The utility of saliva for the assessment of anti-pneumococcal antibodies: investigation of saliva as a marker of antibody status in serum

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This is an Accepted Manuscript of an article published by Taylor & Francis Group in *Biomarkers* on 12 Dec 2016, available online: http://www.tandfonline.com/10.1080/1354750X.2016.1265009.

Abstract

Context: Salivary antibodies may act as non-invasive marker of systemic immunity enabling assessment of vaccination and protection against bacterial infections.

Objective: To assess if levels of anti-pneumococcal antibodies in saliva reflect concentrations in serum and determine whether saliva can accurately identify protective concentrations in serum.

Methods: IgG, IgA and IgM antibody levels in paired saliva and serum samples were measured against 12 pneumococcal polysaccharide antigens in 72 healthy adults.

Results: Antibody levels in saliva correlated positively with serum across immunoglobulin classes, most strongly for IgA. Individuals who had protective antibody levels in serum demonstrated significantly higher IgG and IgA salivary antibody concentrations/ secretion rates. Salivary IgG and IgA pneumococcal antibodies were able to distinguish between those with/ without protective levels in serum for the majority of serotypes. Salivary IgM antibodies were not able to differentiate protective status. Median IgG and IgA pneumococcal salivary parameters were able to identify individuals who had protective levels in serum on ≥ 8/12 serotypes with moderate accuracy: median IgA secretion rates provided the best sensitivity (73%) and specificity (71%).

Conclusions: These findings suggest that IgG and IgA pneumococcal specific antibodies in saliva may be useful surrogate markers of antibody status in serum.

Keywords: Antibody, serum, saliva, bacteria, pneumococcal, protection

Introduction

Bacterial infections are a major cause of mortality across the world and even in high income countries are one of the commonest causes of death. *Streptococcus pneumoniae* (*S pneumoniae*) is a colonising bacterium of the upper respiratory tract capable of causing invasive disease, such as pneumonia. *S pneumoniae* causes up to half of community acquired pneumonia (CAP) (Brown, 2012), one of the leading causes of death in older adults in the UK and USA (Stupka, et al., 2009, Trotter, et al., 2008). Bacterial infections due to *S pneumoniae* are mainly preventable through vaccination programmes, both within vaccinated individuals and the unvaccinated population as result of herd immunity.

Ideally, vaccination efficacy should be tested in a randomised, double-blind, placebo-controlled trial where the number of infections specific to the vaccine pathogen are evaluated between treatment and placebo arms. An example is the recent Community-Acquired Pneumonia Immunization Trial in Adults (CAPITA) that assessed the 13-valent polysaccharide conjugate vaccine (PCV13) against preventing first episodes of vaccine-type CAP (Bonten, et al., 2015). This trial included nearly 85,000 adults with a surveillance period spanning 5 years: trials such as these are extensive and accordingly expensive and not always viable. Consequently, serum antibody levels have become an accepted surrogate marker of vaccine efficacy and also a method of determining if an individual is protected against certain pathogens. Pneumococcal serotype protective cut-offs were derived from a trial involving the 7-valent conjugate vaccine in infants (Black, et al., 2000). Protective efficacy of the vaccine was 97.3% and > 97% of infants achieved > 0.15 µg/mL; from this a non-specific serotype threshold of 0.20 µg/mL was allocated (Balmer, et al., 2007, Jodar, et al., 2003). Subsequently, the WHO (2005) proposed a threshold of 0.35 µg/mL through associating ELISA data with clinical efficacy against invasive disease.

There are a number of practical considerations for acquiring blood to assess specific antibodies in serum. These may be most pertinent for children and in poor resource settings, where risk of bacterial infection is often highest (Flanagan, et al., 2010). A non-invasive method of assessing protection against bacterial disease and vaccination responses could be particularly beneficial in countries introducing new vaccination programmes/aiming to increase coverage. Saliva is an attractive method of specimen collection, particularly for children and the elderly, field research, or where repeated measures are required. Saliva requires small sample volumes and is not considered a class II biohazard (US Centre for Disease Control), further collection requires no specialist training or equipment and may be more cost-effective (Mittal, et al., 2011).

In infants, the 7-valent pneumococcal polysaccharide CRM197 protein conjugate vaccine (7VPnC) has been shown to induce salivary IgG and IgA antibody responses, but only following a booster vaccination (Choo, et al., 2000, Nurkka, et al., 2001). In addition, infants vaccinated with 7VPnC still had significantly higher IgG and IgA pneumococcal salivary antibodies at 20–22 months post-vaccination compared with unvaccinated controls (Rodenburg, et al., 2012). In these studies, IgG pneumococcal antibodies in saliva correlated with antibody levels in serum (Choo, et al., 2000, Nurkka, et al., 2001, Rodenburg, et al., 2012). These findings in infants suggest vaccination may confer anti-bacterial antibodies in saliva and that salivary antibodies may correlate well with systemic levels. However, few studies have investigated salivary antibodies in adults.

Saliva testing for specific antibodies has been advocated as a potential method of assessing systemic immunity and to monitor vaccination (Chiappin, et al., 2007, Rodenburg, et al., 2012), although the utility of saliva for these purposes has yet to be determined. In addition, when multiple vaccine strains are assessed, such as in the case of pneumococcal vaccine, certain serotypes may be indicative of overall levels of

protection thus removing the need to assess all analytes. The aims of the present study were to: assess to what extent levels of pneumococcal antibodies in saliva reflect concentrations in serum; determine whether antibody levels in saliva can accurately identify protective concentrations in serum; evaluate the relationship between different pneumococcal antibody serotypes in both saliva and serum.

Materials and methods

Participants

Participants were 72 adults (34 females), aged 21–80 (median 51 years, SD ± 21 years) from Birmingham, UK. All participants gave written informed consent prior to the study, which had the appropriate Ethics Committee approval. All participants reported having good overall health, including oral health with no known teeth or gum problems. None suffered from any acute illness in the two weeks prior to, or during the study.

Serum and saliva collection

Participants were asked to refrain from exercise and alcohol 24 h prior, and food and caffeine 12 h prior to arriving at the laboratory between 7–9am. A 6mL venous blood sample was collected from an ante-cubital vein and an unstimulated whole-saliva sample was collected over 4 min into tubes weighed pre- and post-collection to determine saliva volume. Samples were centrifuged and the separated serum and saliva supernatant were stored at –20°C until assay.

Salivary and serum antibody measurements

Saliva and serum were analysed using a multi-plex assay (Luminex-200, Bio-plex systems, BioRad Laboratories, California, USA) that simultaneously measured antibody concentrations against 12 pneumococcal (Pn) antigens (serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F). This assay has been described in detail previously (Heaney, et

al., 2015, Whitelegg, et al., 2012). Serum and saliva samples were assayed three times each to measure IgG, IgM and IgA antibody classes.

Data Analysis

For saliva, the following parameters were assessed: antibody concentrations, salivary flow rate (volume/collection time) and antibody secretion rate (flow rate x antibody concentration). Saliva secretion rates are reported to reflect the availability of immunoglobulin at the oral surface and to control for hydration status (Oliver, et al., 2007). As data were not normally distributed, Spearman's rank correlation was used to assess relationships between salivary antibody concentrations/secretion rates and serum antibody concentrations for each serotype for IgG, IgA and IgM, respectively. Correlation coefficients were classed as weak (.1–.39), moderate (.4–.6) or strong (≥.6). To examine if salivary IgA could be used as a proxy marker for IgG in serum - the dominant antibody classes in each of the specimens - the relationship between IgG serum and IgA saliva Pn antibodies were directly assessed. Correlations were also used to evaluate relationships between Pn serotypes within serum and saliva.

For IgG protective levels in serum, the following cut-offs were applied: Pn antibody serotypes, 0.35 μg/mL for protection (WHO, 2005) (minimum protective level) and 1.3 μg/mL for the prevention of colonization and infection (Bonilla, et al., 2005) (optimal protective level). Mann Whitney U tests were used to compare saliva IgG, IgM and IgA antibody concentration and secretion rates between individuals who did/did not exhibit protective levels in serum for Pn serotypes (≥ 0.35 μg/mL vs < 0.35 μg/mL).

Receiver operating characteristic (ROC) curves where generated to assess if salivary antibody levels can identify protective levels in serum. When significant results were observed, accuracy was classified using area under the curve (AUC) with the following values: > .7 (moderate), .8–9 (good) or > .9 (excellent). Salivary antibody concentration

and secretion rates of IgG, IgM and IgA antibodies specific for Pn serotypes were explored in relation to minimum protective levels in serum (≥ 0.35 μg/mL). Composite saliva parameters were then created to provide an overall representation of Pn antibody levels in saliva: median concentration and median secretion rates for all Pn serotypes combined. These composite measures were examined in relation to protective status, classified as minimum protective levels (≥ 0.35 μg/mL) in serum for ≥ 8/12 Pn serotypes, using ROC curves. This serum variable, protection for 2/3rds of serotypes, was selected as it represents a normal adult response to the 23-valent polysaccharide pneumococcal vaccine (PPV23; Pneumovax® 23). The ability of composite saliva measures to identify optimal protective levels (≥ 1.30 μg/mL) was also assessed using ROC curves. For ROC analyses in relation to composite saliva parameters, the best salivary cut-offs to discriminate between protective status in serum were identified using the points on curve closest to the (0, 1); distance was calculated for each observed cut-off point from the curve (distance = $\sqrt{[(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2]}$ and the point where the distance was minimum was selected. All analyses were conducted using IBM SPSS statistics version 21.

Results

Relationship between serum and saliva

Figure 1 illustrates the relationship between IgG, IgM and IgA anti-Pn antibodies in serum and saliva. In general higher antibody concentrations in serum were associated with higher concentrations in saliva; however, variability existed between serotypes and immunoglobulin classes. Table 1 displays each specific correlation coefficient: the strongest relationships were observed for IgA antibodies. Similar positive associations emerged for saliva concentration and secretion rates, where the majority were moderate and statistically significant. When examining serum IgG antibodies in relation to saliva IgA

concentration and secretion rates, significant correlations emerged for most serotypes for both saliva parameters, although these were mainly weak-moderate with coefficients <.5 (Table 2).

[Figure 1 and Tables 1 and 2 here]

Saliva concentration and secretion rates in relation to protective levels in serum

A total of 70.8% of participants had IgG serotype specific levels of ≥ 0.35 μg/mL for ≥ 8/12 Pn serotypes. Only 18.1% of participants achieved levels of ≥ 1.3μg/mL on ≥ 8/12 Pn serotypes; however, 41.7% achieved this level for ≥ 6/12 serotypes. Individuals with protective concentrations in serum had higher salivary secretion rates compared to those without, significantly so for 9/12 IgG, 1/12 IgM and 8/12 IgA antibody serotypes (Table 3). No statistics could be performed for Pn19A as all participants displayed protective levels. Similar outcomes were observed for saliva concentration: higher antibody levels were observed for protected individuals for 9/12 IgG and 8/12 IgA antibody serotypes (same serotypes as Table 3, except Pn4 was significant rather than Pn18C); no significant differences were found in IgM saliva concentration.

[Table 3 here]

ROC curve analyses to identify if salivary antibody levels can identify protection in serum

The ability of Pn salivary antibodies to distinguish between those with and without minimum protective levels (≥ 0.35 μg/mL) in serum for each serotype is shown in Table 4. Salivary IgG antibodies were most effective in identifying those with protective levels and significant results were found for 9 serotypes, for both concentration and secretion rates; secretion rates generally returned slightly higher AUC values compared with concentrations. Averaged across serotypes, AUC values were equivalent to a 78%

probability that individuals with protective levels in serum would have higher salivary antibody levels compared to those without protective levels, for both concentration and secretion rate. The most successful serotype for distinguishing protective status based upon saliva parameters was Pn7F, followed by Pn18C, Pn19F then Pn23F; AUCs were > .8 and subsequently classified as displaying good accuracy.

Salivary IgA antibodies also indicated a level of accuracy in distinguishing protective status in serum. Secretion rates generally returned slightly higher AUC values compared to concentration and values were significant for 8 and 7 serotypes for secretion rates and concentration, respectively. Averaged across serotypes, the AUC values were equivalent to a 68% (IgA concentration) and 70% (IgA secretion rate) probability that individuals with protective levels in serum would have higher salivary antibody levels compared to those without protective levels. Similar to IgG, the best serotypes for distinguishing protective status based upon saliva parameters were Pn23F and Pn7F, both classed as moderately accurate (AUC > .7) Salivary IgM antibodies were not informative based upon AUC values and were not able to discriminate between individuals with/without protective antibody levels in serum.

[Table 4 here]

ROC curve analyses to identify if salivary antibody levels can identify protection in serum: composite saliva parameters

The previous analyses of individual pneumococcal serotypes showed IgG and IgA were the most promising antibody classes; these were taken forward to explore composite saliva parameters for identifying protective status in serum. Median salivary IgG and IgA concentration and secretion rates across Pn serotypes all together were calculated for each participant. These composite measures were firstly examined in relation to minimum

protective levels (≥ 0.35 μg/mL) in serum for ≥ 8/12 Pn serotypes and corresponding ROC curves are shown in Figure 2.

Median IgG concentration and secretion rates returned significant AUC values of .69 and .67, respectively. The best-cut off for concentration was ≥ 0.69 ng/mL giving 76% sensitivity 76% and 62% specificity. The best-cut off for secretion rate was ≥ 0.29 ng/min with sensitivity 65% and specificity 71%. For IgA, median Pn antibody concentration generated an AUC of .67 with ≥ 8.54 ng/mL providing the best-cut off: 65% sensitivity and 62% specificity. Median IgA secretion rate AUC was .68 and a cut-off of ≥ 3.00 ng/min provided the best combination of sensitivity and specificity with values of 73% and 71%, respectively.

[Figure 2 here]

As only a minority of individuals exhibited the higher protective threshold of 1.30 μg/mL for ≥ 8/12 serotypes, a lower number of serotypes (≥ 6/12) was selected to evaluate the higher level of protection in serum in relation to composite saliva parameters (Figure 3). Median IgG Pn concentration and secretion rates produced significant AUCs of moderate accuracy for identifying the higher level of protection in serum: AUC .71 and .73, respectively. The best cut-offs were 1.10 ng/mL for concentration (67% sensitivity and 69% specificity) and 0.51 ng/min for secretion rate (57% sensitivity and 81% specificity). Median IgA concentration resulted in an AUC of .66 and the best cut-off occurred at ≥ 74.32 ng/mL with 70% sensitivity and 64% specificity. Median IgA secretion rate AUC was .66 and a ≥ 5.20 ng/min cut-off returned 57% sensitivity and 76% specificity.

[Figure 3 here]

Relationships between pneumococcal serotypes

The relationships between serotypes for IgG in serum are shown in Supplementary Table 1. Pn9V was most representative of the other serotype specific antibody concentrations, which was significantly positively associated with 9 serotypes. The best average coefficients across serotypes were observed for Pn7F and Pn9V and Pn19F. Stronger associations were observed for saliva than serum. The majority of correlations were significant and moderate-strong for IgG secretion rates (Supplementary Table 2). The best average correlation coefficients were observed for Pn9V and Pn19A followed by Pn4 and Pn5. For IgG concentration, inter-serotype correlations were generally significant but not as strong as secretion rates, again 9V and 19A had the strongest correlation coefficients. For IgA secretion rates, all inter-serotype correlations were significant and the majority were strong: Pn9V had the strongest average coefficient, followed by Pn19F and Pn3 (Supplementary Table 3). For IgA saliva concentration, correlations were not as robust as secretion rates, although 8 serotypes correlated significantly with all 11 other serotypes.

Serotype Pn9V was followed-up to determine if a single candidate was able to act as a measure of overall antibody pneumococcal levels within each biological specimen. Using ROC curve analyses, Pn9V concentration was tested against protective status in serum. Concentration of Pn9V in serum was able to identify participants with protective levels for ≥ 8/12 serotypes from those who did not with a good level of accuracy: AUC = .86 (95% CI .74–.97, $p < .001$). The best cut-off (378.9 ng/mL) provided 92% sensitivity and 81% specificity. To assess if Pn9V was related to overall antibody levels within saliva 'higher' and 'lower' median Pn groups were created using median splits of antibody secretion rates. ROC curves were then generated to test the ability of Pn9V secretion rates in saliva to discriminate between higher and lower overall Pn antibody groups. For IgG, Pn9V secretion rates distinguished between those with higher overall Pn antibody secretion from those with lower levels: AUC = .93 (95% CI .88–.98, $p < .001$). The best cut-off secretion

rate at 0.25 ng/min gave 88% sensitivity and 84% specificity. IgA Pn9V secretion rates were also able to successfully identify higher/lower overall Pn antibody secretion. A significant AUC of .92 was observed (95% CI .86–.99, $p < .001$) and the best cut-off occurred at 3.63 ng/min with 89% sensitivity and 92% specificity.

Discussion

Salivary concentration and secretion rates positively correlated with serum antibodies within immunoglobulin class; these findings indicate anti-pneumococcal antibody levels in serum are broadly reflected by those in saliva. This is consistent with previous studies showing positive associations between salivary and serum anti-pneumococcal antibodies in infants (Choo, et al., 2000, Nurkka, et al., 2001, Nurkka, et al., 2004, Rodenburg, et al., 2012) and the present study extends these findings to adults. Interestingly, the strongest relationships between serum and saliva were observed for IgA antibodies, rather than IgG where salivary antibodies are considered to be mainly serum-derived (Kauppi, et al., 1995, Nurkka, et al., 2001). Most IgA in saliva is secreted by local plasma cells and synthesised in dimeric form; pentameric IgM is also secreted locally (Brandtzaeg, 2007). Alternatively, IgG and the majority of monomeric IgA present in saliva is derived from serum rather than local production; these are likely to enter the saliva through gingival crevices, via gingival crevicular fluid (Brandtzaeg, 2007, Hofman, 2001). Although correlations between IgG in serum and IgA in saliva were not convincing, salivary IgA antibodies were linked to protective status in serum. When examining individual anti-serotype antibodies, a level of ≥ 0.35 μg/mL in serum was associated with significantly higher IgG and IgA antibody concentrations and secretion rates in saliva, although not IgM. Overall, the present results demonstrate that protective levels in serum are linked to higher levels of antibodies in saliva. Salivary immunoglobulins have been proposed to comprise a broad defence

system capable of inhibiting bacterial adherence and thus colonisation (Van Nieuw Amerongen, et al., 2004). Stimulation of salivary antibodies may be advantageous as most bacterial pathogens enter the body via mucosal routes (Flanagan, et al., 2010). The higher salivary antibodies observed parallel to serum may offer enhanced protection against pneumococcal infection. Although the importance of mucosal antibodies as a defence mechanism requires further investigation (Nurkka, et al., 2005).

We tried to determine whether saliva parameters are representative of distinct antibody cut-offs in serum deemed to be indicative of protective status (Bonilla, et al., 2005; WHO, 2005). When analysing individual antibodies paired in serum and saliva for pneumococcal serotypes, salivary IgG antibodies were most effective in identifying those with protective levels in serum. This is perhaps to be expected given protective status in serum is based upon IgG. Both concentrations and secretion rates were able to distinguish protective status in serum with moderate accuracy. On average there was > 70% chance that an individual with protective antibody levels in serum would have higher salivary antibodies compared with an unprotected individual. Although AUCs were on average not as high as IgG, salivary IgA antibodies also indicated a degree of accuracy in differentiating protective status in serum for several serotypes. For both IgG and IgA, Pn7F and Pn23F were the most sensitive in identifying protective status.

For paired serum and saliva analysis, IgG and IgA salivary antibodies were largely fair to good indicators of protective status in serum. The observed AUC values can be interpreted as adequate/acceptable discrimination between protection groups. However, saliva was not useful in identifying protective status in all serotypes. Further, the analyses of individual serotypes may not be feasible or appropriate in population studies, where a global indicator of protection from pneumococcal disease or response to vaccination is likely to be of added interest. Composite saliva measures were explored to examine the

sensitivity and specificity from identified best-cut off points. This strategy provided an antibody profile across pneumococcal serotypes to be tested against overall protective status in serum, which due to ease of calculation could be easily applied in large investigations. Identifying protective status in relation to 2/3rds of serotypes by composite saliva parameters approached moderate accuracy. The best balance between sensitivity and specificity was observed for median IgA secretion rates, with values of 73% and 71% associated with the best cut-off. A cut-off of ≥ 3.00 ng/min successfully identified the majority of individuals who met the WHO criteria for protection on ≥ 8/12 serotypes in this sample population, however, a false positive/negative rate of nearly 30% still existed. The strongest AUCs for composite measures were seen for IgG concentration and secretion for identifying the higher threshold for protection for ≥ 6/12 serotypes. Although a range of sensitivity and specificity values were observed for both IgG and IgA salivary cut-offs in relation to distinguishing the higher level of protection.

Depending on the purpose of the test and impact of the results, the importance of sensitivity and specificity may fluctuate with the consequences of false positives/false negatives. If using a saliva-based test to inform clinical decisions at an individual-level a higher level of performance would need to be developed than what was observed in the present study. However, these preliminary results suggest that measuring pneumococcal specific antibodies in saliva may have promise in future epidemiological studies relating to vaccination and protection from pneumococcal disease. The cut-offs identified in this study were specific for the population investigated and our multiplex assay; larger population studies would need to be performed to establish cut-offs in saliva indicative of specific levels in serum. The use of the WHO protective cut-off was employed in this study as a means of assessing if saliva can identify specific serum thresholds. However, it is acknowledged that 0.35 μg/mL is an estimation (Nurkka, et al., 2005) and true protective levels may vary for the different serotypes and in relation to age. Pneumococcal conjugate

vaccines have been shown to induce salivary antibodies in infants (Nurkka, et al., 2004, Nurkka, et al., 2005, Rodenburg, et al., 2012). No vaccinations were administered as part of this cross-sectional study examining salivary antibodies in relation to serum in adults. In order to assess if salivary antibodies can act as a marker of vaccination, the utility of saliva needs to be tracked over time in response to vaccination in different age groups of interest, including children and elderly adults.

If certain serotypes provide good estimation of overall protection against pneumococcal disease then they could be used as a cost and resource effective proxy marker when measuring a range of serotypes is not possible. Selecting key serotypes as universal representatives of antibody levels across serotypes could also help streamline studies and aid interpretation and analysis when investigating multiple analytes. Inter-serotype relationships were stronger within saliva than in serum, with IgA secretion rates showing the strongest correlations. Serotype Pn9V gave the strongest correlations in both serum and saliva and was explored further as an indicator of overall antibody status. IgG and IgA Pn9V secretion rates were able to identify participants with higher median Pn antibody secretion rates with a high degree of accuracy. Therefore, this serotype may provide a good indication of pneumococcal antibodies in saliva. In serum, Pn9V was able to correctly distinguish between protection statuses: levels were highly sensitive and specific in identifying those with protection for 2/3rds of serotypes in serum in this sample. Serotype Pn9V is present in both Pneumovax and Prevnar 13, and thus could be a useful indicator of overall response to these vaccinations. However, this should perhaps be interpreted with some caution. As mentioned previously, in reality precise protective levels could vary between serotypes.

Conclusions

This study provides initial evidence that antibodies against pneumococcal antigens in serum are broadly reflected by saliva across immunoglobulin classes, IgA, IgG and IgM. Individuals who had protective levels of anti-pneumococcal antibodies in serum demonstrated significantly higher IgG and IgA salivary antibody concentrations and secretion rates. Salivary IgG and IgA antibodies showed reasonable accuracy in distinguishing protective status in serum; it was not possible to identify protective levels using IgM. Levels of certain pneumococcal serotypes are interrelated and Pn9V may be a potential candidate to represent overall pneumococcal antibody status. Saliva sampling may be able to help address the challenges of measuring antibodies in certain populations and settings and offer a potential biomarker of systemic immunity and vaccination; future studies in large populations are required to develop, optimise and validate further.

Declaration of interest statement: The authors report no conflicts of interest

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Table 1. Spearman's rank correlation coefficients (r_s) for each pneumococcal (Pn) serotype within immunoglobulin class. Correlations are between serum IgG, IgM and IgA concentrations, respectively, and saliva concentration and saliva secretion rates for IgG, IgM and IgA, respectively; *** $p < .001$, ** $p < .01$, * $p < .05$

	Saliva concentration			Saliva secretion rate		
r^s	**IgG**	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**
Pn1	.35**	.31*	.56***	.38**	.39**	.53***
Pn3	.53***	.55***	.57***	.47***	.53***	.56***
Pn4	.26*	.34**	.52***	.30*	.34**	.55**
Pn5	.42**	.47***	.67***	.52***	.49***	.68***
Pn6B	.48***	.23	.42***	.52***	.24	.42***
Pn7F	.34**	.32**	.49***	.34**	.42***	.49***
Pn9V	.55***	.15	.58***	.52***	.20	.54***
Pn14	.29*	.36**	.43***	.29*	.14	.48***
Pn18C	.40**	.64***	.56***	.69***	.39**	.51***
Pn19A	.48***	.25*	.57***	.45***	.30*	.44***
Pn19F	.25*	.47***	.22	.24*	.51**	.23*
Pn23F	.64***	.27*	.64***	.61***	.34*	.67***

Table 2. Spearman's rank correlation coefficients (r_s) for antibodies against pneumococcal (Pn) serotypes for IgG in serum and salivary IgA concentration and IgG in serum and salivary IgA secretion; *** $p < .001$, ** $p < .01$, * $p < .05$

r^s	**Concentrations of IgG in serum and IgA in saliva**	**Concentration of IgG in serum and IgA secretion rate in saliva**
Pn1	.30*	.33**
Pn3	.44**	.47***
Pn4	.37**	.38**
Pn5	.12	.24*
Pn6B	.29*	.30*
Pn7F	.37**	.34**
Pn9V	.32**	.30*
Pn14	.47**	.35**
Pn18C	.26*	.31*
Pn19A	.28*	.29*
Pn19F	.15	-.01
Pn23F	.48**	.46***

Table 3. Salivary IgG, IgM and IgA antibody secretion rates specific for pneumococcal (Pn) serotypes (ng/min), for those who did/did not demonstrate minimum protective antibody levels in serum for each serotype, respectively. All individuals had protective titres for Pn19A; *** $p < .001$, ** $p < .01$, * $p < .05$

	IgG		**IgM**		**IgA**	
	Protected	**Not protected**	**Protected**	**Not protected**	**Protected**	**Not protected**
Pn1	.55 (.08–5.65)**	.24 (.02–.04)	1.30 (0.3–10.45)	.93 (.01–8.09)	1.82 (0.16–16.54)**	1.0 (.11–10.04)
Pn3	.13 (.02–1.49)**	.06 (.01–.63)	.20 (.02–1.76)	.12 (0–5.33)	16.72 (2.08–261.16)***	10.56 (1.01–65.47)
Pn4	.12 (.01–1.75)	.09 (0–.96)	.47 (.01–4.68)	.41 (.01–4.38)	4.14 (.72–138.67)	3.78 (.29–31.07)
Pn5	.44 (.07–71.55)**	.23 (.01–.38)	1.71 (.04–16.98)	.55 (.14–9.59)	1.36 (.12–7.53)	.98 (.10–6.03)
Pn6B	.26 (.03–56.64)**	.10 (.01–3.35)	4.61 (.12–26.77)*	2.44 (.13–22.45)	6.56 (.23–31.31)*	2.0 (.33–14.43)
Pn7F	.89 (.02–11.16)**	.12 (.03–.63)	.68 (.03–18.26)	.46 (.10–1.70)	2.51 (.12–20.94)*	.80 (.18–3.14)
Pn9V	.34 (.02–3.78)**	.10 (.01–1.39)	1.10 (.04–11.25)	.71 (.06–6.31)	5.03 (.15–31.79)*	1.98 (.20–31.72)
Pn14	.50 (.02–47.19)	.17 (0.1–13.24)	.34 (0–35.49)	.17 (0–14.79)	9.35 (1.10–420.24)*	3.77 (.87–27.43)
Pn18C	.24 (.02–51.06)***	.05 (0–.17)	.48 (0.1–6.02)	.33 (.09–1.56)	2.14 (.12–38.48)*	.57 (.11–4.91)
Pn19A	2.18 (.11–21.21)	–	1.34 (.06–3.61)	–	8.65 (1.17–58.91)	–
Pn19F	1.96 (.08–31.31.37)**	.37 (0.6–1.72)	5.19 (.09–91.17)	1.64 (.30–15.51)	10.42 (.97–85.33)	6.10 (2.61–10.27)
Pn23F	.28 (.02–22.15)***	.07 (0–.82)	2.14 (.13–18.86)	1.8 (.08–16.63)	3.74 (.24–19.39)***	1.05 (.21–18.41)

Table 4. Area under curve (AUC (95% confidence intervals)) results for receiver operating characteristic (ROC) curves for saliva IgG, IgM and IgA concentrations and secretion rates to identify protective IgG antibody levels in serum (≥ 0.35 µg/mL) from those who did not have protective levels (< 0.35 µg/mL) for each pneumococcal (Pn) serotype. All individuals had protective titres for Pn19A; *** $p < .001$, ** $p < .01$, * $p < .05$

	Saliva concentration			**Saliva secretion rate**		
AUC (95% CI)	**IgG**	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**
Pn1	.70 (.58–.83)**	0.51 (.38–.65)	.66 (.53–79)*	.68 (.55–.80)*	0.58 (.45–.72)	.68 (.56–.81)**
Pn3	.77 (.66–.88)***	0.55 (.42–.69)	.70 (.58–.82)**	.81 (.70–.91)***	0.59 (.46–.73)	.71 (.60–.83)**
Pn4	.57 (.42–.71)	0.60 (.46–.74)	.66 (.53–.79)*	.59 (.45–.73)	0.59 (.45–.73)	.59 (.46–.73)
Pn5	.80 (.67–.92)**	0.60 (.42–.79)	.59 (.41–.77)	.79 (.64–.94)**	0.69 (.51–.88)	.62 (.42–.81)
Pn6B	.73 (.62–.85)**	0.60 (.47–.74)	.65 (.52–.77)*	.70 (.58–.83)**	0.67 (.54–.80)*	.67 (.55–.80)*
Pn7F	.90 (.80–1.00)**	0.54 (.32–.75)	.74 (.57–.92)*	.88 (.76–.99)**	0.62 (.42–.83)	.77 (.62–.93)*
Pn9V	.76 (.63–.88)**	0.59 (.45–.73)	.71 (.57–.85)**	.80 (.65–.91)***	0.62 (.47–.77)	.68 (.54–.81)*
Pn14	.62 (.46–.78)	0.57 (.40–.73)	.67 (.53–.81)*	.60 (.45–.76)	0.60 (.45–.75)	.69 (.55–.82)*
Pn18C	.85 (.75–.96)***	0.44 (.28–.59)	.68 (.50–.86)	.83 (.73–.94)**	0.52 (.37–.68)	.75 (.59–.90)*
Pn19F	.85 (.70–1.00)**	0.55 (.31–.78)	.63 (.36–.90)	.84 (.70–.98)*	0.62 (.35–.88)	.75 (.59–.91)
Pn23F	.84 (.75–.93)***	0.47 (.33–.60)	.78 (.67–.89)***	.85 (.76–.94)***	0.53 (.39–.66)	.76 (.64–.87)***

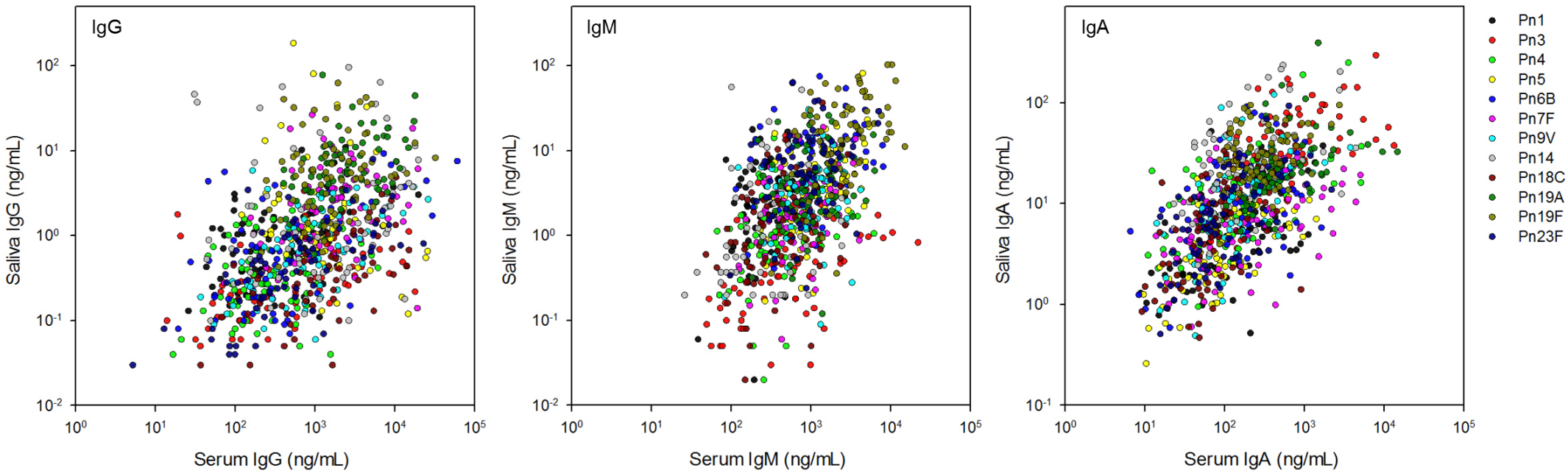

Figure 1. IgG, IgM and IgA (from left to right) pneumococcal antibody levels in serum (x axis) and saliva (y axis) for 12 pneumococcal serotypes.

Data illustrate overall positive correlations between serum and saliva and variability between serotypes

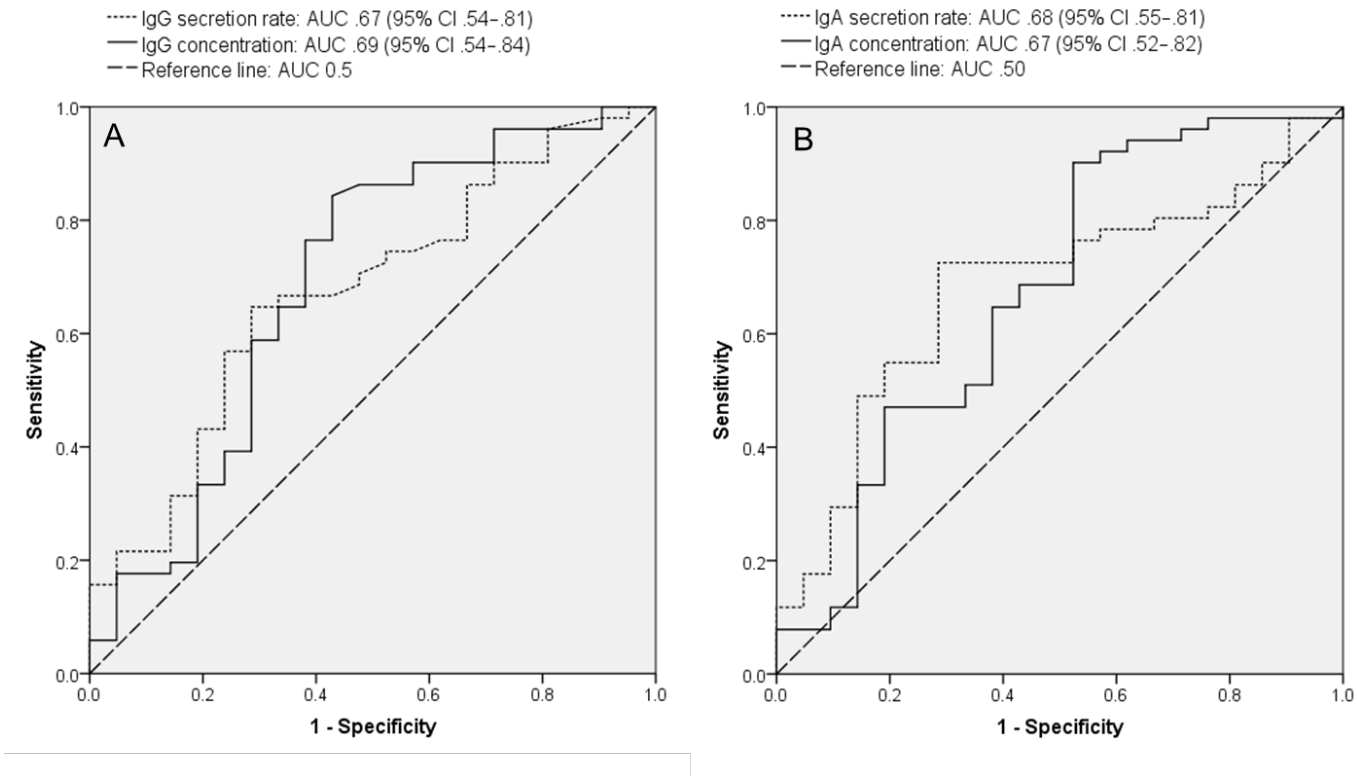

Figure 2. Receiver operating characteristic curves of salivary IgG (A) and salivary IgA (B) antibody parameters for identifying minimum protective antibody levels (≥ 0.35 µg/mL) in serum on ≥ 8/12 pneumococcal serotypes. Saliva measures are median antibody concentration and secretion rate overall for all pneumococcal serotypes combined.

Panel A: Median IgG concentration (solid line) AUC = .69 (95% CI .54–.84), p = .012; best cut-off 0.69 ng/mL, sensitivity 76% and specificity 62 %. Median IgG secretion rate (dotted line) AUC = .67 (95% CI .54–.81), p = .022; best cut-off 0.29 ng/min, sensitivity 65% and specificity 71%.

Panel B: Median IgA concentration (solid line) AUC = .67 (95% CI .52–.82), p = .022; best cut-off 8.54 ng/mL, sensitivity 65% and specificity 62%. Median IgA secretion rate (dotted line) AUC = .68 (95% CI .55–.81), p = .017; best cut-off 3.00 ng/min, sensitivity 73% and specificity 71%.

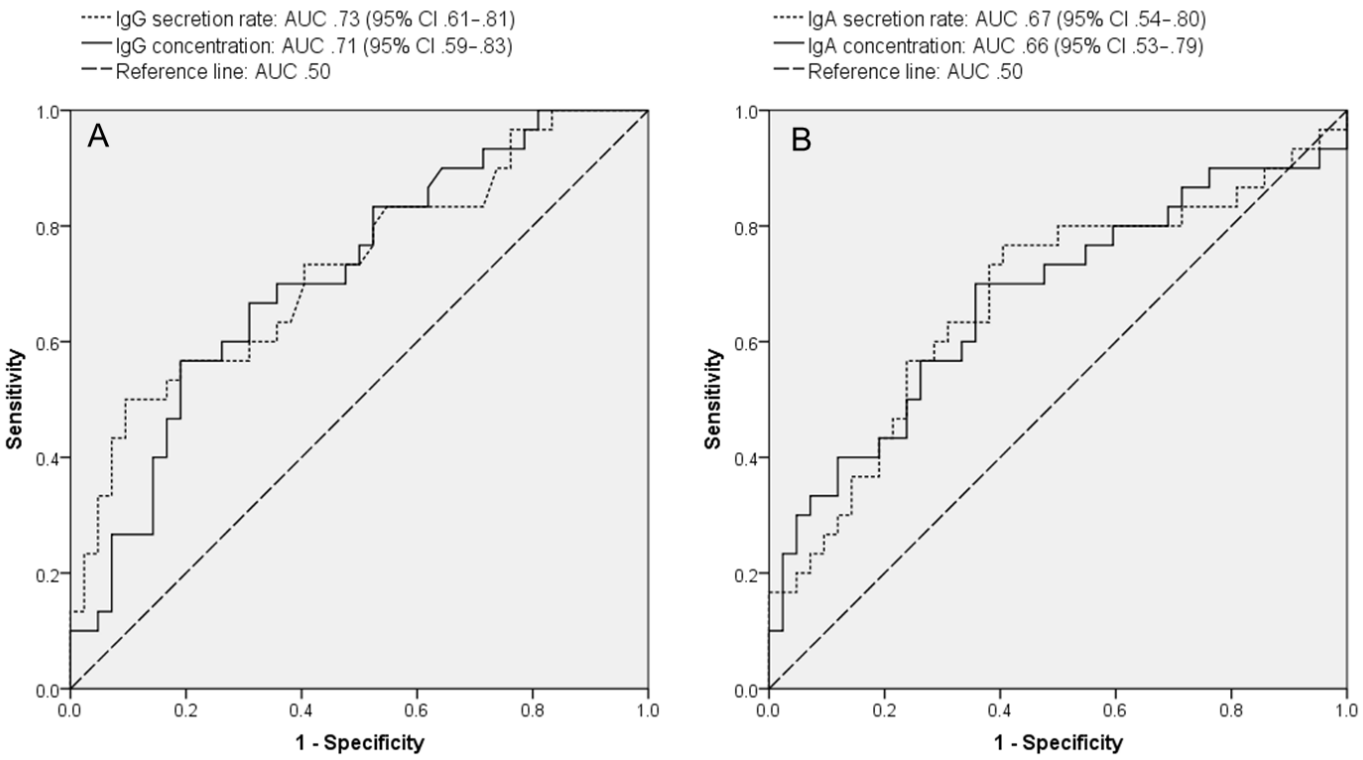

Figure 3. Receiver operating characteristic curves of salivary IgG (A) and salivary IgA (B) antibody parameters for identifying optimal protective antibody levels (≥ 1.30 µg/mL) in serum on ≥ 6/12 pneumococcal serotypes. Saliva measures are median antibody concentration and secretion rate overall for all pneumococcal serotypes combined.

Panel A: Median IgG concentration (solid line) AUC = .71 (95% CI .59–.83), *p* = .001; best cut-off 1.10 ng/mL, sensitivity 67% and specificity 69 %. Median IgG secretion rate (dotted line) AUC = .73 (95% CI .61–.81), *p* = .002; best cut-off 0.51 ng/min, sensitivity 57% and specificity 81%.

Panel B: Median IgA concentration (solid line) AUC = .66 (95% CI .53–.79), *p* = .025; best cut-off 74.32 ng/mL, sensitivity 70% and specificity 64%. Median IgA secretion rate (dotted line) AUC = .67 (95% CI .54–.79), *p* = .013; best cut-off 5.20 ng/min, sensitivity 57% and specificity 76%.

Supplementary Table 1. Correlations between for pneummococcal (Pn) serotypes for IgG antibody levels in serum. Light gery shading indicates a moderate relationship between serotypes; ***$p < .001$, **$p < .01$, *$p < .05$

r_s	Pn1	Pn3	Pn4	Pn5	Pn6B	Pn7F	Pn9V	Pn14	Pn18C	Pn19A	Pn19F	Pn23F
Pn1		.20	.13	.43***	.33**	.15	.38**	.23	.33*	.13	.38**	.30*
Pn3	.20		.10	.04	.23	.40**	.31*	-0.03	.19	.02	.27*	-0.06
Pn4	.13	.10		.27*	.30*	.27*	.16	.24*	.08	.26*	.35**	.17
Pn5	.43***	.04	.27*		.29*	.26*	.33**	.34**	.22	.41**	.25*	.15
Pn6B	.33**	.23	.30*	.29*		.22	.50***	.20	.18	.18	.19	.30*
Pn7F	.15	.40**	.27*	.26*	.22		.43***	.19	.23	.25*	.52***	.23
Pn9V	.38**	.31*	.16	.33**	.50***	.43***		.21	.28*	.42***	.45***	.37**
Pn14	.23	-.03	.24*	.34**	.20	.19	.21		.35**	.24*	.17	.23
Pn18C	.33*	.19	.08	.22	.18	.23	.28*	.35**		-.09	-.10	.29*
Pn19A	.13	.02	.26*	.41**	.18	.25*	.42***	.24*	-.09		.49***	.21
Pn19F	.38**	.27*	.35**	.25*	.19	.52***	.45***	.17	-.10	.49***		.23
Pn23F	.30*	-.06	.17	.15	.30*	.23	.37**	.23	.29*	.21	.23	

Supplementary Table 2. Correlations between pneummococcal (Pn) serotypes for IgG antibody saliva secretion rates. Light gery shading indicates a moderate relationship and darker shading indicates a strong relationship between serotypes; $p < .05$ for all correlations except Pn18C vs Pn 3,14 and 19F and Pn14 vs 6B

r_s	Pn1	Pn3	Pn4	Pn5	Pn6B	Pn7F	Pn9V	Pn14	Pn18C	Pn19A	Pn19F	Pn23F
Pn1		.45	.62	.74	.69	.68	.80	.23	.49	.82	.62	.77
Pn3	.45		.73	.38	.31	.51	.48	.60	.19	.48	.74	.45
Pn4	.62	.73		.71	.50	.76	.70	.57	.33	.75	.84	.60
Pn5	.74	.38	.71		.67	.82	.84	.32	.52	.82	.61	.72
Pn6B	.69	.31	.50	.68		.53	.72	.14	.55	.66	.43	.69
Pn7F	.68	.51	.76	.82	.53		.87	.29	.41	.85	.65	.64
Pn9V	.79	.48	.70	.84	.72	.87		.28	.53	.88	.67	.71
Pn14	.23	.60	.57	.32	.14	.29	.28		.07	.32	.65	.30
Pn18C	.49	.19	.33	.52	.55	.41	.53	.07		.40	.18	.56
Pn19A	.82	.48	.75	.82	.66	.85	.88	.32	.40		.72	.74
Pn19F	.61	.74	.84	.61	.43	.65	.67	.65	.18	.72		.50
Pn23F	.77	.45	.60	.72	.69	.64	.71	.30	.56	.74	.50	

Supplementary Table 3. Correlations between pneummococcal (Pn) serotypes for IgA antibody saliva secretion rates. Light gery shading indicates a moderate relationship and darker shading indicates a strong relationship between serotypes; $p < .001$ for all correlations

r_s	Pn1	Pn3	Pn4	Pn5	Pn6B	Pn7F	Pn9V	Pn14	Pn18C	Pn19A	Pn19F	Pn23F
Pn1		0.55	0.71	0.64	0.55	0.56	0.60	0.56	0.57	0.48	0.57	0.56
Pn3	0.55		0.69	0.67	0.56	0.66	0.70	0.70	0.56	0.61	0.67	0.57
Pn4	0.71	0.69		0.59	0.54	0.63	0.67	0.65	0.56	0.55	0.71	0.53
Pn5	0.64	0.67	0.59		0.67	0.57	0.64	0.58	0.66	0.64	0.63	0.57
Pn6B	0.55	0.56	0.54	0.67		0.61	0.68	0.49	0.76	0.67	0.57	0.66
Pn7F	0.56	0.66	0.63	0.57	0.61		0.74	0.63	0.65	0.64	0.65	0.49
Pn9V	0.60	0.70	0.67	0.64	0.68	0.74		0.58	0.63	0.70	0.61	0.53
Pn14	0.56	0.70	0.65	0.58	0.49	0.63	0.58		0.54	0.62	0.79	0.53
Pn18C	0.57	0.56	0.56	0.66	0.76	0.65	0.63	0.54		0.59	0.50	0.69
Pn19A	0.48	0.61	0.55	0.64	0.67	0.64	0.70	0.62	0.59		0.67	0.53
Pn19F	0.57	0.67	0.71	0.63	0.57	0.65	0.61	0.79	0.50	0.67		0.53
Pn23F	0.56	0.57	0.53	0.57	0.66	0.49	0.53	0.53	0.69	0.53	0.53	