major OMPs in healthy adults and adults suffering from COPD. It is also found in small amounts in blood [32]. This may explain the relatively low levels of IgA found in this study, as serum antigen-specific IgA levels were measured and not secretory IgA [33-34].

The IgM levels to all 7 OMPs were also relatively low throughout the study period. IgM antibodies appear early in the course of an infection and usually reappear, to a lesser extent, after further exposure. In contrast to IgG, IgM (and also IgA) antibodies do not pass across the human placenta.

Though an antibody response was generated against our *M. catarrhalis* OMP vaccine candidates in our focus cohort group during the first 2 years of life, the relatively constant level of *M. catarrhalis* nasopharyngeal colonization observed within the cohort suggests that the antibody response measured did not provide significant protection against *M. catarrhalis* nasopharyngeal colonization up to 2 years of age, with the exception of antigen $MID^{962-1200}$. Non-colonized children showed significantly higher IgG levels for $MID^{962-1200}$ compared to

colonized children at 24 months of age. $MID^{962-1200}$ represents the IgD-binding domain of the *M. catarrhalis* IgD-binding protein (MID), a 200-kDa outer membrane protein comprising 2,139 amino acids that has been shown to display a unique and specific affinity for human IgD. This result provides preliminary evidence that antibodies raised against $MID^{962-1200}$ could offer protection against *M. catarrhalis* colonization. If indeed confirmed, then a $MID^{962-1200}$ vaccine may possibly be used to boost immunity levels at or before 2 years of age, in order to provide protection against *M. catarrhalis* colonization, and hence disease. However, further research is required to investigate this hypothesis.

The factors influencing *M. catarrhalis* colonization and elimination are not yet fully understood, though genetic variation and adhesion to mucosal receptors appear to play an important role in colonization dynamics [35]. For example, several studies have shown that children acquire and eliminate a number of different strains throughout the first 2 years of life by the ability of these strains to evade the host immune system, caused by phase variation and antigenic variation [36]. Under "immune pressure", antigenic variation due to sequence changes in virulence genes may provide a selective advantage for bacterial isolates expressing novel sequence variants. Alternatively, mutations may generate phase variable gene expression, switching off genes that are recognized by the immune system. Specifically, *M. catarrhalis* OMPs UspA1, UspA2 and Hag/MID are known to undergo phase-variation, with antigenic variation reported in the target region of monoclonal antibody (MAb) 17C7 (a conserved UspA1 and UspA2 binding site) [37-42]. The relatively constant level of *M. catarrhalis* nasopharyngeal colonization observed within the cohort could also be related to host factors, for example relatively low levels of antibody at 2 years of age, lack of effective antibody neutralizing activity, evasion of the host innate immune defence by several virulence factors involved in adherence to the respiratory tract, or complement resistance [31, 43-44].

Further research is required in order to determine whether increased IgG levels against the OMPs UspA1, UspA2, and Hag/MID (induced for example via vaccination) would significantly reduce the incidence of *M. catarrhalis* colonization and infection in infants up to 2 years of age (and in later life). In this respect, further studies are being planned at 5 years of age.

CONCLUSIONS

Though further research is required, our results indicate that at 2 years of age, the antibody response to *M. catarrhalis* is still developing, and is largely based on an IgG isotype of antibodies raised against 3 major OMPs (i.e. UspA1, UspA2 and MID/Hag). We also provide preliminary evidence to suggest that antibodies directed against Hag/MID may be associated with the prevention of *M. catarrhalis* colonization, though natural variation in amino acid sequences of this protein may act to limit the potential of vaccines created to generate an immune response against Hag/MID.

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All authors declare that they have no conflicts of interest.

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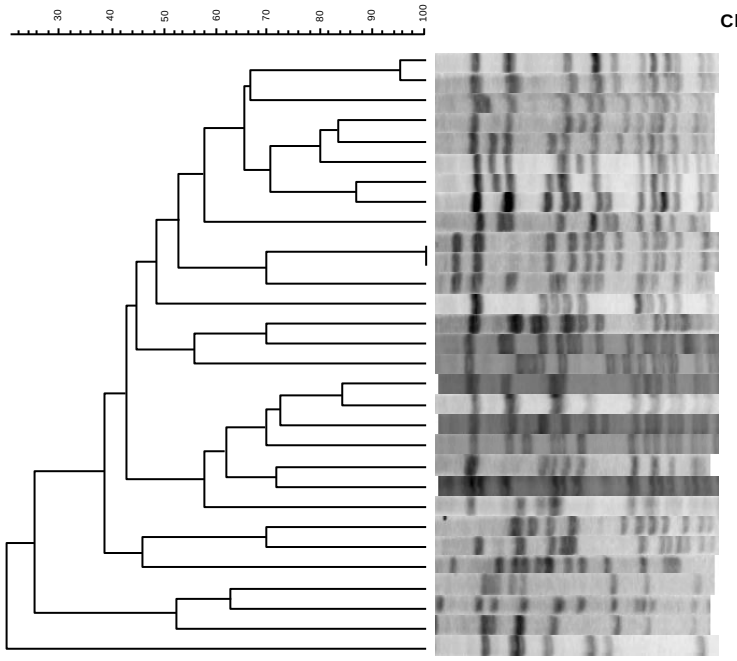
TABLES

Table 1. Prevalence of virulence genes for 30 *M. catarrhalis* isolates for which PFGE genotyping was performed.

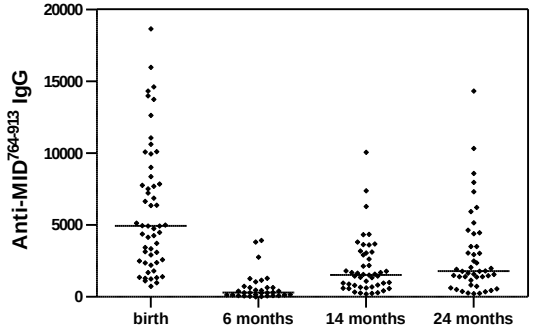
Virulence genes	positive (%)
uspA1	97
uspA2	90
hag/mid	87
16S lineage 1	87
16S lineage 2	13

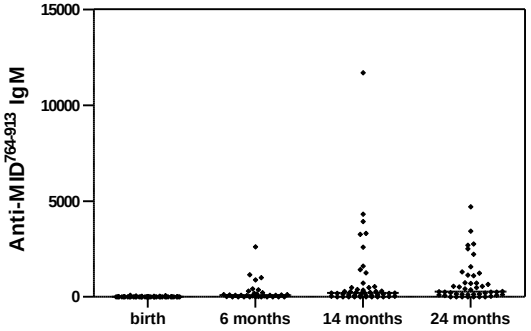
FIGURES

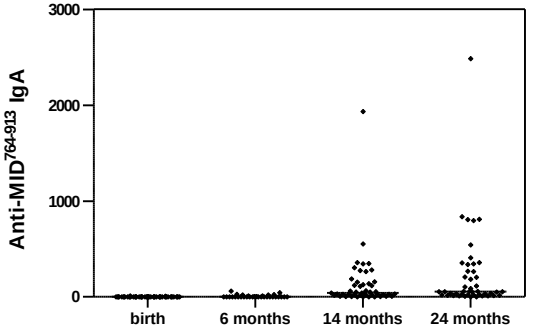


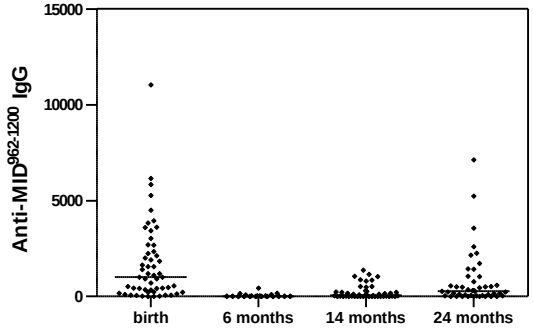
Child nr.	PFGE type	Year	Hag 385-863	MID 764-913	MID 902-1200	UspA1 557-704	UspA2 165-318	Age	Month
G19	1	2006	168	1350	21	5112	3988	14	2
G12	1	2004	693	1067	3	1587	629	6	9
G15	2	2005	48	94	0	194	421	6	1
G02	3	2005	76	1394	38	413	1563	24	6
G21	4	2005	653	138	0	228	101	6	8
G24	5	2006	77	607	8	263	170	14	5
G10	6	2006	39	366	48	1196	368	24	1
G17	7	2006	0	3189	1070	1399	2782	14	2
G27	8	2005	175	294	0	83	148	6	11
G15	9	2004	62	1568	118	5612	224	14	12
G08	9	2004	10	7382	311	971	1740	14	12
G06	10	2004	183	10073	488	484	10939	14	12
G20	11	2006	247	3631	279	1411	252	14	4
G14	12	2004	10	402	0	871	1098	6	12
G14	13	2006	81	571	13	*	9773	24	7
G26	14	2006	28	978	533	2481	322	14	6
G09	15	2006	137	3518	1044	6207	4949	24	1
G13	16	2006	415	6240	270	3003	3695	24	5
G11	17	2006	77	1178	0	3838	7379	24	2
G23	18	2006	98	673	51	397	409	14	5
G07	19	2005	419	3041	62	3468	2467	24	11
G28	20	2005	21	157	22	172	188	6	12
G18	21	2007	142	1568	175	1953	2532	24	4
G13	22	2004	183	1310	38	243	257	6	10
G03	23	2004	489	981	0	459	963	14	10
G16	24	2005	428	3937	0	476	995	6	2
G01	25	2005	360	4461	322	1883	3082	24	6
G22	26	2005	71	668	171	1319	2210	6	8
G04	27	2005	125	4658	779	1664	8099	24	8
G25	28	2007	3	1424	0	988	516	24	3

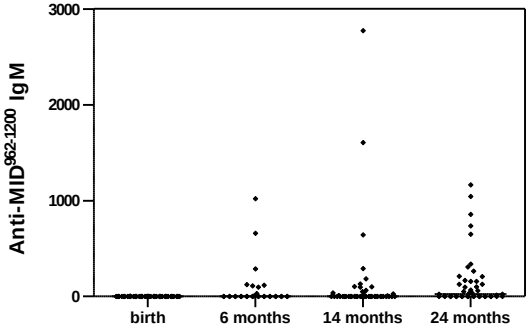
Figure 1. Relationship between *M. catarrhalis* genotypes (representing all positive *M. catarrhalis* nasopharyngeal swab cultures isolated at 6, 14 and 24 months of age) and vaccine candidate serum MFI values. No relationship between MFI value and genetic relatedness was observed for these isolates. Key: Age nasopharyngeal swabs were taken; 6 = 6 months, 14 = 14 months, 24 = 24 months, after birth. The month and year of isolate culture from the nose of children is also shown.

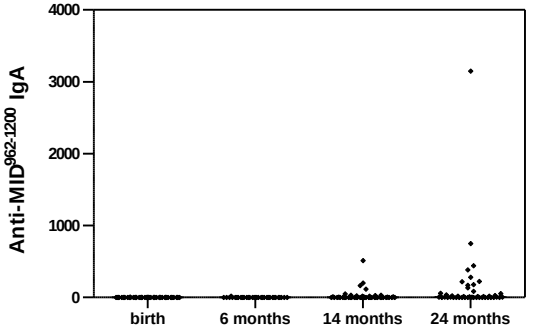

Anti-MID764-913 IgG
20000
15000
10000
5000
0
birth
6 months
14 months
24 months


Anti-MID764-913 IgM
15000
10000
5000
0
birth
6 months
14 months
24 months


Anti-MID764-913 IgA
3000
2000
1000
0
birth
6 months
14 months
24 months


Anti-MID962-1200 IgG
15000
10000
5000
0
birth
6 months
14 months
24 months


Anti-MID962-1200 IgM
3000
2000
1000
0
birth
6 months
14 months
24 months


Anti-MID962-1200 IgA
4000
3000
2000
1000
0
birth
6 months
14 months
24 months

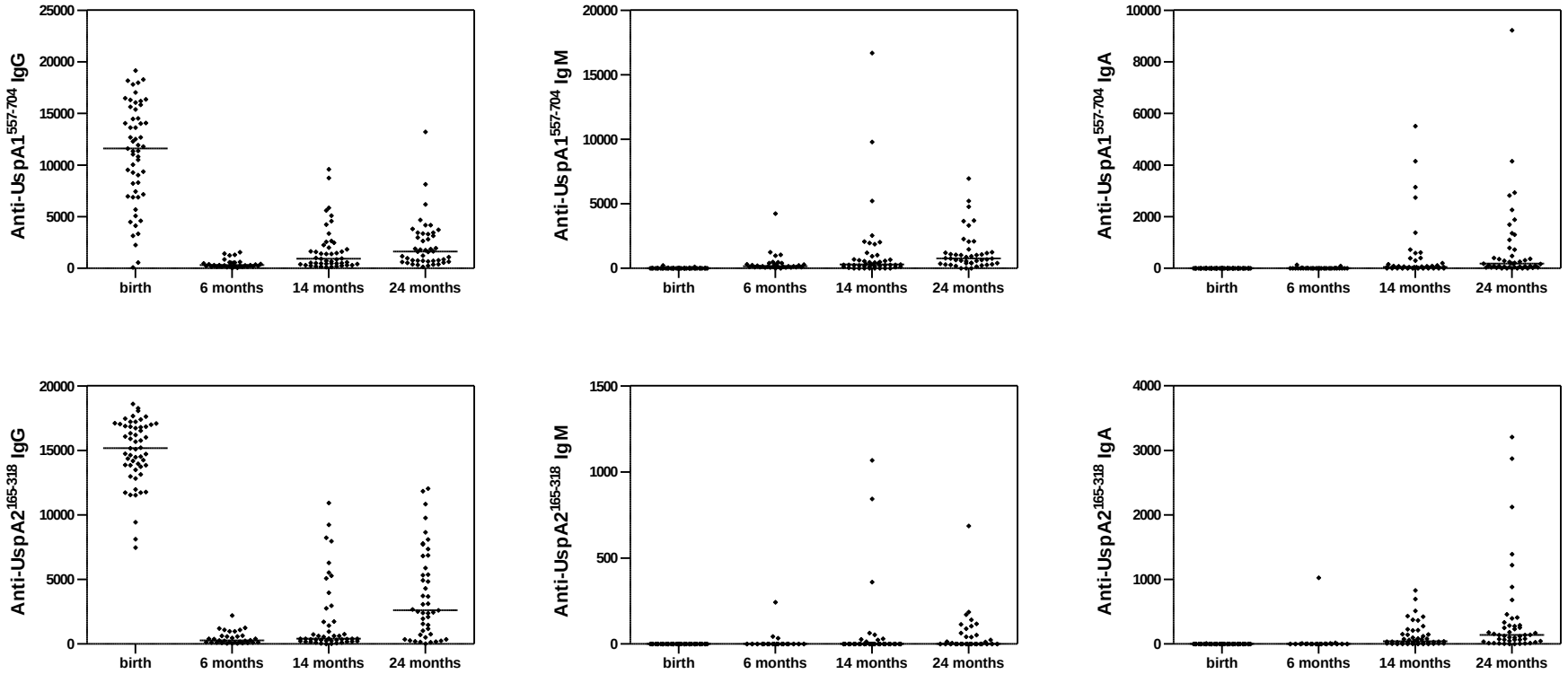
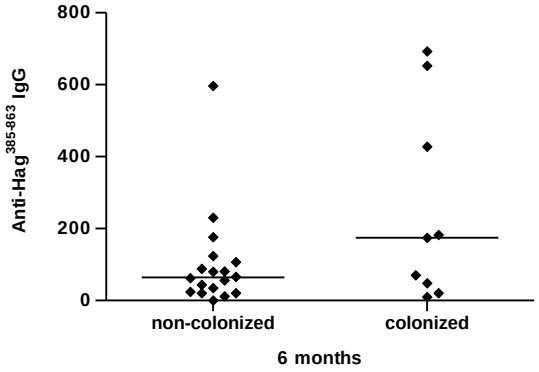
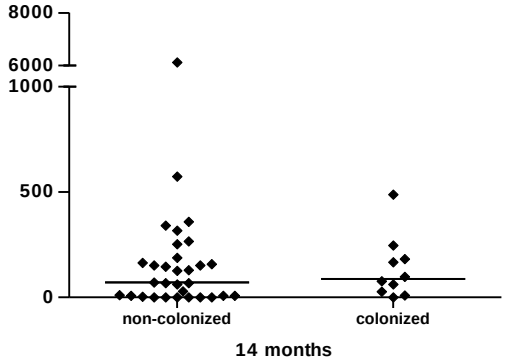
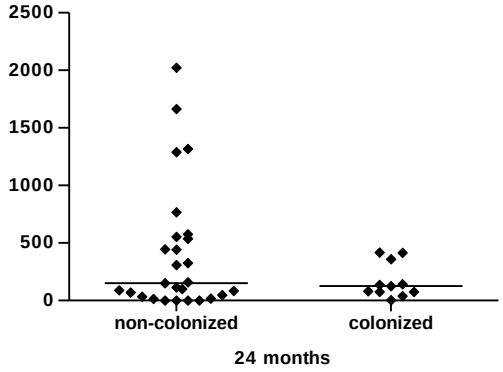


Figure 2. Levels of IgG, IgM and IgA directed against *M. catarrhalis* immunoglobulin D-binding protein (MID) and ubiquitous surface proteins A1 (UspA1) and A2 (UspA2) in 57 children at birth, 6 months, 14 months and 24 months. Antibody levels are reflected by MFI values. Each dot represents a serum sample. Median values are indicated by a horizontal line.

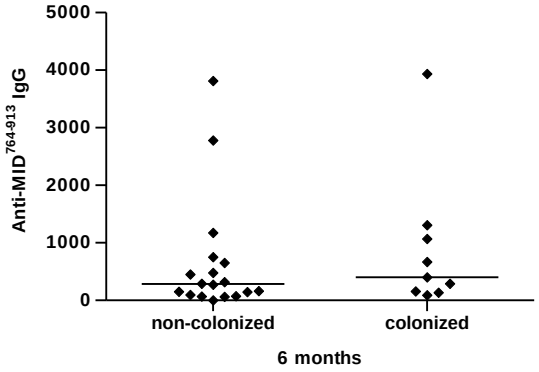
23

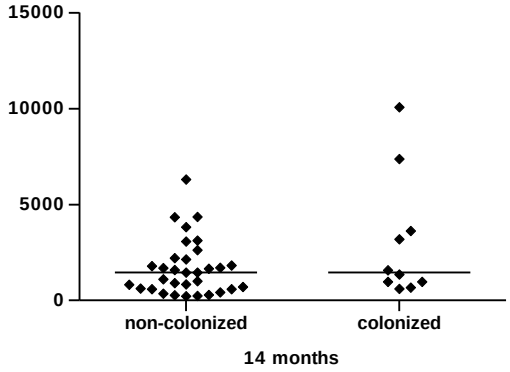

Anti-Hag 385-863 IgG
800
600
400
200
0
non-colonized
colonized
6 months

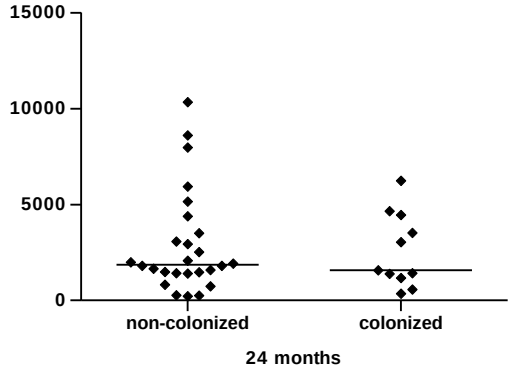

8000
6000
1000
500
0
non-colonized
colonized
14 months


2500
2000
1500
1000
500
0
non-colonized
colonized
24 months

24

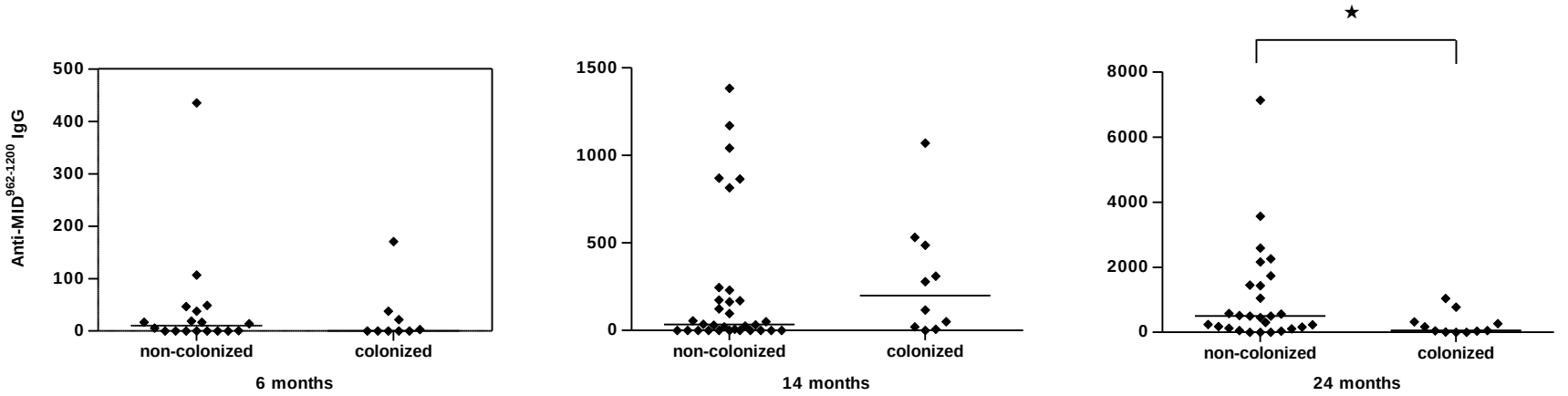

Anti-MID 764-913 IgG
5000
4000
3000
2000
1000
0
non-colonized
colonized
6 months


15000
10000
5000
0
non-colonized
colonized
14 months

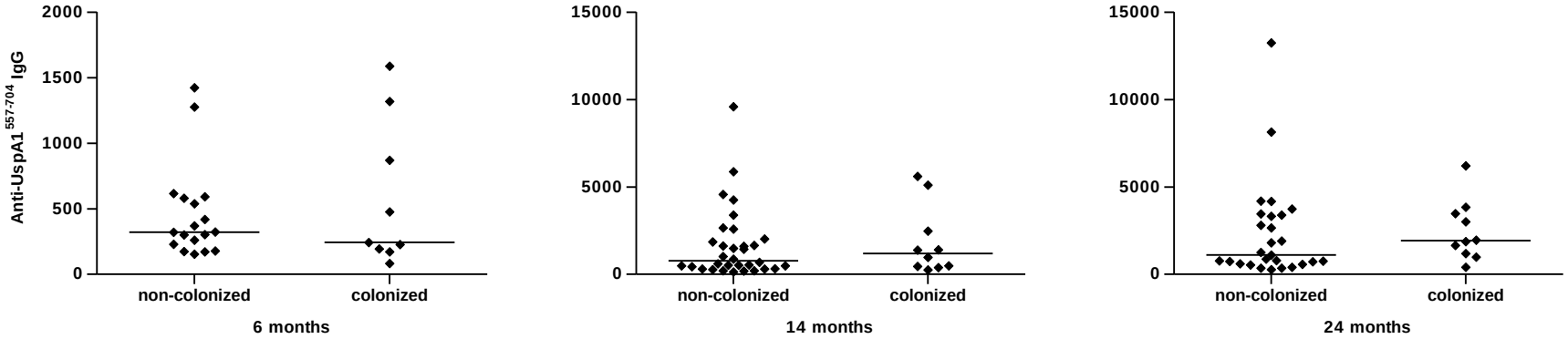

15000
10000
5000
0
non-colonized
colonized
24 months

25

26


Anti-MID962-1200 IgG
500
400
300
200
100
0
non-colonized
colonized
6 months
1500
1000
500
0
non-colonized
colonized
14 months
★
8000
6000
4000
2000
0
non-colonized
colonized
24 months

27


Anti-UspA1557-704 IgG
2000
1500
1000
500
0
non-colonized
colonized
6 months
15000
10000
5000
0
non-colonized
colonized
14 months
15000
10000
5000
0
non-colonized
colonized
24 months

28

29

30

31

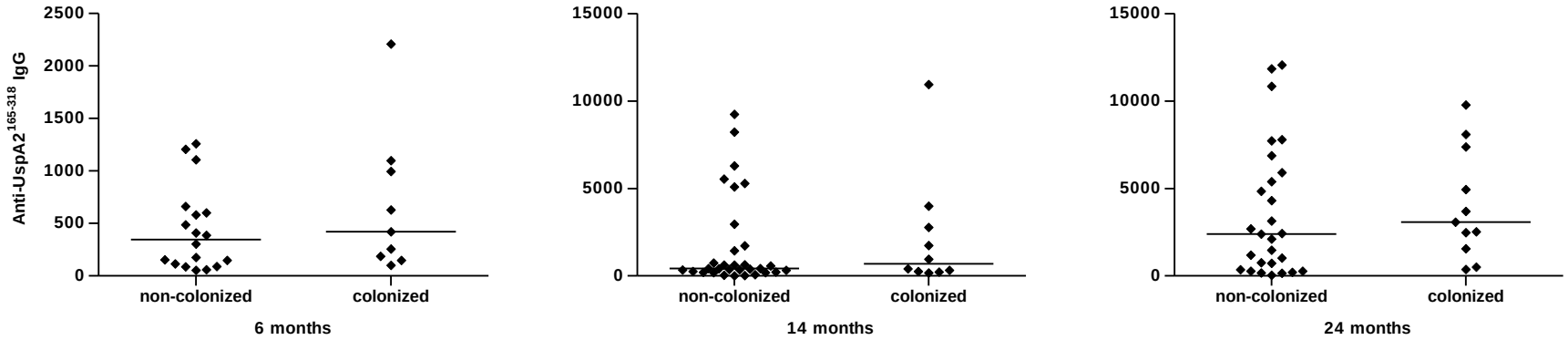


Figure 3. Relationship between *M. catarrhalis* colonization and anti-Hag/MID, UspA1 and UspA2, IgG levels at 6, 14 and 24 months of age, as reflected by MFI values. Median values are indicated by a horizontal line. A significant difference between non-colonized and colonized children was observed for $MID^{962\text{-}1200}$ at 24 months of age (P=0.04).

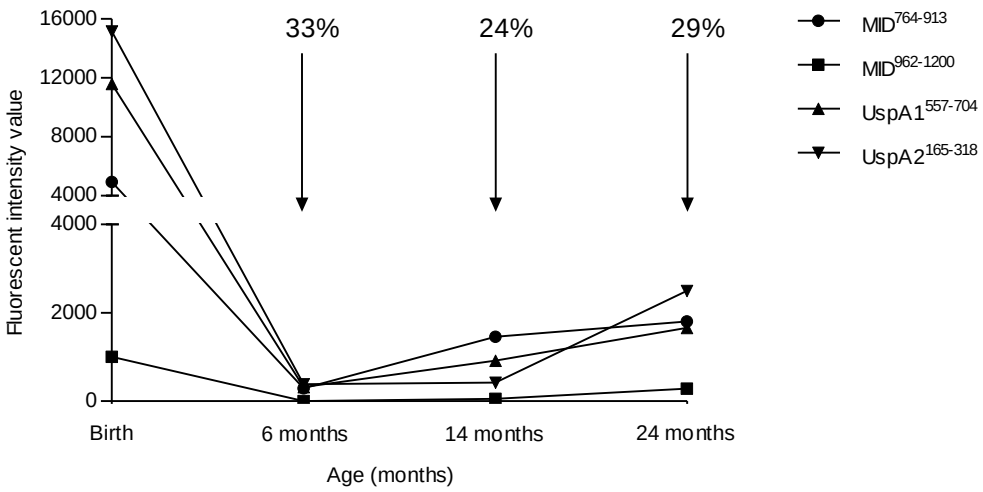


Figure 4. Relationship between *M. catarrhalis* colonization, (colonized children as a percentage of total number of children tested), and anti-*M. catarrhalis* IgG levels at birth, 6, 14 and 24 months of age, as reflected by median fluorescence intensity values. The total number of children tested at birth, 6, 14 and 24 months was 54, 32, 46 and 45, respectively.