

1 **Title**

2 Evaluation of pneumococcal serotyping in nasopharyngeal carriage isolates by latex  
3 agglutination, whole genome sequencing (PneumoCaT) and DNA microarray in a high  
4 pneumococcal carriage prevalence population in Malawi

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22

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24 *Streptococcus pneumoniae*, serotyping, latex agglutination, microarray, whole genome  
25 sequencing, methodology, Africa

### 26 **Running title**

27 Serotyping for pneumococcal carriage

### 28 **Summary of the article's main points**

29 Assessment of pneumococcal serotype distribution associated with colonization and disease  
30 is essential for evaluation of pneumococcal vaccines. Latex serotyping is adequate for  
31 surveillance, but whole genome sequencing and microarray should be considered at  
32 regional reference laboratories.

## 33 **Abstract**

34 **Background.** Accurate assessment of the serotype distribution associated with  
35 pneumococcal colonization and disease is essential for the evaluation and formulation of  
36 pneumococcal vaccines and informing vaccine policy.

37

38 **Methods.** We evaluated pneumococcal serotyping concordance between latex  
39 agglutination, PneumoCaT by whole genome sequencing (WGS) and DNA microarray using  
40 samples from community carriage surveillance in Blantyre, Malawi. Nasopharyngeal swabs  
41 were collected, following WHO recommendations, between 2015 and 2017, using stratified  
42 random sampling among study populations. Participants included healthy children 3–6 years  
43 old (PCV13 vaccinated as part of EPI), healthy children 5–10 years (age-ineligible for  
44 PCV13), and HIV-infected adults (18–40yrs) on ART. For phenotypic serotyping we used a  
45 13-valent latex kit (SSI, Denmark). For genomic serotyping we applied PneumoCaT pipeline  
46 to whole genome sequence libraries. For molecular serotyping by microarray we used the  
47 BUGS Bioscience DNA microarray.

48

49 **Results.** 1347 samples were analysed. Concordance was 90.7% (95% CI: 89.0–92.2)  
50 between latex and PneumoCaT; 95.2% (93.9–96.3) between latex and microarray; and  
51 96.6% (95.5–97.5) between microarray and PneumoCaT. By detecting carried vaccine  
52 serotype (VT) pneumococcus in low relative abundance (median 8%), microarray increased  
53 VT detection by 31.5% compared to latex serotyping.

54

55 **Conclusion.** All three serotyping methods were highly concordant in identifying dominant  
56 serotypes. Latex serotyping is accurate in identifying vaccine-serotypes and requires the  
57 least expertise and resources for field-implementation and analysis. However, WGS, which  
58 adds population structure, and microarray, which adds multiple-serotype carriage, should be

59 considered at regional reference laboratories while investigating the importance of VT in low  
60 relative abundance in transmission and disease.

## 61 Introduction

62 *Streptococcus pneumoniae* colonises the nasopharynx of healthy individuals. Although  
63 carriage is usually asymptomatic, nasopharyngeal (NP) colonization is a prerequisite for  
64 disease including otitis media, sinusitis, pneumonia, bacteraemia, and meningitis. (1) The  
65 pneumococcus is estimated to be responsible for over 318 000 (uncertainty ratio [UR]: 207  
66 000–395 000) deaths every year in children aged 1 to 59 months, with the highest mortality  
67 burden among African children.(2) Evidence also shows that HIV-infected children and  
68 adults are at significantly higher risk of invasive pneumococcal disease (IPD) than their HIV-  
69 uninfected counterparts. (3, 4)

70

71 Current multivalent pneumococcal conjugate vaccines (PCV) target subsets of the nearly  
72 100 capsular serotypes known to be expressed by the pneumococcus. PCV reduces  
73 nasopharyngeal carriage of the pneumococcal serotypes they contain, known as vaccine  
74 serotypes (VT). With reduced carriage among the vaccinated there is then reduced risk of  
75 VT-IPD (direct protection) and reduced transmission, therefore reduced risk of VT-IPD  
76 among those PCV-unvaccinated (indirect protection). However, non-vaccine serotypes  
77 (NVT) have the potential to fill the ecological niche, becoming more common in carriage and  
78 disease. (5-7) This phenomenon, known as serotype replacement, may be more  
79 pronounced in low-income settings because of higher prevalence, density and diversity of  
80 pneumococcal carriage, and represents a considerable risk to the global pneumococcal  
81 immunisation strategy.(8) Serotype distribution differs between continents as well as  
82 individual countries. (9) Given these differences, accurate assessment of the serotype  
83 distribution associated with both pneumococcal colonization and pneumococcal disease is  
84 needed in the evaluation, formulation and delivery of pneumococcal vaccines.

85

86 A pneumococcal serotyping method suitable for use in robust carriage and surveillance  
87 studies should therefore, at minimum, be accurate in its serotype assignment, particularly in

88 relation to VTs. Additional desirable parameters include detection of most or all serotypes,  
89 ability to detect multiple serotypes in carriage (common in high burden settings (10, 11),  
90 more in-depth information on genotype, suitable to scale up for large projects, and practical  
91 for resource-poor settings. Unfortunately, work in resource-poor settings can too often limit  
92 the number of these parameters that can be achieved.

93

94 The gold-standard serotyping method, the Quellung reaction, was developed in the early  
95 1900s and is performed by testing colonies with a set of type-specific antisera. (12) Bacteria  
96 are observed by microscopy, with serotype defined by observing apparent capsular swelling  
97 in reaction to the type-specific antisera. It is laborious, requires frequent use to maintain  
98 skills, requires a complete set of type-specific antisera, and is therefore mainly performed by  
99 reference laboratories. The PneuCarriage project, a large, multi-centre study, was  
100 established with the aim of identifying the best pneumococcal serotyping methods for  
101 carriage studies. (13) The Project identified microarray with a culture amplification step as  
102 the top-performing method. While robust and systematic, their decision algorithm did not  
103 take into account parameters such as cost, skill level and resources needed for assay  
104 implementation and maintenance, as well as output processing and interpretation.

105

106 Here we describe, in the context of an ongoing field-based study, (14) the level of  
107 concordance between three methods commonly used during ongoing routine pneumococcal  
108 surveillance activities in our work: latex agglutination, microarray and serotyping-by-  
109 sequencing (using the PneumoCaT pipeline). We also address parameters that researchers  
110 and policymakers can consider when deciding which assay to implement in their local  
111 setting.

112

## 113 **Materials and Methods**

### 114 **Study Setting**

115 Blantyre is located in Southern Malawi with an urban population of approximately 1.3 million.

116

### 117 **Study Population and Recruitment**

118 Samples were collected as part of a larger 3.5-year pneumococcal carriage surveillance  
119 project, as described elsewhere. (14) In brief, this was a prospective rolling cross-sectional  
120 observational study using stratified random sampling to measure pneumococcal  
121 nasopharyngeal carriage in Blantyre, Malawi. Samples used in this analysis were collected  
122 during the first two years of twice-annual cross-sectional surveys, from June 2015 to April  
123 2017. Recruitment included three groups: i) healthy children 3–6 years old who received  
124 PCV13 as part of routine EPI, ii) healthy children 5–10 years old who were age-ineligible to  
125 receive PCV13 as part of EPI, and iii) HIV-infected adults (18–40yrs) on ART.

126

### 127 **Sample Selection**

128 For concordance analyses between the three methods, all samples were included that had  
129 serotyping results available from each of the three methods (latex, microarray, serotyping-  
130 by-sequencing). From the total nasopharyngeal swab (NPS) samples collected during the  
131 larger surveillance project (including 1,044 from children 3–6 years old [PCV-vaccinated],  
132 531 children 5–10 years old [PCV-unvaccinated, age-ineligible] and 428 HIV-infected adults  
133 on ART), 1347 samples were culture-confirmed for *S. pneumoniae* and also had results  
134 available from the microarray and serotyping-by-sequencing. The final concordance analysis  
135 included 846 children 3–6 years old (PCV13-vaccinated), 422 children 5–10 years old (age-  
136 ineligible for PCV13 vaccination) and 79 adults (HIV-infected and PCV13-unvaccinated).  
137 (Figure 1) Sample selection for microarray and serotyping-by-sequencing was done  
138 independently and blind to latex serotype data.

139

### 140 **Nasopharyngeal Swab Collection**

141 Collection of NP swabs is described elsewhere. (14) In brief, an NP swab sample was  
142 collected from each participant using a nylon flocked swab (FLOQSwabs™, Copan

143 Diagnostics, Murrieta, CA, USA) and then immediately placed into 1.5mL skim milk-tryptone-  
144 glucose-glycerol (STGG) medium and processed at the Malawi–Liverpool–Wellcome Trust  
145 (MLW) laboratory in Blantyre, according to WHO recommendations. (15) Samples were  
146 frozen on the same day at  $-80^{\circ}\text{C}$ . (Figure 2)

147

#### 148 **NPS Culture for Pneumococcal Screening & Serotyping**

149 30uL NPS–STGG was plated on a clean sheep blood-gentamicin (SBG; 7% SBA, 5  $\mu\text{l}$   
150 gentamicin/mL) agar plate (primary plate) and incubated overnight at  $37^{\circ}\text{C}$  in  $\sim 5\%$   $\text{CO}_2$ .  
151 Plates showing no *S. pneumoniae* growth were incubated overnight a second time before  
152 being reported as negative. *S. pneumoniae* was identified by colony morphology and  
153 optochin disc (Oxoid, Basingstoke, UK) susceptibility. The bile solubility test was used on  
154 isolates with no or intermediate (zone diameter  $<14\text{mm}$ ) optochin susceptibility. A single  
155 colony of confirmed pneumococcus was selected and grown on a clean SBG plate  
156 (secondary plate), following the same process as the primary plate. (Figure 1)

157

#### 158 **Latex Serotyping**

159 Pneumococcal growth from secondary plates was used for serotyping by latex agglutination  
160 (ImmuLex™ 7-10-13-valent Pneumotest; Statens Serum Institute, Denmark), following  
161 manufacturer guidelines. Using a reaction card and a sterile inoculation loop, a small sweep  
162 of an overnight bacterial culture was mixed with saline and a series of individual  
163 Pneumotest-Latex reagents in suspension. The card was rocked manually and observed for  
164 agglutination. A Pneumotest-Latex chessboard was used to determine which serotype is  
165 associated with the observed set of agglutination reactions. The kit allows for differential  
166 identification of each PCV13 VT (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F). Other  
167 than for a limited number of serogroups (6, 7, 9, 18, 19, 23) for which the kit provides  
168 serogroup differentiation, there is no further differential identification of NVT serogroups or  
169 serotypes. NVT and non-typeable isolates were reported as NVT. Samples were batch-  
170 tested on a weekly basis, with technicians blinded to the sample source. After serotyping



171 was complete, the remaining growth from each secondary plate was archived at -80°C in  
172 sterile STGG. Refer to Appendix 1 for a more detailed description of latex serotyping.

173

#### 174 **Microarray Serotyping**

175 For samples with culture-confirmed pneumococcal carriage, the original inoculated STGG  
176 was thawed and vortexed. Aliquots of 100µl were shipped in 1.8mL cryovials to BUGS  
177 Bioscience (BUGS Bioscience Ltd., London, United Kingdom) on dry ice. (Figure 1) The  
178 remaining steps for microarray serotyping (including sample processing, culturing, DNA  
179 extraction, molecular serotyping and analysis) were completed entirely by BUGS Bioscience.  
180 (16, 17) Final microarray results were retrieved by the study team from BUGS Bioscience's  
181 web-based SentiNet platform and imported into STATA 13.1 (StataCorp, College Station,  
182 TX, USA) for analysis. Refer to Appendix 1 for a more detailed description of microarray  
183 serotyping.

184

#### 185 **DNA Extraction and WGS**

186 Archived secondary growth isolates were used to develop sequence libraries for serotyping-  
187 by-sequencing (via PneumoCat). To optimise total retrieved DNA, 30µl of thawed isolate-  
188 STGG was incubated overnight in 6mL THY (Todd Hewitt broth + yeast) enrichment culture.  
189 DNA was extracted from the overnight culture using the Qiagen® QIAamp™ DNA Mini Kit,  
190 following manufacturer guidelines for bacterial DNA. Quality control (QC) measures, as  
191 required by the guidelines of the sequencing institution, included DNA quantification  
192 (Qubit™, Thermo Fisher Scientific, Massachusetts, USA) of all DNA samples and gel  
193 electrophoresis imaging on 0.7% agarose to assess DNA integrity. After attaining quantity  
194 and quality requirements, 100µL of extracted DNA were aliquoted into skirted 96-well  
195 microwell plates and stored at -80°C until shipped on dry ice to the Oxford Genomics Centre  
196 (University of Oxford, United Kingdom) for sequencing. Whole genome sequencing was  
197 performed at the Oxford Genomics Centre on a HiSeq4000 platform (Illumina™), with  
198 paired-end libraries and a read length of 150 pb.

199

## 200 **Serotyping-by-sequencing**

201 WGS data was retrieved by the study team from a web-based FTP link. Serotype was  
202 inferred from the isolates' genome sequences using the PneumoCaT software pipeline, an  
203 opensource bioinformatic tool. (18) PneumoCaT requires raw sequencing reads for each  
204 isolate which were trimmed and cleaned. Reads were trimmed of the illumina adapters and  
205 cleaned of low-quality ends using Trimmomatic (ver. 0.38; available at  
206 <http://www.usadellab.org/cms/?page=trimmomatic>). Minimum read length after trimming was  
207 80 base pairs (bp), with the minimum average quality for a sliding window of 4 nucleotide  
208 being 15. A subset of 700,000 reads per end (1.4 million total) was used for any subsequent  
209 analysis. XML result files were parsed with ad hoc bash scripts, in order to extract and  
210 tabulate the serotyping result for each isolate. PneumoCaT was installed and used on a  
211 Linux machine at the MRC Cloud Infrastructure for Microbial Bioinformatics (CLIMB;  
212 <https://www.climb.ac.uk/>). Each serotype identification required an average 5-8 minutes.  
213 Refer to Appendix 1 for a more detailed description of serotyping-by-sequencing.

214

## 215 **Definitions**

216 Concordance was calculated with all samples aggregated and according to the level of  
217 discrimination provided by the method. Concordance is reported using two criteria: i) a  
218 criterion based on whether both assays reported NVT or both reported VT (VT/NVT criterion)  
219 and ii) a criterion based on whether the final serotype reported by each assay is equivalent  
220 (serotype-specific criterion).

221

222 Concordance between latex and serotyping-by-sequencing (PneumoCaT): Other than a  
223 limited number of serogroups (6, 7, 9, 18, 19, 23) for which the latex kit provides serogroup  
224 differentiation, there is no further differential identification of NVT serogroups to serotype.  
225 NVT and non-typeable isolates were reported as NVT. Concordance at serotype level  
226 (serotype-specific criterion) was reported only if latex reported VT carriage. If latex reported

227 NVT, any NVT reported by PneumoCaT was considered concordant. For example: 23F  
228 reported by both latex and PneumoCaT was considered concordant, as was NVT and 15B.  
229 However, 19F and 19A was considered discordant, as was NVT and 6B.

230

231 Concordance between latex and microarray: Concordance at serotype level (serotype-  
232 specific criterion) was reported only if latex reported VT carriage. If latex reported NVT, any  
233 NVT reported by microarray was considered concordant. Because microarray reports  
234 multiple serotype carriage, 23F reported by latex and 23F+34 reported by microarray was  
235 considered concordant, as was NVT and 18C+33D. However, 19F and 33D+19A was  
236 considered discordant, as was NVT and 3+7F. Note that for microarray, some closely related  
237 serotypes were reported as a group, with the individual serotype call in brackets (e.g., 6A/B  
238 [6B]). In this case, results were analysed to the level of the individual serotype call. For  
239 simplicity of analysis, if a method did not claim to detect a serotype (e.g. 23F) but the sample  
240 contained that serotype, this result was deemed discordant.

241

242 Concordance between microarray and serotyping-by-sequencing (PneumoCat): Microarray  
243 and PneumoCat both differentiate VT and NVT to serotype level, allowing concordance to be  
244 calculated on serotype concordance (serotype-specific criterion) for both VT and NVT *S.*  
245 *pneumoniae*.

246

## 247 **Statistical analysis**

248 The formula for percent increase in VT prevalence was:  $([VT \text{ prevalence using latex} - VT$   
249  $\text{prevalence using microarray}] / VT \text{ prevalence using latex}) * 100\%$ . Confidence intervals are  
250 binomial exact. Statistical significance was inferred from two-sided  $p < 0.05$ . Participant data  
251 collection was completed using Open Data Kit (ODK) Collect open source software.  
252 (v1.24.0). Statistical analyses were completed using Stata 13.1 (StataCorp, College Station,  
253 TX, USA).

254

255 **Ethics Considerations**

256 The study protocol was approved by the College of Medicine Research and Ethics  
257 Committee, University of Malawi (P.02/15/1677) and the Liverpool School of Tropical  
258 Medicine Research Ethics Committee (14.056). Adult participants and parents/guardians of  
259 child participants provided written informed consent; children 8-years and older provided  
260 informed assent. This included consent for publication.

261

262 **Results**

263 Pneumococcal carriage prevalence results from the larger surveillance project are reported  
264 elsewhere. (14) Comparing latex and PneumoCat, the adjusted concordance of correctly  
265 identifying pneumococcal carriage as VT or NVT was 90.7% (1216/1341; 95% CI: 89.0–  
266 92.2). (Figure 3) Based on the serotype-specific criterion, concordance between latex and  
267 PneumoCaT was 87.5%; (1174/1341) (95% CI: 85.7–89.3). Comparing latex and  
268 microarray, the concordance based on correctly identifying pneumococcal carriage as VT or  
269 NVT was 97.3% 1311/1347 (95% CI: 96.3–98.1). Based on a serotype-specific criterion, the  
270 concordance was 95.2% (1282/1347; 95% CI: 93.9–96.3). Comparing microarray and  
271 PneumoCaT, concordance based on correctly identifying pneumococcal carriage as VT or  
272 NVT was 96.6% (1295/1341; 95% CI: 95.5–97.5). Based on a serotype-specific criterion, the  
273 concordance was 82.8% (1110/1341; 95% CI: 80.6–84.8).

274

275 **Increased VT Detection Using Microarray**

276 Using a larger study database of 1,949 samples from the same study, we evaluated latex  
277 and microarray data. Aggregating all ages (i.e. child and adult), there was an increase of  
278 31.5% in VT prevalence by microarray compared to latex serotyping: 43.0% increase in VT  
279 carriage among children 3–6 years old, 21.7% among children 5–10 years old and 10.8%  
280 among HIV-infected adults on ART (Table 1). This was due to samples reporting NVT by  
281 latex but that also carried VT, as detected by microarray. These VT, undetected by latex,  
282 were carried in lower relative abundance (median 8%, range: 2% - 48%). The prevalence of

283 multiple serotype carriage (range 2-6 serotypes) was 35.2% (686/1949). The prevalence  
284 among respective age groups was 44.4% (457/1029), 32.8% (169/515), and 14.8%  
285 (60/405). Among samples with multiple serotype carriage, latex identified the dominant  
286 serotype in 85.3% (585/686; 95%CI: 82.4–87.8) of samples. Despite the overall increase in  
287 detection of VT carriage, the proportion of individual VT serotypes detected is not different  
288 when comparing microarray to latex (Figure 4).

289

### 290 **Key Parameters of selected serotyping methods.**

291 Table 2 presents key parameters to further consider when deciding which assay is  
292 appropriate for a particular setting. Estimated costs and feasibility of implementation and  
293 maintenance are specific to the setting in Malawi at the Malawi-Liverpool-Wellcome Trust  
294 Clinical Research Programme in Blantyre. Extrapolation would need further validation  
295 outside the scope of this evaluation. Though more limited in its reporting only a single  
296 serotype, latex is highly accurate while being less costly and requiring less expertise and  
297 resources for field-implementation and analysis. While microarray is the costliest option, it  
298 provides greater accuracy of total pneumococcal carriage, including multiple serotype  
299 carriage and relative abundance of individual serotypes in carriage. Whole genome  
300 sequencing is a strong alternative to latex and would be nearly cost-free if the sequence  
301 libraries were already available. In addition, WGS provides opportunity for further analyses,  
302 including population structure and antibiotic resistance.

303

### 304 **Discussion**

305 We report high concordance between three serotyping techniques applicable to routine  
306 pneumococcal surveillance. Importantly, we have extended the analysis to include relevant  
307 parameters beyond accuracy including cost, time to result, and measures of input required  
308 for assay implementation and maintenance. These are parameters that researchers and  
309 policy makers should consider when deciding which assay to implement. All three assays  
310 appear accurate and concordant in identifying the dominant serotype.

311

312 While latex agglutination is accurate and requires the least expertise and resources for field-  
313 implementation and analysis and provides rapid results, standard latex approaches is not  
314 optimal for optimal surveillance of vaccine impact, including the detection of multiple  
315 serotype carriage and VT in low relative abundance. (19) There have been attempts to  
316 implement latex for detection of multiple serotype carriage. Gratten et al. serotyped up to six  
317 colonies from nasal-swab culture plates and found multiple-serotype carriage in 29.5% of  
318 Papua New Guinean children. (20) The authors went on to serotype at least 50 colonies  
319 from 10 selected nasal-swab cultures and concluded that the minor carried serotype  
320 accounted for 4 to 27% of the total pneumococcal population. A review of published data on  
321 multiple carriage concluded that, to detect a minor carried serotype it would be necessary to  
322 serotype at least five colonies to have a 95% chance of detecting the serotype if it accounted  
323 for 50% of the total pneumococcal population, and one would need to examine 299 colonies  
324 if the serotype was present at a relative abundance of 1%. As part of the PneuCarriage  
325 project, to thoroughly characterise samples, up to 120 colonies from each sample were  
326 selected to achieve >99% power to detect a minor serotype of 5% abundance. (13) This  
327 approach would not be cost- or time-effective. Though dependent on technical capacity to  
328 develop in-house reagents, researchers in The Gambia developed a latex agglutination  
329 technique in which colonies from the primary culture plate are suspended in saline and  
330 serotyped by latex agglutination. (21) While not differentiating NVT serotypes, they did show  
331 that up to 10.4% of pneumococcal acquisitions were found to be of multiple serotypes in a  
332 longitudinal infant cohort study. While latex is limited in its output, the process can be  
333 leveraged for additional endpoints including, for example, measuring carriage density  
334 through counting of colony-forming units (CFU) on agar culture plates.

335

336 With opensource bioinformatic tools such as PneumoCaT, serotyping-by-sequencing can be  
337 less costly than microarray, even accounting for costs of DNA extraction and WGS, while  
338 still being able to differentiate non-typeable and nearly every known VT and NVT. Though

339 we would not recommend initiating DNA extraction and WGS for the use of PneumoCaT  
340 alone, sequence libraries can be further leveraged for extensive informative bioinformatic  
341 analyses, useful in population biology, antimicrobial resistance investigations and vaccine  
342 monitoring. Moreover, using PneumoCaT for serotyping would be essentially cost-free if the  
343 sequence libraries were already available, apart from the limited bioinformatic skills needed.  
344 While microarray is more costly, it differentiates NVT and multiple serotype carriage with  
345 relative abundance, as well as non-*S. pneumoniae* contaminants (i.e. *S. mitis*, *S. salivarius*,  
346 *Staphylococcus aureus*) with a degree of precision. This technique stands out for its  
347 sensitivity, being able to detect serotypes in low relative abundance, which is of critical  
348 importance for understanding the transmission patterns of *S. pneumoniae*.

349  
350 There are a number of limitations to mention, including the number of serotyping methods  
351 which were not evaluated, including PCR and the SeroBA pipeline. SeroBA is a relatively  
352 new serotyping-by-sequencing software. With similar accuracy to PneumoCaT, SeroBA  
353 does have operational advantages. (22) SeroBA can correctly call a serotype with a read  
354 coverage as low as 10X (20X is required for PneumoCaT). Using a k-mer based approach,  
355 rather than the raw sequence alignment, SeroBA requires much lower computational power  
356 and time. On the other hand, the PneumoCat source code can be easily adapted to the  
357 operator needs, and both software are likely to run on a standard server configuration.  
358 Alternative culture-independent methods, such as PCR, could be important for confirming  
359 carriage when re-culturing of original NP swab samples is not feasible. Though PCR has  
360 been successfully applied on DNA extracted directly from NPS-STGG, evidence suggests  
361 that the best way to apply PCR serotyping is after culture enrichment, returning a higher  
362 sensitivity and ability to identify multiple serotype carriage. (9) PCR limitations include the  
363 need for region-specific reaction protocols, implementing a high number of primer pairs to  
364 identify the same range of serotypes identified by microarray or WGS, and the increased risk  
365 of detecting non-viable pneumococci. As there is no evidence of a viable but non-culturable  
366 (VBNC) state in *S. pneumoniae*, (23) identifying non-viable pneumococci could be

367 disadvantageous for field-based research. While a formal economic analysis of the methods  
368 would be justified, we were unable to extrapolate the individual costing components between  
369 sites. Such components would include local salaries and additional labour costs,  
370 procurement and shipping of equipment and consumables, equipment maintenance, local  
371 health and safety requirements, and institutional costs. For this reason, comparative costing  
372 is grossly categorized. Though we did not include invasive isolates (from blood or cerebral  
373 spine fluid, for example), it is important to identify serotypes associated with IPD, including in  
374 post-PCV impact studies. For invasive isolates, with a single-serotype sample, microarray  
375 would have limited advantage. Application of serotyping-by-sequencing would then be the  
376 most informative option, including insight into population structure, antimicrobial resistance  
377 patterns and serotype replacement disease.

378

## 379 **CONCLUSION**

380 Selection of the appropriate assay should be based on the intended analysis and endpoint.  
381 While accuracy and concordance is high between the three assays, parameters of field-  
382 implementation and cost vary significantly. In a setting of limited resources, as is true  
383 throughout much of sub-Saharan Africa, latex is the best overall option for decentralised  
384 surveillance of vaccine impact. However, WGS, which adds population structure, and  
385 microarray, which adds multiple-serotype carriage, should be considered at regional  
386 reference laboratories while investigating the importance of VT in low relative abundance in  
387 transmission and disease.

388

## 389 **Appendixes**

390 An appendix is available online. Consisting of data provided by the authors to benefit the  
391 reader, the posted materials are not copyedited and are the sole responsibility of the  
392 authors, so questions or comments should be addressed to the corresponding author.

393



394 **Data availability.** The data supporting the findings of this study has been deposited in the  
395 Figshare repository. (24)

396

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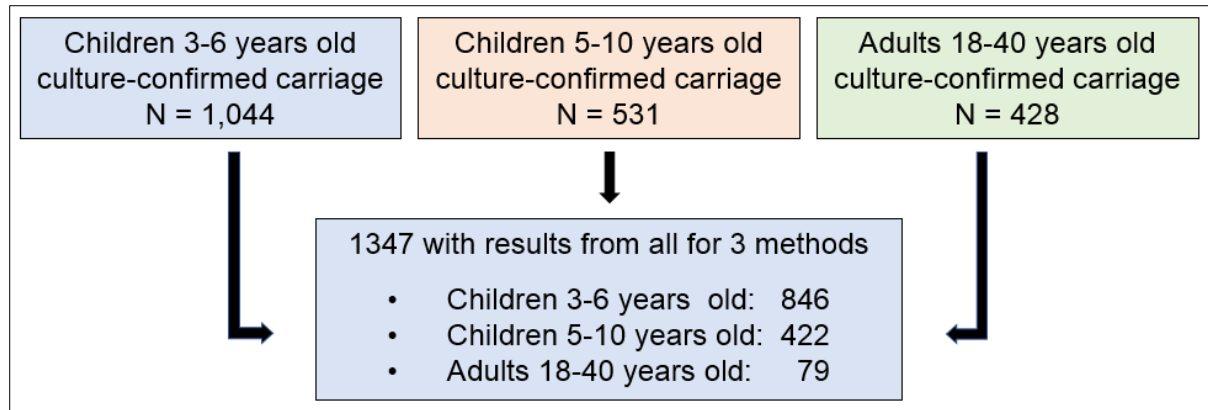
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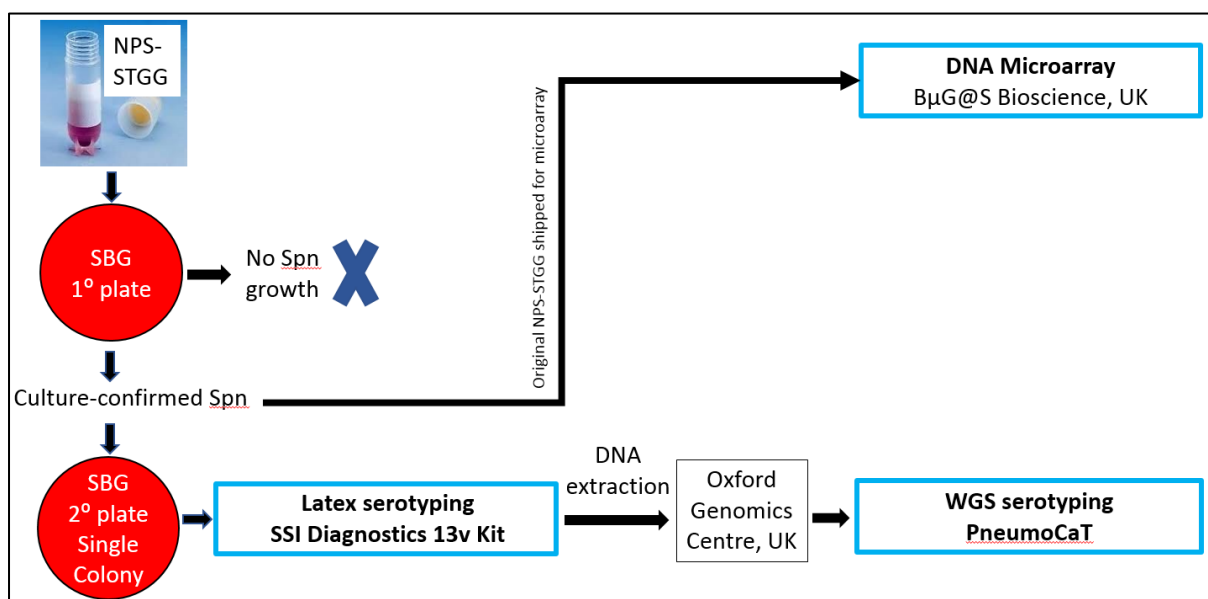


513

514 **Figure 1** Sample selection for analysis. Sample were collected during 4 rolling cross-sectional  
 515 surveys from June 2015 to April 2017. From the total nasopharyngeal swab samples collected 1347  
 516 samples had results available from the three assays under review. The method of selection for  
 517 microarray and PneumoCaT was done independent of available latex serotype data.

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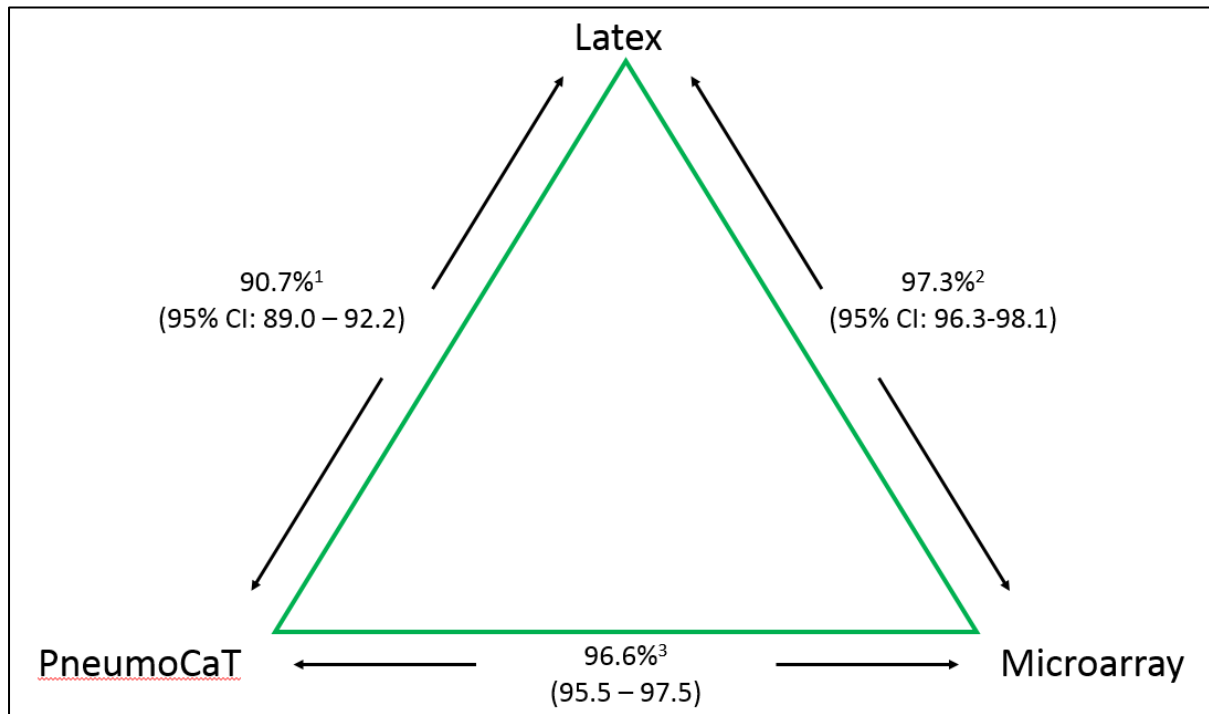


520

521 **Figure 2** Laboratory procedures. Nasopharyngeal swabs (NPS) were inoculated into STGG and  
 522 subsequently plated on a growth agar of sheep blood and gentamycin. Bacteria growth (from single-  
 523 colony picks) from samples culture-confirmed for *Streptococcus pneumoniae* were used for latex  
 524 serotyping. Remaining pure-growth isolates, retained at -80°C in sterile STGG, were later grown for  
 525 DNA extraction and WGS. Aliquots of original samples (NPS-STGG) that were culture-confirmed for  
 526 *Streptococcus pneumoniae* were assessed by microarray. NPS=nasopharyngeal swabs,  
 527 STGG=skim-milk-tryptone-glucose-glycerol, WGS=whole-genome sequencing, Spn=*Streptococcus*

528 *pneumoniae*, SBG=sheep blood and gentamycin, SSI= Statens Serum Institute, 13v=13-valent, NSP-  
529 STGG=NPS inoculated into STGG.

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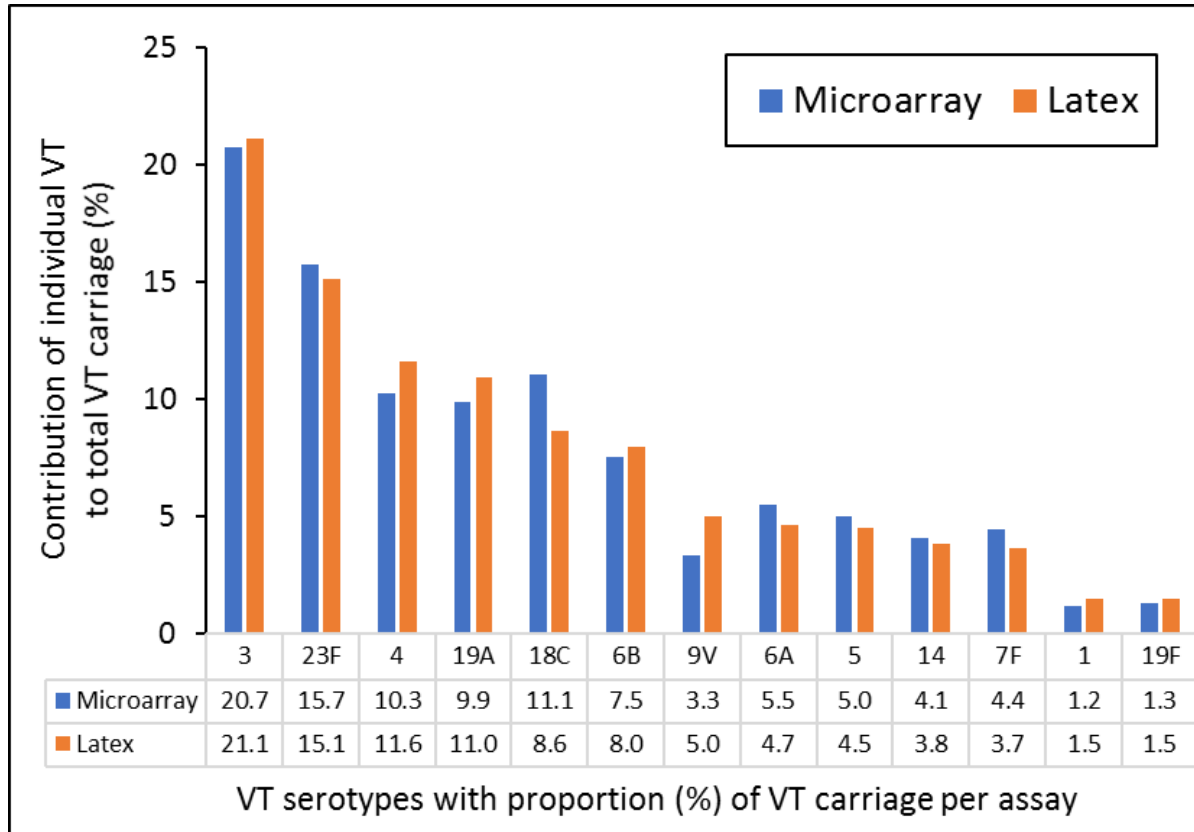


531

532 **Figure 3** Concordance between assays. Concordance between two assays was defined as both  
533 assays identifying pneumococcal carriage as VT or both as NVT. Latex and PneumoCaT reported  
534 one result per sample, both using the same pure-growth culture. Microarray, using an aliquot of the  
535 original NPS-STGG, differentiated individual serotypes in multiple serotype carriage, when present.  
536 When comparing the three assays, concordance was based on serotype if latex reported VT carriage.  
537 If latex reported NVT, this was considered concordant to any NVT reported by PneumoCaT and  
538 microarray, as long as PneumoCaT and microarray reported the same NVT.



539



540

541 **Figure 4** Proportion of individual VT serotypes contributing to total VT carriage. The proportion of  
 542 individual VT serotypes detected is not significantly different when comparing microarray to latex.  
 543 VT=vaccine serotype.

544

545 **Table 1** Increased detection of VT carriage, latex vs microarray

	Latex VT prevalence (n) 95% CI	Microarray VT prevalence (n) 95% CI	% Increase in VT prevalence
Children 3-6 years, PCV-vaccinated (n=1360)	20.0% (272) 17.9, 22.2	28.6% (389) 26.2, 31.1	43.0%
Children 5-10 years, PCV-unvaccinated (n=904)	21.1% (191) 18.5, 23.9	26.5% (240) 23.7, 29.6	21.7%
Adults, 18-40 years, HIV-infected, PCV-unvaccinated (n=963)	14.2% (137) 12.1, 16.6	16.6% (160) 14.3, 19.1	10.8%
Total (n=3227)	18.6 (600) 17.3, 20.0	24.4 (789) 23.0, 26.0	31.5%

546 PCV=pneumococcal conjugate vaccine, VT=vaccine serotype, CI=confidence interval.

547 **Table 2** Key comparative parameters of serotyping methods.

	Latex (phenotypic)	Microarray (genomic)	PneumoCaT (genomic)
<b>Assay implementation</b>			
Sample used in assay	• Pure growth from single isolate	• Original sample in STGG	• Pure growth from single isolate
Cost estimate <sup>1</sup>	• Lowest of three assays	• Highest of three assays	• Middle of three assays
Implementation of assay	• Least difficult (relatively simple)	• Most difficult	• Moderate difficult
Training required for implementation	• Minimal	• Advanced	• DNA extraction: moderate • WGS library manipulation: advanced • PneumoCaT tool: moderate
Training required for processing and interpretation of results	• Minimal	• Moderate	• Moderate
<b>Assay output and interpretation</b>			
Serotypes reported	• Single	• Multiple, if present	• Single
NVT differentiation	• No <sup>3</sup>	• Yes	• Yes
Relative abundance of individual serotypes reported	• No	• Yes	• No
Additional outputs	• Isolates archived and available for further analyses	• AMR Profile <sup>2</sup> • NT differentiation	• WGS library accessible for further analyses, including population structure and AMR
Conclusion	• Adequate for surveillance • Limited resolution for optimal VE estimation	• Cost and technique limits ability to de-centralise implementation • Detection of VT in low relative abundance is of critical importance • Sentinel sites should be considered for regional NVT & VT resolution for optimal VE estimation	• Limited resolution for optimal VE estimation • No benefit over latex unless WGS library already available

548

549 <sup>1</sup>Estimated costs and feasibility of implementation and maintenance are specific to the setting in Malawi at the Malawi-Liverpool-Wellcome Trust Clinical  
 550 Research Programme in Blantyre.

551 <sup>2</sup> AMR profile cannot be assigned to a single strain in a sample with multiple-serotype or multiple-pathogen carriage.

552 <sup>3</sup>NVT & NT reported as NVT. STGG=skim-milk-tryptone-glucose-glycerol, WGS=whole genome sequencing, VT=vaccine serotype, NVT=non-vaccine

553 serotype, AMR=antimicrobial resistance, NT=non-typeable, VE=vaccine efficacy.

