Risk of invasive *Haemophilus influenzae* type b (Hib) disease in adults with secondary immunodeficiency in the post-Hib vaccine era

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Abstract

Prior to the introduction of *Haemophilus influenzae* type b (Hib) conjugate vaccines, invasive Hib disease affected almost exclusively children. According to some recent studies, in the post-vaccine era, adults, the elderly and immunocompromised persons can be affected more often than children. As the production of type-specific anti-capsular polysaccharide antibodies is the major defense mechanism against Hib, individuals with defects in humoral immune responses have a high susceptibility to infections caused by Hib. We hypothesized that non-vaccinated adults with chronic conditions causing immunosuppression may lack protective antibody to Hib. We assessed serum anti-Hib IgG levels and bactericidal activity in 59 patients with chronic renal failure, 30 patients with type 2 diabetes mellitus, 28 patients with chronic obstructive pulmonary disease (COPD), and 20 patients with multiple myeloma, compared to 32 healthy controls of similar age. Considering antibody >0.15 µg/ml as the protective correlate in unvaccinated individuals, we detected below-protective Hib antibody levels in 29% of chronic renal failure, 20% of diabetes, 14% of COPD, and 55% of myeloma patients, compared to 3% of healthy controls. Additionally, 70% of myeloma and 58% of chronic renal failure patients did not have detectable serum bactericidal activity against Hib. Among individuals with severe diseases causing secondary immunodeficiency, patients with multiple myeloma and chronic renal failure are at an increased risk of invasive Hib disease. Considering that Hib continues to circulate in the population, this study provides rationale for the immunization of some adult patients with secondary immunodeficiency with the pediatric Hib vaccine to achieve protective immunity.

Keywords

*Haemophilus influenzae* type b, Secondary immunodeficiency, Antibody, Serum bactericidal activity
Introduction

*Haemophilus influenzae* is a common Gram-negative human-restricted bacterial pathogen, which frequently colonizes the nasopharynx of healthy individuals and can cause local infections, such as otitis media, sinusitis, pneumonia, or exacerbations of COPD. When the bacteria breach the epithelial barriers, they are able to cause invasive disease, including meningitis, sepsis, and epiglottitis (28, 29). Most invasive infections are caused by encapsulated strains, in particular *H. influenzae* type b (Hib) characterized by a polyribosylribitol phosphate (PRP) capsule, which is an important virulence factor. In immune individuals, circulating anti-PRP antibodies effectively protect against the disease by activating the classical complement pathway, as well as opsonizing bacteria for phagocytosis (41).

Prior to the introduction of vaccination against Hib, this pathogen was the major cause of bacterial meningitis in children (43). A dramatic decrease in the incidence of invasive Hib disease has rapidly followed the introduction of Hib-protein conjugated vaccines in Western countries since the beginning of the 1990s (48). In Canada, a conjugate Hib vaccine became first available in 1988 for children over 18 months of age; the routine vaccination of infants beginning at 2 months of age with the current vaccine (PRP conjugated to tetanus toxoid) started in 1992 (2). In the post-Hib vaccine era, invasive Hib disease affects adults, especially the elderly and immunocompromised individuals, more often than children (13, 35, 39, 44). As adults born before the 1990s have not been vaccinated, their natural immunity may be insufficient to prevent invasive disease if they have an immune defect. In addition, low Hib circulation rates due to the vaccine’s “herd effect” may account for reduced maintenance of natural anti-Hib immunity in non-vaccinated populations (15). Severe cases of invasive Hib disease affecting adults have been reported (7, 40). As Hib continues to circulate in countries with high pediatric Hib vaccine coverage (32), the public health guidelines recommend adult vaccination for some high-risk groups (anatomical or functional asplenia, congenital antibody or complement deficiency, etc.) (1).

In modern Western society, the number of adults with secondary immunodeficiency states resulting from aging, severe chronic diseases, or an immunosuppressive therapy is increasing. Such individuals are not routinely immunized against Hib and it is unclear whether they may be at risk of developing invasive Hib disease if exposed to the pathogen. To address this question, we
studied a group of patients with common clinical conditions known to lead to immunosuppression. Because circulating antibodies to Hib capsular polysaccharide are the major defense mechanism against invasive Hib disease, we studied the antibody levels and functional activity as an indicator of protection.

Methods

Patient population

We recruited 59 patients with chronic renal failure, 30 patients with type 2 diabetes mellitus, 28 patients with COPD, 20 patients with multiple myeloma, and 32 age-matched healthy controls. All patients with chronic renal failure were undergoing hemodialysis at the Renal Services, Thunder Bay Regional Health Sciences Centre (TBRHSC). The COPD patients were recruited at a time when they did not have disease exacerbation and were undergoing the outpatient respiratory rehabilitation program at St. Joseph’s Care Group (Thunder Bay). Patients with diabetes and multiple myeloma were attending the outpatient clinics in Thunder Bay and Sault Ste. Marie, Ontario (Algoma District Cancer Program), respectively. All patients undergoing hemodialysis at the Renal Services, or attending the involved physician’s offices at the time of the study (May-August 2009) were invited to participate, and those who were able to give the informed consent, were included. Most of the multiple myeloma patients were on intermittent chemotherapy (pulsed therapy given every three weeks), COPD patients received inhaled corticosteroids, but not oral corticosteroids; the remaining patients did not receive immunosuppressive therapies at the time of the study. None of the study participants have been immunized against Hib. Clinical data of all the patients have been analyzed with respect to duration of disease, co-morbidities, and history of infections. Demographic characteristics of the studied groups are shown in Table 1. No statistically significant differences in age between any patient group and controls were present. Serum samples were obtained from the participants under informed consent and stored at -80°C prior to use. This study was approved by Research Ethics Boards of all involved institutions.

Anti-Hib ELISA

Serum anti-PRP IgG antibody concentrations were determined by using a VaccZyme™ Human Anti Haemophilus influenzae type b ELISA kit (The Binding Site, Birmingham, UK) according to the manufacturer’s protocol. Briefly, serum samples diluted 1:100 were added in duplicate to
microwells pre-coated with PRP conjugated to human serum albumin. The reaction was developed
using peroxidase-conjugated anti-human IgG secondary antibody; the bound IgG was detected
using a colorimetric substrate read at 450 nm with an automated microplate reader (BioTek
Powerwave XS, Vermont). Concentrations of anti-PRP IgG antibody were determined using
standard calibrators supplied with the kit and expressed in µg/ml. The lower and upper limits of
detection were 0.11 and 9 µg/ml, respectively. For statistical analysis, antibody concentrations
below the lower limit of quantitation were assigned half the lower limit of detection, i.e. 0.055
µg/ml. Samples with higher than 9 µg/ml antibody levels were further diluted and re-run to obtain
accurate results. Following dilution, the upper limit of detection was 90 µg/ml.

**Serum Bactericidal Assay**

For this assay, we used a Hib strain isolated from the cerebrospinal fluid of an infant with
meningitis, kindly provided by Dr. R.S.W. Tsang. Bacteria were grown on brain heart infusion
agar supplemented with 10 µg/ml hemin chloride (factor X) and 1 µg/ml nicotinamide adenine
dinucleotide (factor V) in a humidified incubator at 37°C and 5% CO2 overnight. The serum
bactericidal assay (SBA) was essentially performed as previously described (11, 37). Briefly, Hib
was cultured, harvested and diluted to a concentration of approximately 10^4 CFU/ml in SBA buffer
which consisted of Hank’s buffered salt solution supplemented with 10 µg/ml factor X and 1
µg/ml factor V. Serum samples were incubated in a water bath at 56°C for 30 minutes to inactivate
complement and serially diluted two-fold eleven times beginning at a ratio of 1:8. Ten microlitres
of each serum dilution were mixed with 20 µl of bacterial suspension and incubated for 10 minutes
at 37°C and 5% CO2. Next, 10 µl of baby rabbit complement (Pel-Freeze, AR, USA) and 40 µl of
SBA buffer were added to the mixture and incubated for 1 hour at 37°C and 5% CO2. The viable
bacteria were determined by drop plating following overnight incubation at 37°C and 5% CO2.
The SBA titre was defined as the reciprocal of the highest dilution able to kill >50% of bacteria
compared to a negative serum control, which contained SBA buffer in place of serum. Titres
below the low detection limit (of 8) were reported as 4 for statistical analysis. As a positive control
we used anti-Hib reference serum 96/536 (National Institute for Biological Standards and Control,
Potters Bar, UK). For quality control purposes, a post immunization serum of the same healthy
adult control was included in each experiment and consistently yielded a SBA titre of 1024 ± 1
dilution.
Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The sample size was calculated based on 20% difference in means and 30% coefficient of variation (two-sided alternatives) with 80% statistical power and 5% significance (49). Geometric mean antibody concentrations (GMC), SBA geometric mean titres (GMT), and 95% confidence intervals (CI) were calculated for each group. Serum antibody levels and SBA results were compared between patients and controls using Mann-Whitney rank sum test and Student’s t-test as appropriate. Analysis of variables was performed using Pearson’s $\chi^2$ test. Associations were assessed by linear regression analysis or Spearman’s correlation. P values of $<$0.05 were considered significant.

Results

To assess naturally acquired immunity against Hib, we studied IgG anti-PRP antibody levels in sera of unvaccinated adult individuals. Among healthy controls, 97% had anti-PRP IgG levels $>$0.15 $\mu$g/ml, which has been considered as an immunological correlate of natural protection against invasive Hib disease in unvaccinated individuals (4, 20, 34), in comparison to 71% of chronic renal failure, 80% of diabetes, 86% of COPD and 45% of myeloma patients (Table 2). The relative risk of having anti-PRP IgG antibody levels below 0.15 $\mu$g/ml was significantly higher in all the patient groups compared with healthy controls with the exception of COPD (p=0.059) (risk ratio ranging from 6.4 in diabetes to 17.6 in myeloma patients) (Table 3).

Among all patients, the multiple myeloma group had the lowest GMC of IgG antibody, i.e. of 0.22 $\mu$g/ml (95%CI 0.09-0.45) that was significantly lower than in healthy controls (1.50 $\mu$g/ml, 95%CI 1.06-2.13, p=0.0001), followed by chronic renal failure (0.56 $\mu$g/ml, 0.34-0.90, p=0.02). In one myeloma and two chronic renal failure patients, high anti-PRP antibody levels were detected ($>$10 $\mu$g/ml), potentially reflecting a recent exposure to the pathogen (Figure 1a). In patients with diabetes mellitus and COPD, the antibody levels did not significantly differ from controls, i.e. 0.93 $\mu$g/ml (0.50-1.75), p=0.4 and 1.12 $\mu$g/ml (0.56-2.23), p=0.5, respectively (Table 2, Figure 1a).

A decline in antibody levels against bacterial polysaccharide antigens may occur with aging. However, we observed a significant negative correlation of antibody level with age in ≥60 year-old
controls ($r=-0.45$, $p=0.03$), but not in patients ($r=0.023$, $p=0.42$) (Figure 2) suggesting that a lack of naturally acquired antibody in severely ill individuals is attributed to their general immunosuppression, rather than to an advanced age. No differences in antibody levels between male and female in any group were detected (data not shown). Further analysis indicated that IgG anti-PRP antibody levels did not depend on the length of dialysis or co-morbidities (in chronic renal failure), or number and severity of infectious episodes (in all patient cohorts) (data not shown).

Antibody detected by ELISA may have different functional capabilities due to their specific chemical and genetic characteristics. To assess the functional activity of the anti-PRP antibody we employed a serum bactericidal assay, which measures the killing of bacteria by anti-Hib specific antibody mediated by the complement activation (37). Among healthy controls, 56% of serum samples were able to kill > 50% of Hib in the presence of baby rabbit complement, in contrast to only 30% of sera from myeloma patients ($p=0.032$) (Table 2). The relative risk of the lack of bactericidal antibody against Hib in myeloma patients was 1.6 ($p=0.032$). The SBA GMT was significantly lower in myeloma patients compared to the controls, i.e. 12.55 (5.2-30.33) versus 36.44 (16.31-81.44), respectively ($p=0.048$). As well, patients with chronic renal failure had lower SBA GMT compared to controls, i.e. 20.72 (12.22-35.14), $p=0.029$.

However, no statistically significant differences in SBA GMT were found among the diabetes mellitus, COPD, and control group (Table 2, Figure 1b). No correlation was detected between the SBA titres and IgG anti-PRP antibody concentrations measured by ELISA for healthy controls ($r=-0.3$, $p=0.05$) or patients ($r=0.05$, $p=0.56$) (Figure 3). Because aging is associated with decreased functional antibody activity (14), we compared SBA titers between older and younger individuals. No significant differences in SBA GMT between individuals <60 and $\geq$60 years of age among healthy controls or patient groups were detected (data not shown).

Hence, the results of our study have demonstrated that among individuals with severe chronic conditions causing secondary immunodeficiency, patients with multiple myeloma and chronic renal failure show both decreased IgG anti-PRP antibody levels and a defect in antibody functional capabilities; however, no association of antibody deficiency with age was found.
Discussion

In 97% of healthy unvaccinated adults, circulating IgG antibodies against Hib capsular polysaccharide were above the level ensuring long-term protection against invasive Hib disease, i.e. 0.15 μg/ml (4); in 56% of them, functionally active serum antibodies were detectable. Moreover, 81% of healthy individuals had antibody levels ≥1 μg/ml, a correlate of protection in the vaccinated population (20). These data suggest that the general adult population is well protected against invasive Hib disease, which is indeed extremely rare in healthy adults (46). Because none of the subjects has been vaccinated against Hib, natural anti-Hib antibodies may have been induced by the exposure to some common environmental bacteria, which carry antigens cross-reacting with PRP, such as Escherichia coli K100 (16).

In compliance with earlier studies, we considered serum IgG anti-PRP levels as the major indicator of protection against Hib invasive disease (20). However, multiple factors may contribute to clinical protection against this infection, i.e. antibody affinity/avidity, IgG subclass distribution, GM allotype, or idiotype. A specific idiotype (Hibld-1, a marker of the VκII-A2 chain) has been identified as the prevalent idiotype in the post-vaccination adult anti-PRP antibody repertoire (23). According to our previous studies, unvaccinated adults may lack this idiotype despite high levels of natural anti-PRP, potentially induced by some cross-reactive antigens (47). In the present study, we did not see a correlation between anti-PRP IgG levels and serum bactericidal activity. A similar discordance between anti-capsular IgG levels and their functional activity had previously been documented in response to vaccination against Hib and N. meningitidis serogroup C (3, 25). These studies identified antibody avidity as an important source of variability in their functional activity (3). As results obtained from ELISA may be relatively independent of antibody avidity (3), this may explain lack of correlation between IgG antibody levels and SBA. In the case of natural antibody in unvaccinated individuals induced by cross-reactive antigens, the discordance between antibody levels and their functional activity may depend on the usage of different V genes rendering varying avidity. Discordance between IgG anti-PRP and SBA was also observed in unvaccinated Alaska adults and healthy elderly individuals (12, 22).

In contrast to healthy individuals, a proportion of our patients (ranging between 14% in COPD and 55% in myeloma) lacked protective anti-Hib antibody levels. Secondary immunodeficiency could
be due to multiple reasons, e.g. profound metabolic disturbances and malnutrition due to uremia as well as immunosuppressive effect of hemodialysis in chronic renal failure (10, 19), impaired respiratory function, chronic inflammation, and therapy with corticosteroids in COPD (9, 42), or metabolic disorders and associated obesity in type 2 diabetes mellitus (17). The lowest anti-Hib immunity was detected in myeloma patients likely caused by a decreased synthesis of normal immunoglobulins due to both malignant transformation of plasma cells and the immunosuppressive effect of chemotherapy (31). Multiple myeloma patients have a recognized high susceptibility to bacterial infections with common pathogens, such as S. pneumoniae and H. influenzae (31). However, an earlier study found that among 46 patients with multiple myeloma, pre-vaccination anti-PRP levels were comparable to that of the healthy UK adult population (36). A recent paper described that 80% of multiple myeloma patients had anti-Hib IgG > 0.15 μg/ml (18). In contrast, in our study, only 45% of myeloma patients had antibody levels above this cut-off. Such discrepancies may be due to different patient populations, progression/length of disease, or ELISA technique variability. Despite the fact that vaccination of multiple myeloma patients against Hib remains controversial (31), our findings suggest that the majority of such patients lack protective immunity against Hib and should be vaccinated. Chronic renal failure patients also have significantly decreased IgG anti-PRP levels as well as functional antibody activity and hence may have high susceptibility to Hib infection. Cases of peritonitis caused by Hib in chronic renal failure patients undergoing peritoneal dialysis have been reported (8, 30). However, to the best of our knowledge, no specific defects in the immunological defense against Hib have been previously identified in adults with chronic renal failure.

Humoral immune defects represent an important component of immunosenescence (6). Mechanisms behind B-cell defects in the elderly include reduced antibody diversity, defects in isotype switching and somatic mutation resulting in low-affinity antibody production, as well as deficiency in IgM memory B cells (33, 50). However, our data suggest that severe chronic diseases have a larger negative impact on natural immunity against Hib as compared with ageing as we found an age-associated decline in anti-PRP levels in older healthy adults, but not in patients. This implies that general immunosuppression may play a greater role than senescence in the observed lack of protective anti-Hib antibody levels. However, the correlate of protection against invasive Hib disease in older adults is unknown. Several studies indicate that antibody against bacterial
capsular polysaccharides present in the elderly may lack functional activity (22, 33). On the other hand, the existence of B memory cells, a result of previous exposure to Hib or cross-reactive bacteria, may contribute to protection against invasive disease in older individuals.

Although the antibody levels required to prevent nasopharyngeal carriage have not been accurately determined, it has been suggested that they may be as high as 5-10 µg/ml (21, 38). Considering that only 3% of healthy controls in our study have \( \geq 5 \) µg/ml of IgG anti-PRP, most of them are potential carriers. Although only a small fraction will be susceptible to developing Hib invasive disease, such individuals may nevertheless transfer the pathogen supporting Hib circulation within the population. According to recent studies, invasive Hib disease still exists in countries with high pediatric anti-Hib vaccination coverage and the disease affects adults more often than children (5, 24, 39, 44).

This study has several limitations. The multiple myeloma group was smaller than an estimated sample size because a limited number of patients was available at the time of our study. Also, the small sample size could potentially influence the analysis of the effect of age and sex. In general, the interpretation of immunological data with regard to clinical protection against Hib disease is complex. The analysis of humoral immunity does not account for the presence of immunological memory against Hib that can develop following natural exposure to whole bacteria expressing protein antigens along with PRP. Under these circumstances, the anti-polysaccharide immune response can acquire T-cell dependent properties that may potentially contribute to long-lived populations of memory B cells capable of developing a secondary immune response following repeated exposure to the pathogen. However, despite these limitations, our findings point to the lack of immunological protection in a substantial proportion of adults with severe chronic conditions causing secondary immunodeficiency, particularly in multiple myeloma and chronic renal failure patients.

**Conclusion**

Our study has demonstrated that in the era of universal pediatric immunization against Hib, healthy adult individuals typically have protective immunity against invasive Hib disease, but over
90% of them have the potential for pathogen carriage. In contrast, we have found a lack of protective immunity against Hib in adults suffering from multiple myeloma and chronic renal failure. Such individuals may be at risk of developing invasive Hib disease if exposed to the pathogen.

Considering that Hib continues to circulate in Western countries despite the high vaccine coverage of infants and that individuals born before the beginning of the 1990s have not been immunized, adult patients with multiple myeloma and chronic renal failure can benefit from immunization with a Hib conjugate vaccine. Previous studies have established that immunization of adults, including immunodeficient individuals, with pediatric Hib vaccines is safe and highly effective (22, 26, 27). Although it is critically important to maintain herd immunity via adequate and universal pediatric immunization, our findings along with recent data on emerging invasive Hib disease in unimmunized adults provide rationale for extended indications for immunization of vulnerable groups of adults against Hib (39, 45).

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## References


Table 1
Demographic characteristics of studied groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean Age (Median) ±SD</th>
<th>Age range</th>
<th>Female ≥60 years of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic renal failure</td>
<td>59</td>
<td>62.6 (63) ±13.5</td>
<td>29-91</td>
<td>23 (38%) 24 (41%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>30</td>
<td>60.5 (61) ±11.2</td>
<td>33-80</td>
<td>18 (60%) 16 (53%)</td>
</tr>
<tr>
<td>COPD</td>
<td>28</td>
<td>69.8 (72) ±8.8</td>
<td>45-81</td>
<td>19 (68%) 25 (89%)</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>20</td>
<td>68.7 (70) ±10.1</td>
<td>43-84</td>
<td>9 (45%) 16 (80%)</td>
</tr>
<tr>
<td>Controls</td>
<td>32</td>
<td>63 (61) ±8.2</td>
<td>53-80</td>
<td>19 (59%) 19 (59%)</td>
</tr>
</tbody>
</table>

SD: Standard deviation, COPD: Chronic obstructive pulmonary disease

a No statistically significant difference between any patient group and controls; P<0.05 between chronic renal failure and COPD; diabetes and COPD patients

Table 2
Serum antibody levels and bactericidal activity against Hib in patients with secondary immunodeficiency states and healthy controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Anti-PRP IgG</th>
<th>SBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GMC (95%CI)</td>
<td>GMT (95%CI)</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>0.56 *</td>
<td>20.72 *</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0.93</td>
<td>55.72</td>
</tr>
<tr>
<td>COPD</td>
<td>1.12</td>
<td>36.22</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>0.22 **</td>
<td>12.55 *</td>
</tr>
<tr>
<td>Controls</td>
<td>1.50</td>
<td>36.44</td>
</tr>
</tbody>
</table>

GMC: Geometric mean concentration (µg/ml), CI: Confidence interval, SBA: Serum bactericidal assay, GMT: Geometric mean titre, MDA: Minimum detectable activity, COPD: Chronic obstructive pulmonary disease, PRP: Polyrribosylribitol phosphate. * P < .05 and ** P < .01 compared with control group (see precise P values in the text).
Table 3

Relative risk of having anti-PRP IgG antibody levels below 0.15 µg/ml (minimum concentration required for protection against invasive disease) among patients with secondary immunodeficiency.

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative risk (95% CI)</th>
<th>P value (Pearson’s χ² test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic renal failure</td>
<td>9.22 (1.29-66.17)</td>
<td>0.002</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6.4 (0.82-50.12)</td>
<td>0.018</td>
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<tr>
<td>COPD</td>
<td>4.57 (0.54-38.56)</td>
<td>0.059</td>
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<tr>
<td>Multiple myeloma</td>
<td>17.6 (2.46-126.2)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

COPD: Chronic obstructive pulmonary disease, CI: Confidence interval, PRP: Polyribosylribitol phosphate.
Figure Legends

Fig. 1
(a) Distribution of individual anti-PRP IgG antibody levels in patients with secondary immunodeficiency and healthy controls (geometrical mean concentrations are indicated for each group). Dashed line indicates correlate of protection (0.15 μg/ml). (b) Antibody mediated bactericidal activity in patients and controls. Dashed line indicates the lower limit of detection. CRF: Chronic renal failure, DM: Diabetes mellitus, COPD: Chronic obstructive pulmonary disease, MM: Multiple myeloma, PRP: Polyribosylribitol phosphate. *p=0.029 for CRF and p=0.048 for MM compared to controls respectively. The number of individual samples with antibody levels or SBA scores below the lower limit of detection are indicated on each graph.

Fig. 2
Correlation of anti-PRP IgG antibody levels with age among ≥60 year-old individuals: (a) healthy controls, (b) all patient cohorts. PRP: Polyribosylribitol phosphate.

Fig. 3
Correlation of anti-PRP IgG antibody levels with serum bactericidal assay geometric mean titres: (a) healthy controls, (b) all patient cohorts. PRP: Polyribosylribitol phosphate; SBA: Serum bactericidal assay.