Haemophilus influenzae type b (Hib) anti-PRP antibody concentration and avidity index in 3-5 year old children since start of infant Hib immunisation in the UK

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**Abstract**

Introduction of routine infant immunisation with Hib conjugate vaccines in the UK in 1992 led to a significant reduction in invasive disease due to this organism. Subsequently, between 1999 and 2003 there was an increase in the number of immunised children with Hib infection. We investigated whether the rise in cases was related to changes in anti-polyribosylribitol phosphate (PRP) antibody concentration or avidity. Using stored sera we analysed temporal changes in antibody levels among 3-5 year old children immunised between 1991 and 2000. Anti-PRP antibody concentrations were higher in 3-5 year olds who received infant immunisation in 1991 than in subsequent years. This difference may be related to changes in either the mode of administration of Hib conjugate vaccines or rates of Hib nasopharyngeal carriage. This study emphasises the factors affecting anti-PRP antibody concentration following immunisation with conjugate vaccines and the importance of these in long-term protection from invasive disease.

Key words: *Haemophilus influenzae* type b, immunisation, antibody, conjugate, infant, carriage, immunological memory
Introduction

In 1992 the United Kingdom began the routine immunisation of infants with Hib conjugate vaccines given at 2, 3 and 4 months without a booster dose later in childhood(13). The annual incidence of invasive Hib disease fell from a pre-immunisation rate of 28.3 per 100,000 children under 5 years of age to 0.97 per 100,000 by 1998(28). Subsequently between 1999 and the end of 2003 there was a year on year increase in the number of fully immunized children who developed invasive Hib disease with the overall incidence rising to 3.8/100,000 under 5 years of age (28).

Within the UK the introduction of a DTaP-Hib vaccine was temporally associated with this increase in Hib vaccine failures(26) and a case-control study demonstrated a dose response relationship between the number of doses of DTaP-Hib and the probability of vaccine failure(30). Combination aP/Hib vaccines are known to be less immunogenic, in terms of anti-PRP antibody concentrations, than when the Hib component is given separately(16). However, prior to the widespread use of DTaP-Hib, there was already a progressive reduction in the age at which vaccine failures were occurring with successive UK birth cohorts(42). The possibility that there may have been additional factors responsible for changes in direct individual protection due to changes in population levels of serum anti-PRP antibody (antibody against polyribosyl ribitol phosphate, the Hib polysaccharide capsular) and immunological memory needs to be considered. Such additional factors include changes in rates of nasopharyngeal carriage of Hib, an
alteration in methods of administration of Hib vaccine and the use of concomitant serogroup C meningococcal vaccines.

Hib conjugate vaccines reduce Hib carriage rates\(^{(6)}\). Whilst a reduction in carriage will increase herd immunity it may also decrease ‘natural boosting’ of anti-PRP antibody levels and affect the ability of individuals to maintain protective concentrations of antibody\(^{(33)}\).

The efficacy of Hib conjugates was initially demonstrated when the vaccine was given at a separate site to other vaccines given at the same visit\(^{(22)}\). After Hib vaccine introduction in the UK, immunisation practice changed to include routine reconstitution of Hib with DTwP vaccines prior to administration at a single site and an increasing use of combination Hib/DTwP vaccines. The effect on vaccine effectiveness of these practices has not been studied.

The increase in vaccine failures from 1999 onwards was temporally associated with the introduction of universal infant immunization with MenC conjugate vaccine. The effect of concomitant MenC and Hib vaccine administration on anti-PRP concentration was not assessed extensively prior to its introduction. A recent study however demonstrated that in some situations MenC conjugates may reduce the anti-PRP antibody responses to Hib conjugates\(^{(27)}\).
Thus several factors may have contributed to a decrease in the direct protection conferred by Hib conjugate vaccines for children immunised in infancy in the UK. Despite the potential importance of changes in population levels of direct protection there is only one study that has considered changes in anti-PRP antibody levels in children since 1992(41). In that study immunization details for the individuals whose sera were used were not given and in addition that study did not assess changes in any marker of immunological memory. In the current study we have investigated changes in the persistence of anti-PRP antibody concentration and avidity index (as a marker of immunological memory) using the stored sera of children immunized between 1991 and 2000.
Methods

Serum samples

Samples were selected from sera stored at either -20°C or -80°C since their collection as
part of clinical trials conducted by the Oxford Vaccine Group, University of Oxford. The
sera were selected from studies in which children were 3-5 years of age and had received
3 doses of Hib polysaccharide-tetanus toxoid conjugate vaccine (PRP-T) and a whole cell
pertussis-containing (wP) combination vaccine as part of primary immunisation between
1991 – 2000. All children received their primary immunisation at 2, 3 and 4 months of
age. To avoid selection bias, sera from all studies fitting the selection criteria were used.
Sera fitting the selection criteria were available from five studies (table 1, groups A to E).
The Hib vaccine was given at a separate injection site to the DTwP (group A) or was
reconstituted by mixing together prior to administration at a single site (group C) or was
given in the form of a combination vaccine (group D, ACT-Hib DPT, Pasteur-Merieux).
For some children the method of administration of Hib vaccine could not be confirmed
(groups B and E). Some of the children had received an infant schedule of serogroup C
meningococcal vaccine at the same time as the routine immunizations (group C and E).
Ethical approval for the use of these samples was obtained from Oxford Clinical
Research Ethics Committee (reference number C02.013).
Hib anti-PRP ELISA

Anti-PRP antibody concentrations were measured on all serum samples using a standard Hib ELISA(10). 96-well polystyrene plates (NUNC Polysorb) were coated with PRP conjugated to human albumin (obtained from the National Institute for Biological Standards and controls) at a concentration of 2µg/ml in PBS buffer and incubated at 37°C for 90 minutes before storing at 4°C until use. Serum and control samples were diluted and added to the PRP coated plates and incubated for 2 hours. Following this IgG antibodies were detected with goat anti-human IgG alkaline phosphatase conjugated sera (Sigma A9544) left on the plates for 1 hour. IgG binding was quantified by adding p-nitrophenyl phosphate (Sigma 104) diluted in diethonalamine substrate buffer. The reaction was monitored by taking absorbance readings at 450nm (MRX-Tc microplate reader, Revelation). The reaction was stopped by the addition of 3M NaOH at a set absorbance reading for wells containing the anti-PRP reference serum (FDA, lot 1983). The antibody concentrations were determined by reference to a standard curve, generated using four parameter sigmoid curve fitting, from known dilutions of an anti-PRP reference serum (FDA, lot 1983). The minimum sensitivity of the assay was estimated to be an anti-PRP concentrations of 0.1 µg/ml.
**Hib anti-PRP avidity assay**

The anti-PRP antibody avidity was determined using a thiocyanate elution assay described elsewhere(19). Serum samples were first assayed for anti-PRP concentration using the anti-PRP ELISA assay. Due to the sensitivity of the ELISA assay only sera with a titer of at least 0.2mcg/ml were analysed for anti-PRP antibody avidity. A dilution was then calculated that would yield an optical density of either 0.5 or 1.0 at 450nm in the ELISA assay. Serum samples at the predetermined dilution were added to the 96-well plates above and incubated for 2 hours. Following this ammonium thiocyanate of varying concentration (0.8M, 0.4M, 0.2M, 0.1M, 0.05M and 0.25M) was added to duplicate wells of each serum sample tested, with one set of duplicate wells having no ammonium thiocyanate added. After 15 minutes incubation the plate was washed and the ELISA assay method continued to the end from the addition of anti-human IgG conjugate to alkaline phosphatase. The avidity index of anti-PRP antibody in a serum sample was expressed as the millimolar concentration of thiocyanate required to reduce the optical density of the final ELISA reading by 50%.

**Statistical Analysis**

For each group of sera Hib anti-PRP antibody concentration and avidity index were log transformed and geometric means calculated together with 95% confidence intervals. For each group the proportion of individuals with concentrations above those considered necessary for both long and short term protection (1.0 mcg/ml and 0.15mcg/ml
respectively) was calculated. These groups were compared using the Chi squared test for trend. The effect of year of serum collection, concomitant serogroup C meningococcal conjugate vaccine and age on the logarithmically transformed anti-PRP antibody concentration and avidity index was investigated independently by simple linear regression analysis.
**Results**

**Anti-PRP antibody concentrations at 3 - 5 years of age**

The reverse cumulative distribution of anti-PRP antibody concentrations (figure 1) demonstrates that there was a reduction in the proportion of individuals with anti-PRP concentrations greater than 0.15 mcg/ml during the period 1994 - 2003 (Chi squared test for trend, \( p < 0.02 \)). However there was no significant trend for the proportion with concentrations greater than 1.0 mcg/ml across the same period (Chi squared test for trend, \( p > 0.2 \)). Geometric mean anti-PRP concentrations were highest in the earliest periods of serum collection (figure 2). Linear regression analysis of anti-PRP antibody concentration against the variables of age, sex, avidity index, concomitant MenC immunization and year of serum collection (table 2) demonstrated a significant effect for year of serum collection (\( p = 0.03 \)), concomitant MenC vaccine (\( p=0.04 \)). There was a negative linear trend across the time period (\( p =0.003 \)). The direction of effect for concomitant MenC vaccine was for lower anti-PRP antibody concentrations.

**Anti-PRP avidity index at 3 - 5 years of age**

Geometric mean avidity index by year of serum collection is shown in figure 3. Linear regression analysis (table 2) demonstrated a significant effect of age on anti-PRP avidity (\( p=0.004 \)) but there was no significant linear trend (\( p = 0.66 \)) across the time period of the analysis.
The effect of mode of administration of PRP-T vaccine (whether separate injection site, reconstituted with DTwP or combination vaccine) could not be analysed by simple linear regression analysis as there was information from too few groups to allow this to be done independently of other factors (e.g. year of serum collection, concomitant MenC administration)
Discussion

In this study there is a trend for the proportion of immunised 3 – 5 year old children with anti-PRP antibody concentrations >0.15mcg/ml to be greater the earlier they were immunised in relation to the introduction of routine infant immunization. There was no consistent trend in anti-PRP antibody avidity index during the same study period.

In all groups of sera there was a relatively low proportion of children with anti-PRP levels above the threshold for long-term protection (1.0 mcg/ml). This has been well described previously in the UK population. The lack of large numbers of vaccine failures despite low population levels of anti-PRP antibody had been ascribed to the protection conferred by immunological memory(23). However subsequent analyses of effectiveness data have suggested the vulnerability conferred by low anti-PRP concentrations and together with studies of vaccine failures questioned the protection conferred by immunological memory(32).

There is only one previous study of anti-PRP sero-epidemiology in the UK covering the time period before and after the introduction of routine Hib conjugate infant immunization(41). In that study cross-sectional age-specific sera were used for each of five time periods between 1991 and 2003. Except for the effect of the ‘catch-up’ campaign there were no changes in anti-PRP concentration for any age-group. However details of immunisations were not available for these children and in addition that study did not assess any markers of immunological memory. Other authors have noted an apparent decline in the immunogenicity of Hib conjugate
vaccines coincident with the widespread use of infant Hib immunization and some
have speculated on the role of declining Hib carriage rates(20, 37).

Changes in population levels of anti-PRP antibody after the introduction of routine
Hib immunization may be due to:

a) reduction in ‘natural boosting’ due to Hib carriage or organisms with
cross-reactive antigens.

b) changes in the way in which the Hib component of immunization was
given (separately, reconstituted with DTwP-IPV or given as a combination
vaccine).

c) the use of concomitant serogroup C meningococcal conjugate vaccine.

The effect of Hib carriage

In the pre-vaccine era anti-PRP antibody concentration fell in the first few months of
life as maternally acquired antibody was lost(3, 21). The nadir of anti-PRP antibody
coincided with the peak of invasive Hib disease in early childhood. The subsequent
rise in anti-PRP antibody concentration was attributed to the immune response to
nasopharyngeal carriage of Hib or exposure to cross reactive organisms such as E.coli
K-100 (7, 36). This phenomenon of carriage inducing antibody has been termed
‘natural boosting’. A prominent feature of all glyco-conjugate vaccines against the
common encapsulated bacterial pathogens of childhood is that they significantly
reduce nasopharyngeal carriage in an immunized population(8). The advantage of this
reduction in carriage is the resulting herd immunity whereby there is a decrease in
disease even in unvaccinated individuals and enhanced protection of those who have
been vaccinated (21, 25, 35, 38). However as well as generating herd immunity a
decrease in carriage will also reduce the opportunities for ‘natural boosting’ and may
result in lower levels of persistent antibody than would have otherwise been the case. In Oxfordshire carriage rates in young children declined from 3.98% in an unvaccinated population, prior to 1992, to 0% by 1997 and 2000 (31). The serum samples in this study span this period and the observed reduction in proportion of individuals with protective anti-PRP antibody concentrations is consistent with a reduction in natural boosting. There are already some data from the UK demonstrating that anti-PRP antibody concentrations in unvaccinated adults decline with decreasing rates of carriage and that this is associated with an increase in rates of invasive disease(33).

**Method of administration of Hib vaccine**

The reconstitution of PRP-T with DTP prior to administration was given official support by the Department of Health in early 1999(1) but was also used prior to this. Reconstituting Hib glyco-conjugate vaccines with DTwP can result in lower anti-PRP concentrations immediately following infant immunization as compared to the vaccines being administered concurrently at different sites(4, 17, 18, 24). However, other studies have found no significant difference between groups receiving mixed or separate vaccines(5, 9, 34, 39). Almost all of the comparative studies relate to anti-PRP concentrations at 1 month after primary immunization and there is little information on whether differences in immunogenicity are reflected in subsequent antibody persistence.

Hib-DTwP combination vaccines were introduced into the UK from 1996 onwards and this was associated with a decline in the use of separate Hib vaccine (figure 4).
Information is limited regarding the relative immunogenicity of Hib combination vaccines compared to other modes of administration. A pre-licensure study had shown comparable immunogenicity for a DTwP-PRP-T combination vaccine in comparison to a PRP-T vaccine reconstituted with DTwP immediately prior to immunisation(2). However the data comparing combination vaccines to vaccines given separately relates to HibOC (a CRM\textsubscript{197} conjugated oligosaccharide Hib conjugate vaccine). Two studies demonstrated a lower anti-PRP response for the HibOC vaccines given separately as opposed to in combination form. Once again it is not clear whether differences in initial immunogenicity are reflected in subsequent long-term antibody persistence. There is little data allowing direct comparison of antibody persistence between Hib conjugate vaccines given as part of a combination vaccines as opposed to separately.

In the current study there were too few groups with complete information on method of administration of Hib vaccine to allow an analysis to be undertaken independently of other factors (such as study period and concomitant MenC administration). This was due to the fact that the only group who had Hib vaccine confirmed as given separately also had no concomitant MenC vaccine and was the earliest group in relation to the introduction of routine Hib immunization in the UK.

**Concomitant serogroup C meningococcal glyco-conjugate vaccine**

Interactions may occur between concomitantly administered protein-polysaccharide vaccines. The administration of a CRM\textsubscript{197} conjugated 9-valent pneumococcal polysaccharide-MenC combination vaccine (PnC9-MenC) resulted in reduced MenC
bactericidal titres compared to the MenC component given alone (12). This has been attributed to the phenomenon of carrier induced epitope suppression whereby a limited amount of T-cell help is divided between increasing numbers of antigens and may thereby reduce the magnitude of response to an individual antigen. A similar effect has also been seen for vaccines with similar carrier proteins given at different sites. A study of a tetravalent pneumococcal polysaccharide tetanus toxoid conjugate given at a separate site to the PRP-T vaccine showed reduced anti-PRP concentrations compared to the vaccine given alone (14). In addition non-carrier specific suppression was seen in the PnC9-MenC study reduction of anti-PRP responses to a PRP-T Hib conjugate vaccine when given with the PnC9-MenC conjugate despite not sharing carrier proteins (12). These effects are not easily predictable. In a study involving DTwP-PRP-T given at a separate site to a MenC vaccine conjugated to either CRM$_{197}$ or tetanus toxoid a better anti-PRP response was seen with the tetanus toxoid conjugate (27). A recent review of the effect of concomitant MenC conjugates on the responses to Hib conjugate vaccines, including previously unpublished trial data, indicated that there was a trend to lower anti-PRP antibodies in children given concomitant CRM$_{197}$ conjugated MenC vaccines (15). However the conclusions of the review were drawn from comparisons between studies rather than direct comparisons within a trial. Most of the information on the interference of conjugate vaccines relates to immediate immunogenicity and there is little information on the persistence of antibody concentration.

In groups C and E infant immunization included a CRM$_{197}$ conjugated serogroup C meningococcal polysaccharide vaccine. At 3-5 years of age there was an association of borderline significance between concomitant administration of MenC conjugate
vaccine and lower anti-PRP antibody concentrations. However within study C individuals had been randomized to receive MenC or Hepatitis B vaccine as part of the primary immunization schedule and there was no between group difference in anti-PRP antibody concentrations at three years of age (data not shown). Hence in the current study the evidence is weak that concomitant MenC administration was an important contributor to lower anti-PRP antibody concentrations in the current study.

**Anti-PRP antibody avidity**

Antibody produced following administration of Hib conjugate vaccine undergoes a gradual increase in avidity in the weeks following primary immunization in infancy(19). The increase in antibody avidity occurs due to the selection of B-cells with higher affinity for antigen in germinal centres and avidity has been proposed as a marker of successful induction of immunological memory(19). The avidity of anti-PRP antibody varies with the particular Hib conjugate vaccine used. Such variation has been linked to the function of the antibody, higher levels of avidity corresponding to more functional antibody(40). Hib vaccine failures in the UK occurred despite evidence of immunological memory amongst individuals with vaccine failure(32). However this leaves open the question of whether there were differences in some aspect of immunological memory between vaccine failures and children who remained protected. Immunological studies of a small number of Hib vaccine failures from Holland measured a lower avidity in vaccine failures as opposed to controls who did not have invasive Hib disease(11). This is consistent with work on UK vaccine failures(29). Unlike anti-PRP antibody concentration, there are no
recognized correlates of protection for anti-PRP antibody avidity index, but it has been presumed that a higher avidity is associated with more effective priming(19).

In the current study there was not a significant effect of year of serum collection on anti-PRP avidity index and there was no consistent trend across the time period. Although there was a significant relationship of increasing anti-PRP avidity index with age and the median ages of the groups analysed varied (range of medians 3.2 – 4.5 years) the relationship was relatively weak ($R^2 = 0.055$). There is thus no evidence of a systematic change in avidity, as an additional marker of antibody function and immunological memory, which could explain increasing numbers of vaccine failures.

**Limitations**

In this study a relatively restricted number of groups of serum samples were available in relation to the number of variables that might have affected the anti-PRP concentration and avidity index (e.g. Hib carriage, age mode of administration of Hib vaccine, concomitant MenC vaccine). This limited the power to determine which of these elements were independently responsible for the decline in antibody concentration observed. Following a 2, 3, 4 month schedule of infant immunization with Hib conjugate vaccine, and in the absence of further boosting, an initially peak anti-PRP antibody level declines quickly over the following months(23). UK data indicates that population values for anti-PRP concentrations reach a constant level between 2 – 5 years before gradually increasing again thereafter(41). The sera that are the subject of the current study are from individuals between 3 – 5 years where, in the
absence of other factors, one would expect relatively constant anti-PRP antibody concentrations and thus age is unlikely to have confounded the analysis of anti-PRP concentrations. A further consideration for this study is that serum was obtained from individuals recruited to previous studies rather than being randomly selected from the general population and may thus not be truly representative of trends within the population. However, all the children were recruited from the general population and they were thus exposed to the same opportunities for ‘natural boosting’ through Hib carriage as that population. Furthermore the trend to decreasing use of immunization with separate Hib vaccine seen within the groups studied is reflected in the changes in practice within the wider population.

Conclusions

A reduction in the persistence of anti-PRP antibody concentrations after the introduction of routine Hib immunization in the UK may have been a significant factor in the epidemiology of Hib vaccine failures prior to 1999(42). Despite a decrease in anti-PRP concentrations between successive cohorts of children studied there was no change in a marker of immunological memory, anti-PRP avidity index. Factors that are likely to have influenced anti-PRP antibody concentrations in the children in the current study, such as a reduction in the rate of nasopharyngeal carriage of Hib, may also have exaggerated any decrease in antibody persistence that arose from the subsequent use of aP/Hib combination vaccines and ultimately precipitated an increase in vaccine failures from 1999 onwards.
The accretion of a number of individually small differences in immunogenicity may be relatively un-noticed when licensing is undertaken on comparisons of immunogenicity with currently licensed vaccines rather than efficacy studies. Efficacy studies are impractical in vaccinated populations and it is therefore important to maintain good quality surveillance over a period of many years following the introduction of a vaccine, in order to detect any changes in effectiveness.

These findings are of significance for all protein-polysaccharide conjugate vaccines for which carriage may be an important mechanism for maintaining protective immunity and where there is the potential for interactions with other vaccines.

Acknowledgements

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Conflict of interest statement: AJP has conducted clinical trials on behalf of Oxford University, sponsored vaccine manufacturers and has received assistance from vaccine manufacturers to attend scientific meetings. Industry-sourced honoraria for lecturing or writing are paid directly to an independent charity or an educational/administrative fund held by the Department of Paediatrics, University of Oxford. ERM has received assistance from vaccine manufacturers to attend scientific
meetings and serves on the Scientific Advisory Board of Novartis Vaccines and Diagnostics. DFK and LMY have no conflicts of interest.

References


**Figure 1** Reverse cumulative distribution of anti-PRP antibody concentrations (mcg/ml) for groups of children whose sera was collected between 1994 and 2003. The two vertical lines indicate the cut-off for the titers associated with short term (>0.15 mcg/ml) and long term (>1.0 mcg/ml) protection from invasive Hib disease.
Figure 2 Geometric mean anti-PRP antibody concentration (mcg/ml) with 95% confidence intervals for children aged 3-5 years immunized with a 2, 3, 4 months primary schedule in studies conducted between 1995 and 2003. Studies are identified by year of serum collection and group letter (see Table 1).
**Figure 3** Geometric mean anti-PRP antibody avidity index (Mol of thiocyanate) together with 95% confidence interval by year of serum collection, for children aged 3 – 5 years immunized with a 2, 3, 4 month primary schedule, from sera collected in studies conducted between 1994 and 2002. Studies are identified by year of serum collection and group letter (see Table 1).
Figure 4 Charts of the single Hib and Hib DTwP combination vaccines issued by the Department of Health over the period 1993/4 – 2000/01. (Data from Department of Health, Vaccine Issues)
<table>
<thead>
<tr>
<th>Group</th>
<th>Year of primary immunisation</th>
<th>Year of serum collection</th>
<th>Number of sera</th>
<th>Median Age in Years (range)</th>
<th>PRP-T (Hib) vaccination</th>
<th>Serogroup C meningococcal vaccine in primary schedule</th>
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<tr>
<td>A</td>
<td>1991</td>
<td>1994</td>
<td>87</td>
<td>3.6 (3.5-3.7)</td>
<td>Separate</td>
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</tr>
<tr>
<td>B</td>
<td>1995-1996</td>
<td>2000</td>
<td>79</td>
<td>4.5 (4.0-5.2)</td>
<td>NA</td>
<td>No</td>
</tr>
<tr>
<td>C</td>
<td>1995-1996</td>
<td>2000</td>
<td>64</td>
<td>4.5 (4.1-4.9)</td>
<td>Reconstituted with DTwP</td>
<td>Mixed</td>
</tr>
<tr>
<td>D</td>
<td>1997-1998</td>
<td>2002</td>
<td>72</td>
<td>4.0 (3.6-5.0)</td>
<td>Combination vaccine†</td>
<td>No</td>
</tr>
<tr>
<td>E</td>
<td>1999-2000</td>
<td>2003</td>
<td>42</td>
<td>3.2 (3.0-3.4)</td>
<td>NA</td>
<td>Yes</td>
</tr>
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</table>

**Table 1** Characteristics of the groups of 3 – 5 year old children whose stored sera were used in the study of anti-PRP antibody concentration and avidity. The ‘Group’ designation (A-E) is used to refer to individual groups of children in the text. ‘PRP-T (Hib) vaccination’ refers to whether the Hib vaccine was given at a separate site to other vaccines or was reconstituted with DTwP vaccine prior to giving at the same site or was in the form of a combination vaccine. NA = no confirmation available regarding the mode of administration of PRP-T vaccine. † = 2 children given separate PRP-T vaccine and no confirmation of the mode of administration of PRP-T vaccine for 8 children.
<table>
<thead>
<tr>
<th>CHILDREN AGED 3-5 YEARS</th>
<th>Log anti-PRP antibody</th>
<th>Log anti-PRP avidity index</th>
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<tr>
<td>Log anti-PRP antibody</td>
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<td>Year of serum collection</td>
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<td>Linear trend</td>
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<tr>
<td>Concomitant MenC</td>
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<td>0.09</td>
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</table>

Table 2 Linear regression analysis of factors likely to influence anti-PRP antibody concentrations and avidity index at 3-5 years of age. Values in the table are P values and asterisks indicate those that are significant at P <0.05 level. NA = not applicable.