Magnetic Bead-Based Separation (MBS) of Pneumococcal Serotypes

Anna York[1*], Emily T. Huynh[1], Sidiya Mbodj[1], Devyn Yolda-Carr[1], Maikel S. Hislop[1,2], Haley Echlin[3], Jason W. Rosch[3], Daniel M. Weinberger[1], Anne L. Wyllie[1]

[1]Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, Connecticut, USA
[2]Department of Life Sciences and Chemistry, Utrecht University of Applied Sciences, Utrecht, Netherlands
[3]Department of Infectious Disease, St. Jude Children's Research Hospital, Memphis, TN, United States of America
*Corresponding author: anna.york@yale.edu

SUMMARY

The separation of pneumococcal serotypes from a complex polymicrobial mixture may be required for different applications. For instance, a minority strain could be present at a low frequency in a clinical sample, making it difficult to identify and isolate by traditional culture-based methods. We therefore developed an assay to separate mixed pneumococcal samples using serotype-specific antiserum and a magnetic bead-based separation method. Using qPCR and colony counting methods, we first show that serotypes (12F, 23F, 3, 14, 19A and 15A) present at ~0.1% of a dual serotype mixture can be enriched to between 10% and 90% of the final sample. We demonstrate two applications for this method: extraction of a known pneumococcal serotype from saliva samples and efficient purification of capsule switch variants from experimental transformation experiments. Moreover, this method may have further laboratory or clinical applications when the selection of specific serotypes is required.

Key words: *Streptococcus pneumoniae*, pneumococcus, capsular polysaccharide, serotypes, transformation, capsule switch, saliva

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is an opportunistic pathogen that resides asymptomatically in the upper respiratory tract of many healthy adults and children worldwide. This asymptomatic colonization is a pre-requisite for the development of pneumococcal disease, including upper respiratory tract infections (such as otitis media), lower respiratory tract infections (such as pneumonia), and invasive pneumococcal disease (IPD) (such as meningitis and bacteremia). Pneumococcal disease often occurs in the very young, elderly, or immunocompromised[1]. Pneumococcus is a leading cause of lower respiratory disease, and contributed to 1,189,937 deaths globally in 2016.[2]

The capsular polysaccharide (CPS) is the outermost layer of encapsulated strains of *S. pneumoniae*, and more than 100 antigenically distinct serotypes have been identified.[3] Pneumococcal conjugate vaccines (PCV) are highly effective against pneumococcal disease but only cover up to 20 of these serotypes. While pneumococcal disease declined following the introduction of PCVs, a concomitant increase in disease caused by non-vaccine serotypes occurred. This emergence of non-vaccine serotypes in carriage and invasive disease is called

serotype replacement.[4] Serotype replacement occurs for two reasons, first the opening of a new niche in which existing strains expressing capsules not targeted by the vaccine can thrive. Second, vaccine-targeted strains can acquire the capsule biosynthesis cassette from a different serotype, allowing them to evade vaccine-induced immunity. Serotype switching occurs when the *cps* locus from one *S. pneumoniae* serotype (or related species) is transferred into the genetic backbone of another *S. pneumoniae* serotype by transformation.[5] Genetic exchange between two *S. pneumoniae* serotypes requires co-colonization of two or more serotypes.

In addition to naturally occurring serotype switches,[6–8] researchers have been generating *cps* switch mutants in the lab for nearly 100 years. The first capsule switch experiments conducted by Griffith in 1928, were accomplished by mixing avirulent, unencapsulated pneumococci with virulent, but killed, encapsulated strains, and injecting this mixture into a mouse. The capsule-switched strains could then be isolated from the mouse.[9] More recently, generating *cps* switch mutants in the lab has been accomplished using various genetic cassettes.[10,11] These types of studies have permitted the generation of a number of capsule switch mutants, and this allows for detailed experimental evaluation of the relative importance of capsule and genetic background for different phenotypes.[8,12,13] Current methods for generating capsule-switched variants require the use of selectable markers, are labor intensive, and not easily scalable. Methods that allow for separation of multiple serotypes could eliminate the need for selection pressure altogether or could be used in combination to conduct higher throughput transformations.

There is also a need to isolate individual pneumococcal strains from clinical samples. Nasopharyngeal swabs have long been considered the gold standard sample type for the detection of carriage of *S. pneumoniae,*[14] but recent studies have demonstrated utility for saliva to improve the detection of carriage in adults.[15,16] Whilst testing saliva improves the detection of pneumococci when using molecular methods (such as qPCR), it can be challenging for the isolation of live pneumococcal colonies due to the density and diversity of bacteria present in saliva. A method that enables the separation of pneumococci, in a serotype-specific manner, from other species present in saliva would be useful for clinical and laboratory studies alike.

We developed a magnetic bead-based separation (MBS) method which requires no selection markers and can be used to extract live pneumococci, of a known serotype, from a mixture of pneumococci or from clinical samples containing other bacteria (such as saliva).

METHODS

Figure 1 summarizes the MBS method; briefly, a mixture of serotypes is incubated with antisera pool(s) unique to the desired serotype, then following wash steps is incubated with secondary antibody conjugated to a magnetic bead. The cells are extracted using the automated Kingfisher Flex Purification System and the eluate plated on blood agar plates. Unless otherwise stated a blood agar plate (BAP) comprises Tryptic Soy Agar (TSA) II supplemented with 5% (v/v) defibrinated sheep blood, and are sometimes referred to as 'plain plates'. BAPs containing the following concentrations of antibiotics/additives for selection were also used: 0.018 μg/mL, 0.036 μg/mL, 0.18 μg/mL and 0.072 μg/mL penicillin, 10 μg/mL gentamycin, 400 μg/mL kanamycin and 800 μg/mL streptomycin with 10% (w/v) sucrose. Unless otherwise stated all overnight incubations occur at 37°C and 5% CO_2.

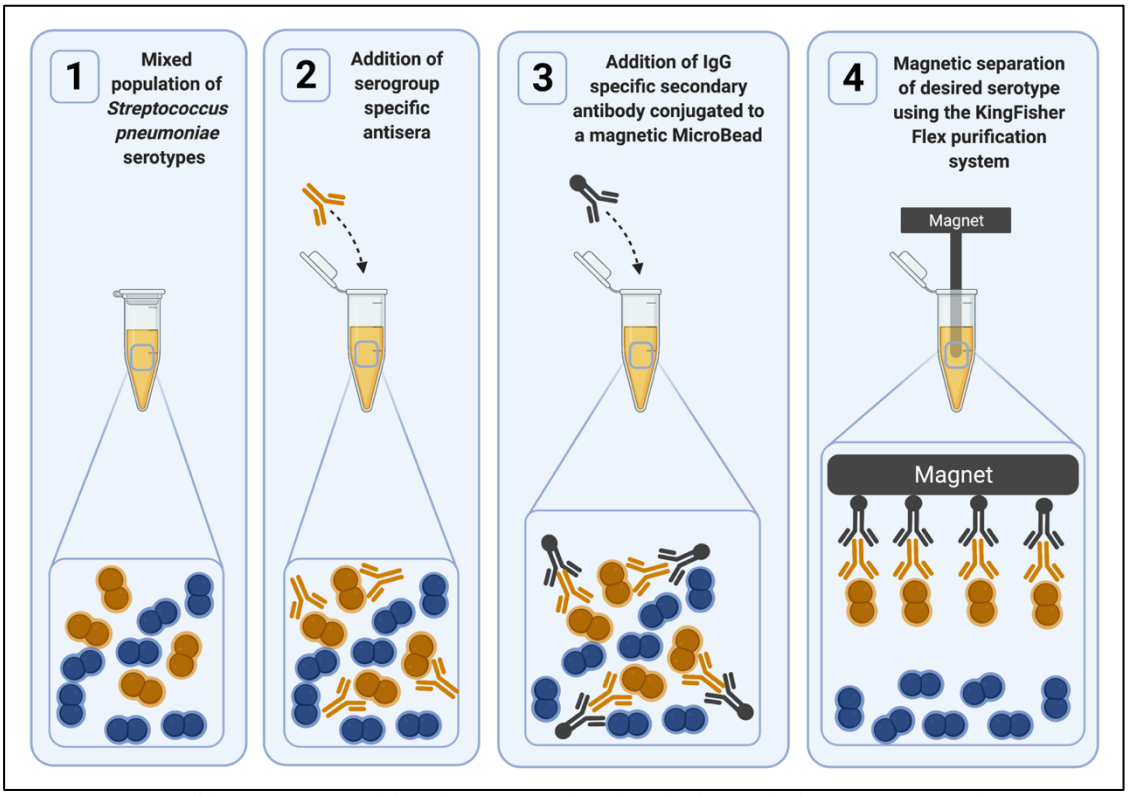


Figure 1. An overview of the Magnetic Bead-based Separation (MBS) method. To a dual serotype mixture (1), antisera specific for the desired serotype are added (2). Following brief wash steps, IgG-specific secondary antibody conjugated to a magnetic bead is incubated (3) and finally the desired cells are extracted using the KingFisher Flex Purification System (4).

Magnetic Bead-Based Separation (MBS) Method

Approximately $1x10^4$ cells and $1x10^7$ cells from two different serogroups of *S. pneumoniae* were mixed together (~0.1% minority serotype). Cells were pelleted by centrifugation at 13,000 rpm and resuspended in 450 µl Buffer 1 (1x Phosphate Buffered Saline with 1% bovine serum albumin (BSA)). The resuspended sample was incubated at 4°C on a shaking platform at 150 rpm for 1 hour. The two antisera pools specific for the minority serogroup were combined in a 1:1 ratio and diluted 50-fold in Buffer 1. Next, 30 µl of antisera mix was added to the sample and incubated at 4°C on a shaking platform at 150 rpm for 1 hour. The sample was centrifuged at 13,000 rpm for 5 mins, the supernatant was discarded, and the pellet was resuspended in 450 µl Buffer 1; this step was repeated again. Next, 20 µl of Anti-Rabbit IgG MicroBeads (Miltenyi Biotech) was added, gently vortexed and incubated at 4°C on a shaking platform at 150 rpm for 30 minutes. The sample was extracted using the KingFisher™ Flex Purification System (ThermoFisher) with the protocol detailed in Table S3. The eluted sample was resuspended by pipetting the sample in the elution well 50-100 times before transferring it to a new Eppendorf tube. Following transfer, the sample was thoroughly mixed by vortexing a minimum of 10 times for 5-10s with 5 sec intervals.

To minimize cell losses, when supernatant was removed from cell pellets, 50 µl of supernatant was always left on top of the pellet. The specific rabbit antiserum pools (SSI Diagnostica, Hillerød, Denmark) used for the MBS method, and the SSI ImmuLex™ Pneumotest Pools used for serotyping are outlined in Table S1.

Proof of concept and primary analysis

To demonstrate proof of concept for the MBS method we used three pairs of six different serotypes where one serotype in each pair was penicillin resistant and the other penicillin sensitive. It is important to note that different penicillin sensitivity is not necessary for separation but was instead used to make the quantification of the efficiency of this method easier. The three pairs were 12F and 23F (Pair 1), 3 and 14 (Pair 2) and 19A and 15A (Pair 3). Serotype 3 exists as two distinct morphologies; small non-mucoid colony variant (SCV) and mucoid variant.[17] We therefore isolated SCV and mucoid variants and chose to work primarily with the SCV for three reasons; SCVs are easier to count, easier to isolate as single colonies (for serotyping) and less easy to distinguish from other serotypes based on morphology, thus reducing selection bias during the colony selection for serotyping. The MIC of each serotype was determined using penicillin E-strips, and then the exact concentration of penicillin for blood agar plates was determined experimentally by varying the penicillin concentration and plating out cells at known CFU/mL. The concentration of penicillin used in the blood agar plates was the concentration at which the resistant serotype grew equally well on a penicillin containing plate, as it did on a plain plate, whilst the susceptible serotype showed no growth on the penicillin containing plate but normal growth on a plain plate. For Pairs 1, 2 and 3, BAPs containing 0.018 μg/mL, 0.036 μg/mL and 0.18 μg/mL penicillin were used, respectively.

For all three pairs, Sample R is when the penicillin resistant serotype is the minority species, and Sample S is when the penicillin sensitive serotype is the minority species. Samples were plated out onto BAPs with and without penicillin, at two stages in the protocol; immediately prior to the first incubation (PRE), and after extraction (POST). In all cases 5 μl of sample was serially diluted in 45 μl PBS, in triplicate. For samples where the minority strain was penicillin resistant, 20 μl of sample at a 10^{-1} dilution was plated on penicillin plates, while 20 μl of sample at a 10^{-4} dilution was plated on plain blood agar plates. In samples where the majority serotype was penicillin resistant, 20 μl of sample at a 10^{-4} dilution was plated on both BAPs with and without penicillin. In addition to the diluted samples, 10 μl of undiluted sample at the PRE and POST stage, and the remaining volume (~40 μl) after elution was plated on BAPs, to provide DNA for qPCR experiments conducted to establish separation efficiency. In all cases 10 μl or 20 μl samples were pipetted onto the BAP and the plate was then tilted to allow the sample to run down the length of the plate. The BAPs were incubated overnight.

Secondary analyses

To establish if separation efficiency was similar for both mucoid (Muc) and single colony variants (SCV) of Serotype 3, two additional pairs; 23F and 3SCV (Pair 4), and 23F and 3Muc (Pair 5) were investigated. These experiments were conducted in duplicate, and efficiency assessed by colony counting and qPCR methods. Pair 4 and 5 used BAPs containing 0.072 μg/mL penicillin.

To investigate the effect of initial proportion of minority serotype on the efficiency of separation, 23F and 12F (Pair 1) were again used. The initial amount of majority serotype (12F) was kept constant at $1x10^7$ CFUs, while the minority serotype (23F) was varied ($5x10^4$, $1x10^4$, $5x10^3$ and $1x10^3$). These experiments were conducted once for each dilution, and efficiency was assessed by colony counting and qPCR methods.

The experiments above were conducted using two pooled antisera that were specific for the minority serotype. We investigated whether a single pool of antisera could also be used. This is important because certain pairs of serotypes can only be distinguished by one pool. Serotype

173 pairs which could not be distinguished based on penicillin sensitivity (and therefore could not
174 be assessed by colony counting methods), were used for this analysis, and for pairs which
175 shared a common antisera pool, only the unique antisera was used. These experiments were
176 conducted once for each condition, and efficiency was assessed by qPCR alone.
177
178 **Colony counting to quantify separation efficiency**
179 Colonies were counted and the mean colony number was determined, which was then used for
180 downstream analysis. The following equations for Sample R and Sample S were used to
181 determine the percentage of the minority serotype present at each time point.
182
183 **Sample R Equation**
184

185 $$\%\, minority = \left(\frac{\#colonies\ (pen\ plate)}{\#colonies\ (plain\ plate)}\right) \times 100$$

186
187
188
189 **Sample S Equation**
190

191 $$\%\, minority = \left(\frac{\#colonies\ (plain\ plate) - \#colonies\ (pen\ plate)}{\#colonies\ (plain\ plate)}\right) \times 100$$

192
193
194 **Serotyping of colonies to confirm separation efficiency**
195 Eight colonies were picked at random from the plain blood agar elution plates and expanded to
196 create a lawn on 1/8th of a BAP and incubated overnight. The serotype of each lawn was
197 confirmed by testing each of the four antisera pools specific to both the majority and the
198 minority serotype in the pair, using ImmuLex™ Pneumotest (SSI Diagnostica) reagents.
199
200 **Real-Time qPCR to confirm separation efficiency**
201 Colonies/lawns from each sample, grown on BAP, were harvested into 200 µl PBS using a
202 cotton swab and the DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN) as
203 per the manufacturers protocol. DNA concentration was measured using Qubit™ as per the
204 manufacturers protocol. A no-template negative control was included for each primer pair used,
205 [18] and a standard curve (positive control) was constructed using genomic DNA from each of
206 the six serotypes under investigation. Each qPCR reaction was 25 µl total volume, consisting
207 of iQ™ SYBR® Green Supermix (BioRad), 5 µl of template DNA and 200 nM of each primer.
208 The real time qPCR was run on a BioRad CFX96™ Touch Real-Time qPCR System. The
209 cycling conditions were 1 cycle of denaturation at 95°C for 10 minutes, followed by 40 cycles
210 of 95°C for 15 seconds and 60°C for 1 minute for amplification, and a melt curve from 65°C
211 to 95°C in increments of 0.5°C. For each sample, amplification with primer pairs from both
212 the minority and majority serotype was conducted in duplicate, the mean of duplicates was
213 used for downstream analysis. The concentration of each serotype in a sample was determined
214 by comparing the C_T value to the standard curve for the corresponding serotype.
215
216 **Applications for MBS method - Generating capsule-switch mutants by transformation**
217 To determine whether the MBS method could be used to improve capsule switching
218 experiments (by reducing workload and scaling-up transformations), genomic DNA (gDNA)
219 from four donor serotypes (12F, 23F, 35B, 11B) was transformed individually and as a mixed

220 sample into the recipient D39SΔcps::SweetJanus.[11] The mixed sample was processed with and
221 without the use of the MBS method. An individual transformation of D39 gDNA into
222 D39SΔcps::SweetJanus was included as a positive control.
223
224 With the exception of using Todd Hewitt supplemented with 0.5% Yeast Extract (THY) media
225 for liquid cultures, gDNA was extracted as outlined previously.[11]
226
227 Frozen stocks of D39SΔcps::SweetJanus were inoculated onto BAP and incubated overnight.
228 Cells harvested from the BAP were used to inoculate THY media to a starting OD_{620} of 0.04
229 AU, and were grown at 37°C and 5% CO_2 until late logarithmic phase (OD_{620} ~0.80). For each
230 of the five individual transformations, 1 mL of culture was transferred into a 1.5mL Eppendorf
231 tube, 3 µg/mL of competence stimulating peptide 1 (CSP1) and 4 µg of the appropriate DNA
232 was added. For the mixed transformation, 4 mL of culture was transferred to a 15 mL falcon
233 tube, 3 µg/mL CSP1 and 4 µg of each of the four gDNA templates was added. Cells were
234 incubated for three hours at 37°C. Subsequently, individual transformation and mixed
235 transformation samples were positively selected for by plating on BAP supplemented with 800
236 µg/mL streptomycin and 10% (w/v) sucrose (Strep/Suc plates), and incubated overnight.
237
238 For the five samples that underwent individual transformations, eight colonies each were
239 selected and expanded onto new Strep/Suc plates and incubated overnight. These expanded
240 samples were re-plated onto both Strep/Suc plates, as well as BAP supplemented with 400
241 µg/mL kanamycin (Kan plates), for negative selection, and incubated overnight. Colonies that
242 grew on Strep/Suc but not Kan plates were serotyped to confirm they have successfully gained
243 the capsule.
244
245 For the mixed transformation sample, all colonies were harvested using a cotton swab and
246 resuspended in 1.5 mL Brain Heart Infusion (BHI) media + 10% (v/v) glycerol. As a control,
247 100 µl of the mixed sample was serially diluted to 10^{-6}, then 100 µl of 10^{-4}, 10^{-5} and 10^{-6}
248 dilutions were plated on BAP, and incubated overnight. Following, 100 µl of the mixed sample
249 was aliquoted into four 1.5 mL Eppendorf tubes, centrifuged at 13,000 rpm resuspended in 500
250 µl Buffer 1 and processed through MBS using the appropriate antisera pool(s) for targeting the
251 appropriate serotype. The elution was plated on BAP and incubated overnight. Thirty-two
252 colonies were selected from the mixed sample that did not undergo MBS, and eight colonies
253 were selected from each of the four samples that had undergone MBS. The serotype of all
254 expanded colonies was determined using SSI latex agglutination.
255
256 **Applications for MBS method - Isolating pneumococci from saliva**
257 De-identified pneumococcus-negative saliva samples were obtained from healthy volunteers
258 (< 30 years of age; IRB protocol number 2000029374). De-identified pneumococcus-positive
259 saliva samples were obtained from healthy volunteers (> 60 years of age; IRB protocol number
260 2000026100). The relationship between qPCR cycle threshold (C_T) value and CFU/mL was
261 determined using pneumococcus-negative saliva, spiked with pneumococci (serotype 19A) at
262 a variety of known CFU/mL. The concentration of the 19A stock was determined to be $5x10^9$
263 CFU/mL, which was then serially diluted 1:10 in pneumococcus-negative saliva. After two
264 hours at room temperature, 100 µl of each sample was plated onto BAP supplemented with 10
265 µg/mL gentamycin (Gent plates) and incubated overnight. The lawn of each culture-enriched
266 saliva sample was harvested into 2100 µl BHI + 10% (v/v) glycerol using an L-shaped spreader.
267 gDNA for each sample was extracted using a standard protocol (Table S4), all DNA templates
268 were tested by qPCR for the pneumococcal gene *piaB* using Luna® Universal One-Step RT-

qPCR mix, 2.5 µl template DNA and 200 nM of each primer and probe (Table S2) in a total reaction volume of 20 µl. The cycling conditions were 1 cycle of denaturation at 95°C for 3 minutes, followed by 40 cycles of 98°C for 15 seconds and 60°C for 30 seconds. C_T values were plotted against CFU/mL of 19A in the raw saliva sample (Figure S1).

Using data from Figure S1 in combination with data from previous studies[19] we were able to determine suitable concentrations for spiked-saliva, that reflect levels commonly found in saliva obtained from the healthy individuals during carriage studies. Pneumococcus-negative saliva was spiked with pneumococci (serotype 19A) at varying concentrations ($5x10^4$, $5x10^3$, $5x10^2$ and $5x10^1$ CFU/mL) and left at room temperature for 2 hours. Following, 100 µl of each sample was plated onto Gent plates and incubated overnight. The lawn of the culture-enriched saliva was harvested into 2100 µl BHI + 10% (v/v) glycerol.

From each culture-enriched saliva sample, 10 µl was added to 490 µl Buffer 1, and cell separated using the MBS protocol, with the following modifications. The primary incubation step was conducted using SSI antisera (~16.8 µg total protein) and SunFire Bio monoclonal antibody (mAb) (~16.8 µg total protein) combined. The secondary incubation was conducted using ~48 µg total protein of anti-mouse IgG MicroBeads (Miltenyi Biotech) to target the mAb only. As a negative control, culture-enriched saliva samples did not undergo MBS and were instead serially diluted to 10^{-6}, the 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated on BAPs and incubated overnight.

Colonies that looked like pneumococci (small, grey, moist colonies with a green zone of alpha-hemolysis), were isolated and expanded onto new BAP: sixteen colonies from each sample (with and without cell separation) that contained $5x10^4$, $5x10^3$, $5x10^2$ CFU/mL of 19A in raw saliva samples, and 24 colonies from the sample (with and without cell separation) that contained $5x10^1$ CFU/mL of 19A in raw saliva. Each expanded colony was optochin tested to confirm whether it was pneumococcus (optochin sensitive) or another oral bacteria (optochin resistant). Where a ring of optochin sensitivity was observed but a second (contaminating) bacteria with optochin resistance was also present or, where satellite colonies of pneumococcus were present within the zone of inhibition, samples were considered 'pneumococcal colonies' since pure pneumococci can be isolated from the contaminant.

RESULTS

The MBS proof of concept experiments showed that for all six serotypes, the minority serotype was successfully enriched from ~0.1% starting percentage to between 13% (serotype 14) and 90% (serotype 3) post MBS, corresponding to a 100-to-900-fold enrichment (Figure 2a). The final percentage of the minority varied between serotypes but was relatively consistent between the three replicates. There was generally good concordance in the estimated MBS efficiency as determined by the qPCR and colony counting (Figure 2b), however efficiency determined by colony counting seemed to be higher and lower than with qPCR for serotype 14 and 3, respectively. Eight colonies from each elution plate were selected at random and in every single case, minority serotype colonies were identified by serotyping (Table 1). This demonstrates that this technique can be used to recover a desired serotype from a dual mixture.

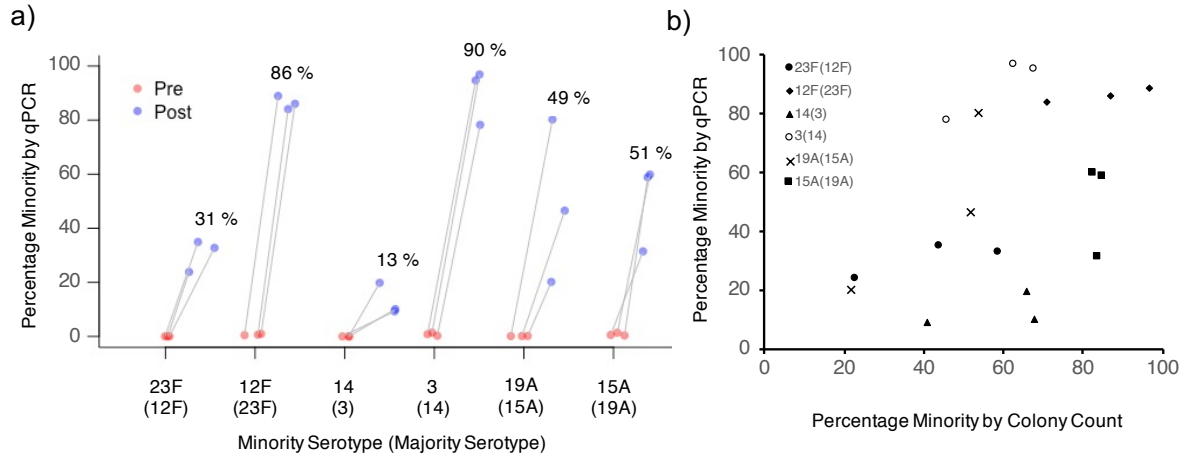


Figure 2. Efficiency of minority strain enrichment using the MBS method on six serotypes. A) Percentage minority serotype present prior to MBS (Pre) and after MBS (Post). The average percentage minority in the post sample is presented above the data points, b) Percentage minority serotype present after MBS (Post) as determined by qPCR and colony counting. Minority and majority serotypes are displayed in the legend in the following format, minority(majority). Results are shown in triplicate for each of three serotype pairs where each serotype of the pair was tested as the minority serotype.

A secondary analysis was conducted to identify whether serotype 3Muc was also enriched with a similar efficiency as serotype 3SCV, and to gain insight into how separation efficiency varies when the majority serotype of the pair is altered. MBS was conducted on Pair 4 (23F and 3SCV and Pair 5 (23F and 3Muc). The results were compared to MBS results obtained previously for enrichment of minority serotypes 23F or 3SCV when paired with another majority serotype (namely serotype 12F and serotype 14 from Pair 1 and Pair 2, respectively). The percentage enrichment for both 23F and 3 remained similar even when the majority serotype of the pair was altered (Figure 3). Furthermore, it demonstrates that the MBS method permits successful enrichment of both SCV and mucoid variants of serotype 3, and that the efficiency is similar regardless of the morphology. In all cases minority serotype single colonies were isolated from the elution plate by selection of single colonies and confirmed to be the desired serotype using SSI latex agglutination (Table 1).

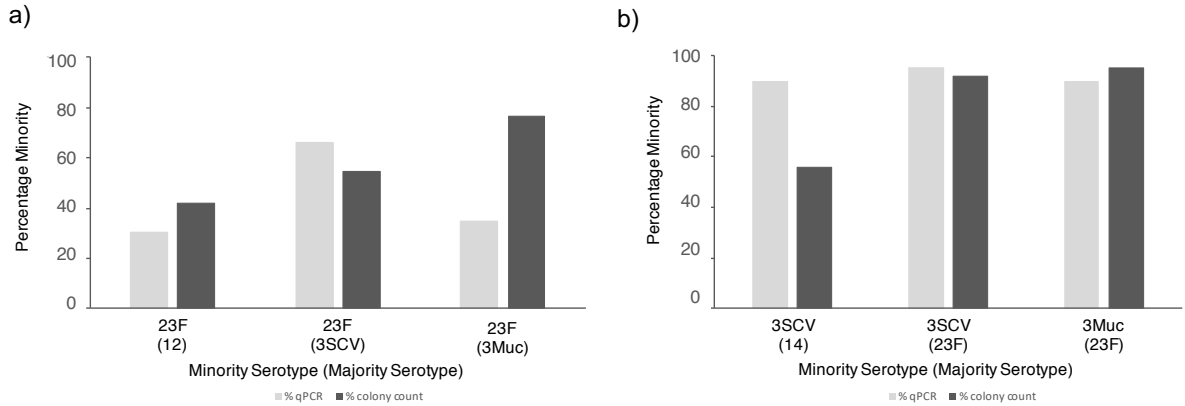


Figure 3. Final percentage of minority serotype after MBS. Averages of triplicate results from Figure 1 are shown for 23F (when with a majority of 12F) and 3 (when with a majority of 14). Averages of duplicate results were plotted for 23F (when with a majority of 3SCV), 23F (when with a majority of 3Muc), 3SCV (when with a majority of 23F) and 3Muc (when with a majority of 23F). Percentage minority from both colony counting and qPCR methods is shown.

Table 1. Total number of colonies on the plain blood agar elution plate (out of eight selected at random) that were positive for the minority serotype by SSI latex agglutination. Positive results reported were those that tested positive with minority serotype antisera and negative with majority serotype antisera.

Serotype Pairs		Number of colonies of the minority serotype following MBS (from eight selected at random from plain blood agar plates)		
Minority Serotype	Majority Serotype	Rep1	Rep2	Rep3
23F	12F	5	5	5
12F	23F	8	6	5
14	3 (SCV)	4	4	3
3 (SCV)	14	7	6	6
19A	15A	1	7	3
15A	19A	7	6	5
23F	3(SCV)	4	2	N/A
3(SCV)	23F	6	8	N/A
23F	3(Muc)	6	8	N/A
3(Muc)	23F	5	7	N/A

The primary analysis specifically used serotype pairs that could be distinguished using two unique pools of antisera. MBS was then tested on eight serotype pairs using only a single antisera pool. A total of six antisera pools (H, P, B, E, R, H and Q) were tested and all were able to successfully enrich a ~0.1% minority serotype to between 10% and 99% in the final sample (Figure 4a).

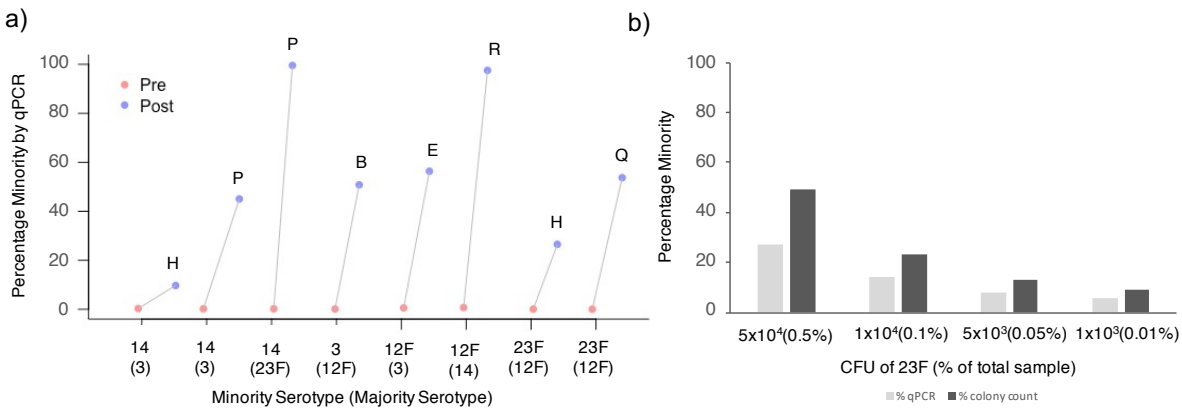


Figure 4. Efficiency of minority enrichment using the MBS method on eight serotypes. A) Percentage minority serotype present prior to MBS (Pre) and after MBS (Post), b) Percentage minority 23F present after MBS (Post) as determined by qPCR and colony counting. Minority and majority serotypes are displayed in the following format, minority(majority). Results shown are singlicate data points only.

Additional analysis aimed to determine whether enrichment was constant at different % minorities. The 23F and 12F pair were used with the majority serotype (12F) remaining constant at $1x10^7$ CFUs and the minority serotype (23F) at four different concentrations in the initial sample. Enrichment of the minority serotype can be achieved even when the starting percentage of a minority serotype is as low as $1x10^3$ CFUs. However, as the initial % minority decreases the percentage minority recovered following MBS also decreases. For initial samples containing $5x10^4$, $1x10^4$, $5x10^3$ and $1x10^3$ CFU's of minority serotype 23F, the corresponding percentage of 23F present in the final samples were 27%, 14%, 8% and 6% respectively as determined by qPCR, or 49%, 23%, 13% and 9% respectively as determined by colony counting (Figure 4b).

366
367 In order to separate serogroups that share reactivity to one antiserum pool, the MBS method
368 should be used with only a single antiserum pool. We therefore investigated outcomes when
369 using one or two antisera Pools and compared the efficiency of antisera pools in the presence
370 of different majority serotypes. MBS of serotype 14 from a majority serotype 3, using both
371 antisera Pool H and Pool P, resulted in the final sample containing ~13% of serotype 14.
372 However, use of only Pool H or Pool P, at an equal final volume to the combined pools, resulted
373 in serotype 14 being 10% and 45% of the final samples respectively. Therefore, in this example,
374 Pool P alone achieves the greatest efficiency of MBS, but in the absence of knowing which
375 antisera is more efficient, and if the serotype pairs permit dual use, it would be prudent to
376 combine both antisera pools. Furthermore, we confirm that the overall efficiency of enrichment
377 achieved by any antisera pool, is not only dependent upon the minority serotype alone, but also
378 the majority serotype. The final percentage of serotype 14 following MBS (using Pool P) from
379 a majority serotype 23F, is 99%, more than double the percentage of serotype 14 present
380 following MBS (using Pool P) from a majority serotype 3.
381
382 **Generating capsule-switch mutants by transformation**
383 For transformations conducted individually, the positive control (D39 gDNA) was successfully
384 transformed into D39SΔcps::SweetJanus at the cps locus, with 8/8 colonies serotyping as
385 serotype 2. gDNA from serotype 23F and serotype 35B was also found to transform into
386 D39SΔcps::SweetJanus at the cps locus, with 7/8 and 8/8 colonies serotyping as 23F and 35B
387 respectively. Conversely, 0/8 colonies were serotyped as 12F or 11B for these transformations,
388 suggesting that transformation may not have occurred or may have occurred at very low
389 efficiency (Table 2).
390

391 *Table 2. Number of positive transformations (per 8 colonies) when individual transformations are conducted using the*
392 *standard transformation procedure and selection methods.*

Serotype	Individual Transformations # of transformants
D39	8/8
12F	0/8
23F	7/8
35B	8/8
11B	0/8

393
394 Transformations conducted in higher throughput (i.e. gDNA from 4 serotypes combined) show
395 that in the absence of cell separation, even when gDNA samples are mixed, it is possible to
396 isolate transformation to 23F and 35B with 9/32 and 11/32 colonized serotyping as these
397 serotypes, respectively. Similarly, to the results seen in the individual transformations,
398 transformants of 12F or 11B were not identified (0/32). The mixed transformations that were
399 subsequently cell separated with MBS to enrich for the desired serotype showed that 23F, 35B
400 and 11B were successfully transformed, with 8/8, 5/8 and 7/8 colonies identified to be 23F,
401 35B and 11B, respectively (Table 3). This confirms that 11B is able to transform into
402 D39SΔcps::SweetJanus at the cps locus but this likely occurs at a lower efficiency, or that
403 acquisition of the *cps* locus requires additional non-cps recombination from other serotypes in
404 the mix. For serotype 12, colonies were observed on the BAP following MBS, however 0/8
405 were identified to be 12F transformants, therefore this transformation may only occur at very
406 low frequencies, under very specific conditions, or not at all. Of the 8 colonies selected from
407 the cell separation enriching for 12F, 6/8 were serotype 23F, 1/8 were serotype 11B and only

1/8 was untransformed. For serotype 35B, 1/3 was serotype 23F and 2/3 were untransformed. For serotype 11B, 1/8 was serotype 23F. The presence of these contaminating serotypes suggests that the antisera/antibodies used in MBS have some non-specific cross-reactivity.

Table 3. Number of positive transformations for each serotype from a mixed transformation containing gDNA of 12F, 23F, 35B and 11B, in the presence of cell separation (8 colonies picked per sample) and in the absence of cell separation picked (total of 32 colonies picked).

Serotype	Control (no cell separation) # of transformants	Cell Separation # of transformants
12F	0/32	0/8
23F	9/32	8/8
35B	11/32	5/8
11B	0/32	7/8

Isolating pneumococci from saliva

To determine if the MBS method could be used to enrich for a known serotype in pneumococcus-positive saliva, we spiked two saliva samples (A and B) which tested qPCR-negative for pneumococcal genes *piaB* and *lytA,* with varying concentrations of serotype 19A and compared the success of identifying pneumococcal colonies in the presence and absence of MBS (Table 4). For both saliva A and saliva B, at all concentrations of 19A, the MBS method resulted in equal or improved isolation of pneumococcal colonies. In saliva A, the MBS method was still able to enrich for pneumococcus when the concentration of 19A was $5x10^1$ CFU/mL in raw saliva, however for Saliva B the MBS method was only successful at a 19A concentration of $5x10^3$ CFU/mL in raw saliva. The sensitivity of this assay is therefore dependent upon not only the concentration of pneumococci in the sample but also the composition of saliva itself, and may vary from sample to sample. The MBS method was then tested on a saliva sample that had tested qPCR-positive for serotype 15B/C but from which we had been unable to isolate pneumococcus using the standard culture-based dilution method.[20] Here, the MBS method successfully enriched for pneumococcus in the sample, and of the 32 colonies selected, 29 were confirmed to be pneumococcus.

Table 4. Isolation of optochin-sensitive pneumococcal colonies using the standard dilution method compared to the MBS method. Two saliva samples (A and B) were spiked with four concentrations of serotype 19A. Pure pneumococcal colonies were identified by a zone of inhibition around the optochin disk, any colonies that were mixed colonies (i.e. those with a zone of inhibition but some secondary growth (a non-pneumococcal contaminant) growing within the zone of inhibition, or, had satellite colonies appearing withing the zone of inhibition) were considered to be successful isolation of pneumococcus).

CFU/mL of *S. pneumoniae* 19A	# of 19A colonies identified in Saliva A		# of 19A colonies identified in Saliva B	
	With MBS method	Standard dilution method	With MBS method	Standard dilution method
$5x10^4$	15/16	15/16	15/16	1/16
$5x10^3$	15/16	5/16	5/16	0/16
$5x10^2$	16/16	2/16	0/16	0/16
$5x10^1$	6/24	0/24	0/24	0/24

DISCUSSION

We developed the MBS method that can enrich for a desired serotype from a mixed-serotype sample in a laboratory setting. Enrichment using the MBS method was demonstrated for six serotypes (23F, 12F, 3, 14, 15A and 19A) including two serotypes with more unique capsules (serotype 3 and serotype 14). We were able to demonstrate two use cases for this method: separation of capsule switch mutants (from mixed transformation experiments) and, enrichment of pneumococcus from saliva samples.

In the primary analysis used to develop the MBS method, we show that all six of the minority serotypes investigated (23F, 12F, 14, 3, 19A and 15A) can be successfully enriched from ~0.1% of an initial mixed serotype sample to up between 13% and 90% in the final sample. The inclusion of Serotype 3 (which exists as single colony and mucoid variants) and serotype 14 (which has an uncharged capsule) in this panel, showed that this method is suitable for serotypes with more unique capsules. Two methods; colony counting, and qPCR were employed in order to assess efficiency of the MBS method. The estimates from both methods were broadly concurrent but there are a few examples where the efficiency estimates do differ. This may be explained by the formation of varying chain lengths in pneumococcus, such that if the two serotypes in a pair form vastly different length chains, the estimations of efficiency may be biased. A serotype that readily forms chains would result in an underestimation of its presence in the sample using the colony counting method, but qPCR would provide a more accurate estimation. Despite some differences in efficiency estimates between colony counting and qPCR methods, we were able to successfully isolate minority serotype colonies post MBS in all cases. This demonstrates a tangible utility for this method in the laboratory setting. When separating a mixture of cells only a small number of colonies must be isolated to identify the desired serotype. This method therefore allows for the easy recovery of serotype-specific *S. pnuemoniae* isolates.

In the secondary supporting analysis, we compared how enrichment of a minority serotype varied when in the presence of different majority serotypes. A minority serotype 23F was paired with a majority serotype of either 12F or 3, and minority serotype 3 was paired with a majority serotype of either 14 or 23F. With minority 23F, some variation in efficiency of MBS was noted when the majority serotype was changed, however for minority serotype 3, the enrichment efficiency remained very similar despite the change in majority serotype pair. This suggests that the serotype with which the minority is mixed may have some impact on the efficiency of MBS, but it is likely primarily determined by the avidity of the antisera for the desired serotype. Unlike the majority of pneumococcal serotypes, serotype 3 utilizes the synthase-dependent pathway for CPS production, resulting in non-covalently bound CPS which can be released from the glycolipids or synthase.[21] The CPS of serotype 3 is not covalently linked to the peptidoglycan and can be released,[22] which leads to a reduction in the protective effect of anti-Type 3 CPS antibodies induced by the PCV13,[23] we were therefore surprised to find that the MBS method can successfully extract serotype 3 from a mixed sample. This success may be explained by the fact that the cells are not actively growing and likely therefore not releasing CPS into the environment. Furthermore, it is intriguing but reassuring that the efficiency of enrichment between mucoid and SCV serotype 3 is very similar; the MBS method can be successfully used on serotype 3 samples, which are of particular interest due to the reduced effectiveness of PCV13 on serotype 3 IPD.[24–26]

We demonstrate that good separation can be achieved with only one unique antiserum, meaning that serotypes with cross-reactivity to one antiserum can still be separated using this method. As expected, we demonstrate that the efficiency of enrichment achieved by each of the two

489 antisera pools is not equal and therefore, depending on the desired serotype one antisera may
490 be preferred over another. Furthermore, enrichment of a serotype can occur even when a
491 serotype is present at only 0.01% of the total sample ($1x10^3$ minority serotype with $1x10^7$
492 majority serotype).
493
494 A key limitation of the MBS method in general, is that due to cross-reactivity within
495 serogroups, SSI antisera Pools can only be used to separate *S. pneumoniae* serotypes belonging
496 to different serogroups. However, use of type-specific antisera or a mAb instead of pooled
497 antisera would circumvent this limitation. Another limitation is the total proportion of minority
498 cells that can be recovered. While enrichment from 0.1% up to >10% has been demonstrated,
499 it is worth noting that only a small proportion (~1%) of the total minority cells present in the
500 initial mixture are successfully extracted. This may be overcome by increasing antibody
501 incubation periods or antibody concentration to increase binding capacity.
502
503 Having optimized the MBS method, we evaluated its potential for laboratory applications. The
504 MBS method allows for higher throughput generation of capsule-switch variants, by combining
505 the method with the existing techniques, such that gDNA from multiple donor serotypes can
506 be transformed into a recipient serotype in a single mixed reaction. After initial selection for
507 transformants on selection media, the MBS method can be used to separate out the individual
508 transformants in a serotype-specific manner. Mixed transformations would permit higher
509 throughput generation of capsule-swapped variants, the potential to determine comparative
510 efficiency and a significant reduction in BAP usage and labor intensity. However, in the
511 absence of MBS, whilst isolation of different serotypes is comparable to that observed in
512 individual transformations, the benefits are offset by the lengthy and time-consuming process
513 of serotype screening each isolate by latex agglutination. Therefore, to harness the true benefit
514 of mixed transformations a simple and easy technique to select for different serotypes is
515 required. The MBS method was used to isolate multiple serotypes, from a mixed sample of
516 four serotypes. The MBS method outperformed the individual transformations and the mixed
517 transformation (without MBS), by successfully isolating an additional serotype (11B) which
518 was not isolated using the other methods. This suggests that the MBS method may be
519 particularly useful to enrich for serotypes which transform with low efficiency. The MBS
520 technique was not 100% specific, and a small amount of cross-reactivity was observed,
521 however, since each sample is enriched for the desired serotype, and the serotype of each
522 colony is confirmed by latex agglutination, these contaminants are of little concern are for this
523 particular application.
524
525 We also show that the MBS method can be modified to successfully enrich for pneumococci
526 of a known serotype from saliva samples. Enrichment is possible even in saliva samples where
527 pneumococci is present at very low concentrations ($5x10^1$ CFU/mL), for which isolation of
528 pneumococci using standard methods is typically very challenging. This permits easy
529 identification and isolation of pneumococci present in saliva at concentrations too low to detect
530 using standard dilution and plating methods. The use of SSI antisera alone on a polymicrobial
531 sample such as saliva was problematic due to antisera reactivity with non-pneumococcal
532 bacteria present in saliva. In general, we found that the SSI antisera outperformed mAbs in
533 terms of total number of pneumococcal colonies isolated, we hypothesize that this is due to the
534 increased avidity of antisera (presence of IgA, IgM) which agglutinates pneumococci
535 increasing the overall yield during MBS. Therefore, to take advantage of the increased avidity
536 of antisera and simultaneously the high specificity of mAbs, we combined both in the primary
537 incubation step, but only targeted the mAb in the secondary antibody step. While the elution
538 from saliva samples was not 100% pure pneumococci, contaminating non-pneumococcal

539 bacteria was reduced, and identification and selection of single pneumococcal colonies was
540 improved when compared to the standard dilution and plating method. The enrichment
541 observed varies depending on concentration of pneumococci present in the sample, but also on
542 the saliva composition itself. The composition of bacterial community in saliva varies between
543 different age groups[27] and so the success of the MBS method will likely vary accordingly.
544 Since the MBS method can work on saliva containing very low concentrations of pneumococci
545 it may be particularly useful for the isolation of minority serotypes in samples obtained from
546 multiply colonized individuals. Previous research shows that 52% of Dutch primary school
547 children tested positive for multiple pneumococcal serotypes,[19] however, conventional
548 serotyping methods often result in an underestimation of multiply colonized individuals.[28]
549 Detection of multiple serotypes is possible using serologic, biochemical (Mass Spectroscopy
550 and nuclear Magnetic Resonance), and genotypic (sequencing, qPCR and microarrays)
551 methods, however, until now, attempting to isolate minority serotypes by conventional
552 methods (single colony selection) has been laborious and time consuming.[21]
553
554 **CONCLUSION**
555 The MBS method allows for the successful enrichment of a minority serotype from a dual
556 sample containing two *S. pneumoniae* serotypes belonging to different serogroups. Using this
557 method, an initial sample containing 0.01-0.1% of a desired serotype, can be enriched to up to
558 90% in the final sample. Enrichment to between 10 and 90% was demonstrated for six minority
559 serotypes, and half of the commercially available antisera pools (Pools B, E, H, P, Q, R and S)
560 were tested. We demonstrate two different applications for this technique: separating capsule-
561 switch variants from mixed transformation experiments and enriching for pneumococci of a
562 known serotype from saliva. The MBS technique can be used successfully to enrich for
563 serotypes which are present at very low-levels in both mixed cultures and more complex
564 polymicrobial sample types (such as saliva), making it a versatile and important technique for
565 a multitude of applications.
566
567 **AUTHOR CONTRIBUTIONS**
568 Conceptualization, DMW and ALW; Methodology, AY, DMW and ALW; Investigation,
569 AY, EH, SM, MH, DYC, HE, JR; Analysis, AY and DW; Writing – initial draft, AY; Writing
570 – Review & Editing, DMW, ALW and DYC; Supervision, ALW, DMW, JR.
571
572 **AUTHOR DECLARATION**
573 DMW has received consulting fees from Pfizer, Merck, GSK, Affinivax, and Matrivax and is
574 PI on research grants from Pfizer and Merck to Yale. ALW has received consulting fees from
575 Pfizer and is PI on research grants from Pfizer to Yale. The other authors declare no conflict
576 of interest.
577
578 **FUNDING**
579 This work was supported by R01AI123208 from NIAID/NIH (DMW). The content is solely
580 the responsibility of the authors and does not necessarily represent the official views of the
581 National Institutes of Health.

582 **Supplemental Information**

583

584 *Table S1. Serotypes and corresponding antisera pools used for MBS (rabbit antiserum; SSI Diagnostica, Hillerød,*
585 *Denmark) and for serotyping (ImmuLex™ Pneumotest; SSI Diagnostica).*

Serotype	Pooled Antisera for Neufeld	ImmuLex™ Pneumococcus Antisera
12F	Pool E #16733 Pool R #16741	Pool E #52394 Pool R #52401
23F	Pool H #16736 Pool Q #16740	Pool H #52397 Pool Q #52400
3	Pool B #16728 Pool R #16741	Pool B #52391 Pool R #52401
14	Pool P #16739 Pool H #16736	Pool P #52399 Pool H #52397
19A	Pool B #16728 Pool P #16739	Pool B #52391 Pool P #52399
15A	Pool H #16736 Pool S #16742	Pool H #52397 Pool S #52402
2 (D39)	Pool A #16725 Pool T #16743	Pool A #52390 Pool T #52403
35B	Pool G #16735	Pool G #52396
11B	Pool D #16731 Pool T #16743	Pool D #52393 Pool T #52403

586

587

588 *Table S2. PiaB qPCR primers and probes.*

Primer/Probe Name	Primer Sequence
PiaB Forward	CATTGGTGGCTTAGTAAGTGCAA
PiaB Reverse	TACTAACACAAGTTCCTGATAAGGCAAGT
PiaB Probe	TGTAAGCGGAAAAGCAGGCCTTACCC

589

590

591 *Table S3. KingFisher Flex MBS Protocol*

Pick up tips
96DW tip comb
Collect Beads
Count 2 seconds 5
Mix
Release Beads Medium mix 0:01:00min Count 3 seconds 1
Release Beads
Fast 00:00:05
Leave
Leave Plate

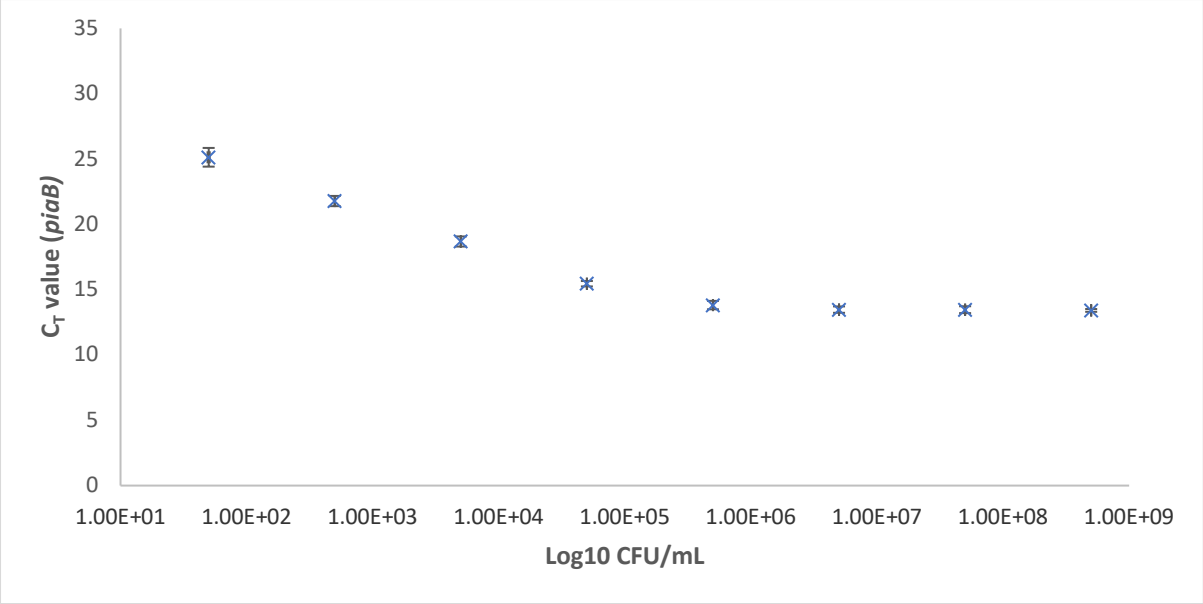
592

593

594 *Table S4. KingFisher Flex DNA extraction protocol*

Pick up tips
96DW tip comb
Predigest
56ºC Preheat on Mixing medium 0:10:00 minutes
Heat kill pk 95 ºC

Preheat off Mixing medium 0:10:00 minutes
Initial Mixing
Release bead 0:00:00 Slow mix 0:01:00min Medium mix 0:04:00min Loop1
Add Proteinase K and Binding
Add 10 µl pro k, 25 µl binding beads and 530 µl binding buffer
PK Digest
Release bead 0:00:00 Preheat on 65ºC Mix Fast 0:15:00 min Collect beads 5 count 1 second Collect beads: Count 5 time 0
Wash 1
Release beads 20 second bottom mix Mixing 0:00:10 bottom mix 0:00:10 Fast mix Loop 3 times Collect beads: Count 5
Wash 2
Release beads 0:00:20 FAST Mixing 0:00:10 bottom mix 0:00:10 Fast mix Loop 2 times Collect beads: 4 count 1 second
Wash 3
Release beads 0:00:00 Mix 0:00:30 Fast Collect beads: Count 5 seconds 0
Dry
0:02:00 Dry type (outside well)
Elution 1
Release Beads 0:00:00 Heating and Cooling Preheat ON 75°C Mixing 0:00:15 bottom mix 0:00:45 Medium mix Loop 6 times Collect Beads Count 1 seconds 0
Final Beads
Mixing 0:02:00 slow
Elution 2
Release Beads 0:00:00 Heating and Cooling Preheat ON 75°C Mixing 0:00:15 bottom mix 0:00:45 Medium mix Loop 6 times Collect Beads Count 1 seconds 0
Final Beads
Mixing 0:02:00 slow
Leave Tip
Tip Plate
HOLD Temp
Elution Plate 1 10°C



599
600 *Figure S1. Detection of pneumococcus gene piaB (C_T value) when culture-enriched saliva samples were tested with qPCR,*
601 *and the corresponding CFU/mL of S. pneumoniae 19A that was spiked into each raw saliva sample. Raw saliva was confirmed*
602 *to be pneumococcus-negative (C_T >40) by qPCR towards piaB. Data shown as mean and standard deviation of biological*
603 *triplicate data.*

604 **Supplemental File - Raw Data**
605

606

607 **References**

608 1. CDC, C. for D. C. and P. Updated Recommendations for Prevention of Invasive
609 Pneumococcal Disease Among Adults Using the 23-Valent Pneumococcal
610 Polysaccharide Vaccine (PPSV23). *MMWR* **59**, 1102–1106 (2010).
611 2. Troeger, C. *et al.* Estimates of the global, regional, and national morbidity, mortality, and
612 aetiologies of lower respiratory infections in 195 countries, 1990–2016: a systematic
613 analysis for the Global Burden of Disease Study 2016. *Lancet Infect. Dis.* **18**, 1191–1210
614 (2018).
615 3. Ganaie, F. *et al.* A New Pneumococcal Capsule Type, 10D, is the 100th Serotype and
616 Has a Large *cps* Fragment from an Oral Streptococcus. *mBio* **11**, e00937-20,
617 /mbio/11/3/mBio.00937-20.atom (2020).
618 4. Weinberger, D. M., Malley, R. & Lipsitch, M. Serotype replacement in disease after
619 pneumococcal vaccination. *The Lancet* **378**, 1962–1973 (2011).
620 5. Nahm, M. H. *et al.* Pneumococci Can Become Virulent by Acquiring a New Capsule
621 From Oral Streptococci. *J. Infect. Dis.* jiz456 (2019) doi:10.1093/infdis/jiz456.
622 6. Chochua, S. *et al.* Invasive Serotype 35B Pneumococci Including an Expanding Serotype
623 Switch Lineage, United States, 2015–2016. *Emerg. Infect. Dis.* **23**, 922–930 (2017).
624 7. Moore, M. R. *et al.* Population Snapshot of Emergent Streptococcus pneumoniae
625 Serotype 19A in the United States, 2005. *J. Infect. Dis.* **197**, 1016–1027 (2008).
626 8. Croucher, N. J. *et al.* Selective and Genetic Constraints on Pneumococcal Serotype
627 Switching. *PLOS Genet.* **11**, e1005095 (2015).
628 9. Griffith, Fred. The Significance of Pneumococcal Types. *J. Hyg. (Lond.)* **27**, 113–159
629 (1928).

630 10. Li, Y., Thompson, C. M. & Lipsitch, M. A Modified Janus Cassette (Sweet Janus) to
631 Improve Allelic Replacement Efficiency by High-Stringency Negative Selection in
632 Streptococcus pneumoniae. *PLoS ONE* **9**, e100510 (2014).
633 11. Echlin, H. & Rosch, J. W. Advancing Genetic Tools in Streptococcus pneumoniae. *Genes*
634 **11**, 965 (2020).
635 12. Kelly, T., Dillard, J. P. & Yother, J. Effect of genetic switching of capsular type on
636 virulence of Streptococcus pneumoniae. *Infect. Immun.* **62**, 1813–1819 (1994).
637 13. Hathaway, L. J. *et al.* Capsule Type of Streptococcus pneumoniae Determines Growth
638 Phenotype. *PLoS Pathog.* **8**, e1002574 (2012).
639 14. Satzke, C. *et al.* Standard method for detecting upper respiratory carriage of
640 Streptococcus pneumoniae: Updated recommendations from the World Health
641 Organization Pneumococcal Carriage Working Group. *Vaccine* **32**, 165–179 (2013).
642 15. Krone, C. L. *et al.* Carriage of Streptococcus pneumoniae in Aged Adults with Influenza-
643 Like-Illness. *PLOS ONE* **10**, e0119875 (2015).
644 16. Wyllie, A. L. *et al.* Molecular surveillance on Streptococcus pneumoniae carriage in non-
645 elderly adults; little evidence for pneumococcal circulation independent from the
646 reservoir in children. *Sci. Rep.* **6**, 34888 (2016).
647 17. Allegrucci, M. & Sauer, K. Characterization of Colony Morphology Variants Isolated
648 from Streptococcus pneumoniae Biofilms. *J. Bacteriol.* **189**, 2030–2038 (2007).
649 18. Pimenta, F. C., Roundtree, A., Soysal, A., Bakir, M. & McGee, L. Sequential Triplex
650 Real-Time PCR Assay for Detecting 21 Pneumococcal Capsular Serotypes That Account
651 for a High Global Disease Burden. *J. Clin. Microbiol.* **51**, 6 (2013).
652 19. Wyllie, A. L. *et al.* Streptococcus pneumoniae in Saliva of Dutch Primary School
653 Children. *PLOS ONE* **9**, e102045 (2014).

654 20. Trzciński, K. *et al.* Superiority of Trans-Oral over Trans-Nasal Sampling in Detecting
655 Streptococcus pneumoniae Colonization in Adults. *PLoS ONE* **8**, e60520 (2013).
656 21. Geno, K. A. *et al.* Pneumococcal Capsules and Their Types: Past, Present, and Future.
657 *Clin. Microbiol. Rev.* **28**, 871–899 (2015).
658 22. Cartee, R. T., Forsee, W. T., Schutzbach, J. S. & Yother, J. Mechanism of Type 3
659 Capsular Polysaccharide Synthesis inStreptococcus pneumoniae *. *J. Biol. Chem.* **275**,
660 3907–3914 (2000).
661 23. Choi, E. H., Zhang, F., Lu, Y.-J. & Malley, R. Capsular Polysaccharide (CPS) Release by
662 Serotype 3 Pneumococcal Strains Reduces the Protective Effect of Anti-Type 3 CPS
663 Antibodies. *Clin. Vaccine Immunol.* **23**, 162–167 (2016).
664 24. Horácio, A. N. *et al.* Serotype 3 Remains the Leading Cause of Invasive Pneumococcal
665 Disease in Adults in Portugal (2012–2014) Despite Continued Reductions in Other 13-
666 Valent Conjugate Vaccine Serotypes. *Front. Microbiol.* **7**, (2016).
667 25. Silva-Costa, C. *et al.* Pediatric Complicated Pneumonia Caused by Streptococcus
668 pneumoniae Serotype 3 in 13-Valent Pneumococcal Conjugate Vaccinees, Portugal,
669 2010–2015. *Emerg. Infect. Dis.* **24**, 1307–1314 (2018).
670 26. Slotved, H.-C. *et al.* The incidence of invasive pneumococcal serotype 3 disease in the
671 Danish population is not reduced by PCV-13 vaccination. *Heliyon* **2**, e00198 (2016).
672 27. Xu, X. *et al.* Oral cavity contains distinct niches with dynamic microbial communities.
673 *Environ. Microbiol.* **17**, 699–710 (2015).
674 28. Huebner, R. E. *et al.* Lack of utility of serotyping multiple colonies for detection of
675 simultaneous nasopharyngeal carriage of different pneumococcal serotypes. *Pediatr.*
676 *Infect. Dis. J.* **19**, 1017–1020 (2000).
677