Title

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

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Running title

Serotyping for pneumococcal carriage

Summary of the article's main points

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

Abstract

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

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

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

Journal of Clinical Microbiology JCM Journal of Clinical Microbiology

Conclusion. All three serotyping methods were highly concordant in identifying dominant serotypes. Latex serotyping is accurate in identifying vaccine-serotypes and requires the least expertise and resources for field-implementation and analysis. However, WGS, which adds population structure, and microarray, which adds multiple-serotype carriage, should be considered at regional reference laboratories while investigating the importance of VT in low relative abundance in transmission and disease.

Introduction

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

Current multivalent pneumococcal conjugate vaccines (PCV) target subsets of the 100 capsular serotypes known to be expressed by the pneumococcus. PCV reduces nasopharyngeal carriage of the sub-set of pneumococcal serotypes they contain, known as vaccine serotypes (VT). With reduced carriage among the vaccinated there is then reduced risk of VT-IPD (direct protection) and reduced transmission, therefore reduced risk of VT-IPD among those PCV-unvaccinated (indirect protection). However, non-vaccine serotypes (NVT) have the potential to fill the

ecological niche, becoming more common in carriage and disease. (5-7) This phenomenon, known as serotype replacement, may be more pronounced in low-income settings because of higher prevalence, density and diversity of pneumococcal carriage, and represents a considerable risk to the global pneumococcal immunisation strategy.(8) Serotype distribution differs between continents as well as individual countries. (9) Given these differences, accurate assessment of the serotype distribution associated with both pneumococcal colonization and pneumococcal disease is needed in the evaluation, formulation and delivery of pneumococcal vaccines.

A pneumococcal serotyping method suitable for use in robust carriage and surveillance studies should therefore, at minimum, be accurate in its serotype assignment, particularly in relation to VTs. Additional desirable parameters include detection of most or all serotypes, ability to detect multiple serotypes in carriage (common in high burden settings (10, 11), more in-depth information on genotype, suitable to scale up for large projects, and practical for resource-poor settings. Unfortunately, work in resource-poor settings can too often limit the number of these parameters that can be achieved.

The gold-standard serotyping method, the Quellung reaction, was developed in the early 1900s and is performed by testing colonies with a set of type-specific antisera. (12) Bacteria are observed by microscopy, with serotype defined by observing apparent capsular swelling in reaction to the type-specific antisera. It is laborious, requires frequent use to maintain skills, requires a complete set of type-specific antisera, and is therefore mainly performed by reference laboratories. The

Journal of Clinical Microbiology

PneuCarriage project, a large, multi-centre study, was established with the aim of identifying the best pneumococcal serotyping methods for carriage studies. (13) The Project identified microarray with a culture amplification step as the top-performing method. While robust and systematic, their decision algorithm did not take into account parameters such as cost, skill level and resources needed for assay implementation and maintenance, as well as output processing and interpretation.

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

Materials and Methods

Study Setting

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

Study Population and Recruitment

Samples were collected as part of a larger 3.5-year pneumococcal carriage surveillance project, as described elsewhere. (14) In brief, this was a prospective rolling cross-sectional observational study using stratified random sampling to measure pneumococcal nasopharyngeal carriage in Blantyre, Malawi. Samples used in this analysis were collected during the first two years of twice-annual cross-

sectional surveys, from June 2015 to April 2017. Recruitment included three groups: i) healthy children 3–6 years old who received PCV13 as part of routine EPI, ii) healthy children 5–10 years old who were age-ineligible to receive PCV13 as part of EPI, and iii) HIV-infected adults (18–40yrs) on ART.

Sample Selection

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

Nasopharyngeal Swab Collection

Collection of NP swabs is described elsewhere. (14) In brief, an NP swab sample was collected from each participant using a nylon flocked swab (FLOQSwabs™, Copan Diagnostics, Murrieta, CA, USA) and then immediately placed into 1.5mL skim milk-tryptone-glucose-glycerol (STGG) medium and processed at the Malawi–

Journal of Clinical Microbiology

Liverpool–Wellcome Trust (MLW) laboratory in Blantyre, according to WHO recommendations. (15) Samples were frozen on the same day at −80°C. (Figure 2)

NPS Culture for Pneumococcal Screening & Serotyping

30uL NPS–STGG was plated on a sterile sheep blood-gentamicin (SBG; 7% SBA, 5 µl gentamicin/mL) agar plate (primary plate) and incubated overnight at 37°C in ~5% CO_2. Plates showing no *S. pneumoniae* growth were incubated overnight a second time before being reported as negative. *S. pneumoniae* was identified by colony morphology and optochin disc (Oxoid, Basingstoke, UK) susceptibility. The bile solubility test was used on isolates with no or intermediate (zone diameter <14mm) optochin susceptibility. A single colony of confirmed pneumococcus was selected and grown on a sterile SBG plate (secondary plate), following the same process as the primary plate. (Figure 1)

Latex Serotyping

Pneumococcal growth from secondary plates was used for serotyping by latex agglutination (ImmuLex™ 7-10-13-valent Pneumotest; Statens Serum Institute, Denmark), following manufacturer guidelines. Using a reaction card and a sterile inoculation loop, a small sweep of an overnight bacterial culture was mixed with saline and a series of individual Pneumotest-Latex reagents in suspension. The card was rocked manually and observed for agglutination. A Pneumotest-Latex chessboard was used to determine which serotype is associated with the observed set of agglutination reactions. The kit allows for differential identification of each PCV13 VT (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F). Other than for a limited number of serogroups (6, 7, 9, 18, 19, 23) for which the kit provides

serogroup differentiation, there is no further differential identification of NVT serogroups or serotypes. NVT and non-typeable isolates were reported as NVT. Samples were batch-tested on a weekly basis, with technicians blinded to the sample source. After serotyping was complete, the remaining growth from each secondary plate was archived at -80°C in sterile STGG. Refer to Text S1 in the supplemental material for a more detailed description of latex serotyping.

Molecular Serotyping by Microarray

For samples with culture-confirmed pneumococcal carriage, the original inoculated STGG was thawed and vortexed. Aliquots of 100µl were shipped in 1.8mL cryovials to BUGS Bioscience (BUGS Bioscience Ltd., London, United Kingdom) on dry ice. (Figure 1) The remaining steps for microarray serotyping (including sample processing, culturing, DNA extraction, microarray and analysis) were completed entirely by BUGS Bioscience. (16, 17) Final microarray results were retrieved by the study team from BUGS Bioscience web-based SentiNET platform and imported into STATA 13.1 (StataCorp, College Station, TX, USA) for analysis. Refer to Text S1 in the supplemental material for a more detailed description of microarray serotyping.

DNA Extraction and WGS

Archived secondary growth isolates were used to develop sequence libraries for serotyping-by-sequencing. To optimise total retrieved DNA, 30µl of thawed isolate-STGG was incubated overnight in 6mL THY (Todd Hewitt broth + yeast) enrichment culture. DNA was extracted from the overnight culture using the Qiagen® QIAamp™ DNA Mini Kit, following manufacturer guidelines for bacterial DNA. Quality control (QC) measures, as required by the guidelines of the sequencing institution, included

DNA quantification (Qubit™, Thermo Fisher Scientific, Massachusetts, USA) of all DNA samples and gel electrophoresis imaging on 0.7% agarose to assess DNA integrity. After attaining quantity and quality requirements, 100µL of extracted DNA were aliquoted into skirted 96-well microwell plates and stored at -80ºC until shipped on dry ice to the Oxford Genomics Centre (University of Oxford, United Kingdom) for sequencing. Whole genome sequencing was performed at the Oxford Genomics Centre on a HiSeq4000 platform (Illumina™), with paired-end libraries and a read length of 150bp.

Serotyping-by-sequencing

WGS data was retrieved by the study team from a web-based FTP link. Serotype was inferred from the isolates' genome sequences using the PneumoCaT software pipeline, an opensource bioinformatic tool. (18) PneumoCaT requires raw sequencing reads for each isolate which were trimmed and cleaned. Reads were trimmed of the illumina adapters and cleaned of low-quality ends using Trimmomatic (ver. 0.38; available at http://www.usadellab.org/cms/?page=trimmomatic). Minimum read length after trimming was 80 base pairs (bp), with the minimum average quality for a sliding window of 4 nucleotide being 15. A subset of 700,000 reads per end (1.4 million total) was used for any subsequent analysis. XML result files were parsed with ad-hoc bash scripts, in order to extract and tabulate the serotyping result for each isolate. PneumoCaT was installed and used on a Linux machine at the MRC Cloud Infrastructure for Microbial Bioinformatics (CLIMB; https://www.climb.ac.uk/). Each serotype identification required an average 5-8 minutes. Refer to Text S1 in the supplemental material for a more detailed description of serotyping-by-sequencing.

Definitions

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

Concordance between latex and serotyping-by-sequencing (PneumoCaT): Other than a limited number of serogroups (6, 7, 9, 18, 19, 23) for which the latex kit provides serogroup differentiation, there is no further differential identification of NVT serogroups to serotype. NVT and non-typeable isolates were reported as NVT. Concordance at serotype level (serotype-specific criterion) was reported only if latex reported VT carriage. If latex reported NVT, any NVT reported by PneumoCaT was considered concordant. For example: 23F reported by both latex and PneumoCaT was considered concordant, as was NVT and 15B. However, 19F and 19A was considered discordant, as was NVT and 6B.

Concordance between latex and microarray: Concordance at serotype level (serotype-specific criterion) was reported only if latex reported VT carriage. If latex reported NVT, any NVT reported by microarray was considered concordant. Because microarray reports multiple serotype carriage, 23F reported by latex and 23F+34 reported by microarray was considered concordant, as was NVT and 18C+33D. However, 19F and 33D+19A was considered discordant, as was NVT and 3+7F.

Journal of Clinical Microbiology

Note that for microarray, some closely related serotypes are reported as a group, with the final individual serotype call in brackets (e.g., 6A/B [6B]). In this case, results were analysed using the individual serotype call. For example, if microarray reported 6A/B [6B], this was considered discordant to a 6A latex result and concordant to a 6B latex result. For simplicity of analysis, if a method did not claim to detect a serotype but the sample contained that serotype, this result was deemed discordant. For example, if microarray detected both serotypes 23F and 19A but latex detected only serotype 3, this result was considered discordant.

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

Statistical analysis

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

Ethics Considerations

The study protocol was approved by the College of Medicine Research and Ethics Committee, University of Malawi (P.02/15/1677) and the Liverpool School of Tropical

Medicine Research Ethics Committee (14.056). Adult participants and parents/guardians of child participants provided written informed consent; children 8-years and older provided informed assent. This included consent for publication.

Results

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

Increased VT Detection Using Microarray

Using a larger study database of 1,949 samples from the same study, we evaluated latex and microarray data. Aggregating all ages (i.e. child and adult), there was an increase of 31.5% in VT prevalence by microarray compared to latex serotyping: 43.0% increase in VT carriage among children 3–6 years old, 21.7% among children 5–10 years old and 10.8% among HIV-infected adults on ART (Table 1). This was due to samples reporting NVT by latex but that also carried VT, as detected by

microarray. These VT, undetected by latex, were carried in lower relative abundance (median 8%, range: 2% - 48%). The prevalence of multiple serotype carriage (range 2-6 serotypes) was 35.2% (686/1949). The prevalence among respective age groups was 44.4% (457/1029), 32.8% (169/515), and 14.8% (60/405). Among samples with multiple serotype carriage, latex identified the dominant serotype in 85.3% (585/686; 95%CI: 82.4–87.8) of samples. Despite the overall increase in detection of VT carriage, the proportion of individual VT serotypes detected is not different when comparing microarray to latex (Figure 4). Refer to Table S1 in the supplemental material for the reported frequency of each vaccine serotype (VT) detected by microarray and latex.

Key Parameters of selected serotyping methods.

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

Discussion

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

While latex agglutination is accurate and requires the least expertise and resources for field-implementation and analysis and provides rapid results, standard latex approaches is not optimal for optimal surveillance of vaccine impact, including the detection of multiple serotype carriage and VT in low relative abundance. (19) There have been attempts to implement latex for detection of multiple serotype carriage. Gratten et al. serotyped up to six colonies from nasal-swab culture plates and found multiple-serotype carriage in 29.5% of Papua New Guinean children. (20) The authors went on to serotype at least 50 colonies from 10 selected nasal-swab cultures and concluded that the minor carried serotype accounted for 4 to 27% of the total pneumococcal population. A review of published data on multiple carriage concluded that, to detect a minor carried serotype it would be necessary to serotype at least five colonies to have a 95% chance of detecting the serotype if it accounted for 50% of the total pneumococcal population, and one would need to examine 299 colonies if the serotype was present at a relative abundance of 1%. As part of the PneuCarriage project, to thoroughly characterise samples, up to 120 colonies from

Journal of Clinical Microbiology

each sample were selected to achieve >99% power to detect a minor serotype of 5% abundance. (13) This approach would not be cost- or time-effective. Though dependent on technical capacity to develop in-house reagents, researchers in The Gambia developed a latex agglutination technique in which colonies from the primary culture plate are suspended in saline and serotyped by latex agglutination. (21) While not differentiating NVT serotypes, they did show that up to 10.4% of pneumococcal acquisitions were found to be of multiple serotypes in a longitudinal infant cohort study. While latex is limited in its output, the process can be leveraged for additional endpoints including, for example, measuring carriage density through counting of colony-forming units (CFU) on agar culture plates. What's more, though less cost- and time-effective, use of both commercial products (including Statens Serum Institute, Denmark) and production of in-house latex serotyping reagents has been well-documented to significantly expand the number of NVT serotypes that can be differentiated by latex, including quality control procedures. (11, 22)

With opensource bioinformatic tools such as PneumoCaT, serotyping-by-sequencing can be less costly than microarray, even accounting for costs of DNA extraction and WGS, while still being able to differentiate non-typeable and nearly every known VT and NVT. Though we would not recommend initiating DNA extraction and WGS for the use of PneumoCaT alone, sequence libraries can be further leveraged for extensive informative bioinformatic analyses, useful in population biology, antimicrobial resistance investigations and vaccine monitoring. Moreover, using PneumoCaT for serotyping would be essentially cost-free if the sequence libraries were already available, apart from the limited bioinformatic skills needed. While microarray is more costly, it differentiates NVT and multiple serotype carriage with

Journal of Clinical Microbiology

relative abundance, as well as non-*S. pneumoniae* contaminants (i.e. *S. mitis, S. salivarius*, *Staphylococcus aureus*) with a degree of precision. This technique stands out for its sensitivity, being able to detect serotypes in low relative abundance, which is of critical importance for understanding the transmission patterns of *S.* pneumoniae. Having the extra counts for each serotype from the same number of samples, as provided by microarray, also has the advantage of adding power to a study's statistics.

There are a number of limitations to mention, including the number of serotyping methods which were not evaluated, including PCR and the SeroBA pipeline. SeroBA is a relatively new serotyping-by-sequencing software. With similar accuracy to PneumoCaT, SeroBA does have operational advantages. (23) SeroBA can correctly call a serotype with a read coverage as low as 10X (20X is required for PneumoCaT). Using a k-mer based approach, rather than the raw sequence alignment, SeroBA requires much lower computational power and time. On the other hand, the PneumoCaT source code can be easily adapted to the operator needs, and both software are likely to run on a standard server configuration. Alternative culture-independent methods, such as isolation-independent conventional multiplex PCR-serotyping (cmPCR) could be important for confirming carriage when re-culturing of original NP swab samples is not feasible. Though cmPCR has been successfully applied on DNA extracted directly from NPS-STGG, evidence suggests that cmPCR serotyping after culture enrichment returns a higher sensitivity and an ability to identify multiple serotype carriage. (9) Nonetheless, cmPCR can be confounded by non-pneumococcal streptococci (including *S. mitis, S. oralis and S. parasanguinis*). (24) Due to a high sequence similarity with target serotype-specific

Journal of Clinical Microbiology

amplicons, cmPCR can overestimate pneumococcal carriage. Carvalho *et al.*, for example, reported that 82.5% of samples (combined nasopharyngeal and oropharyngeal swabs) that were positive for pneumococcus by cmPCR were culture-negative by latex. Similarly, 35.0% of those positive for pneumococcus by cmPCR were negative by lytA-pnemococcal specific PCR. This is particularly problematic when bacterial DNA is extracted from culture-enriched naso- and oropharyngeal samples, requiring PCR results to be confirmed by latex agglutination or other serotyping procedures. (24) Additional PCR limitations include the need for region-specific reaction protocols, implementing a high number of primer pairs to identify the same range of serotypes identified by microarray or WGS, and the increased risk of detecting non-viable pneumococci. As there is no evidence of a viable but non-culturable (VBNC) state in *S. pneumoniae,* (25) identifying non-viable pneumococci could be disadvantageous for field-based research. While a formal economic analysis of the methods would be justified, we were unable to extrapolate the individual costing components between sites. Such components would include local salaries and additional labour costs, procurement and shipping of equipment and consumables, equipment maintenance, local health and safety requirements, and institutional costs. For this reason, comparative costing is grossly categorized. Though we did not include invasive isolates (from blood or cerebrospinal fluid, for example), it is important to identify serotypes associated with IPD, including in post-PCV impact studies. For invasive isolates, with a single-serotype sample expected, microarray would have limited advantage. Application of serotyping-by-sequencing would then be the most informative option, including insight into population structure, antimicrobial resistance patterns and serotype replacement disease.

CONCLUSION

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

Supplemental Material

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

Data availability. The data supporting the findings of this study has been deposited in the Figshare repository. (26)

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References

462 1. Simell B, Auranen K, Kayhty H, Goldblatt D, Dagan R, O'Brien KL, Pneumococcal
463 Carriage G. 2012. The fundamental link between pneumococcal carriage and disease.
464 Expert Rev Vaccines 11:841-55.
465 2. Wahl B, O'Brien KL, Greenbaum A, Majumder A, Liu L, Chu Y, Luksic I, Nair H,
466 McAllister DA, Campbell H, Rudan I, Black R, Knoll MD. 2018. Burden of
467 Streptococcus pneumoniae and Haemophilus influenzae type b disease in children in
468 the era of conjugate vaccines: global, regional, and national estimates for 2000-15.
469 Lancet Glob Health 6:e744-e757.
470 3. von Gottberg A, Cohen C, de Gouveia L, Meiring S, Quan V, Whitelaw A, Crowther-
471 Gibson P, Madhi SA, Whitney CG, Klugman KP. 2013. Epidemiology of invasive
472 pneumococcal disease in the pre-conjugate vaccine era: South Africa, 2003-2008.
473 Vaccine 31:4200-8.
474 4. Nunes MC, von Gottberg A, de Gouveia L, Cohen C, Kuwanda L, Karstaedt AS,
475 Klugman KP, Madhi SA. 2011. Persistent high burden of invasive pneumococcal
476 disease in South African HIV-infected adults in the era of an antiretroviral treatment
477 program. PLoS One 6:e27929.
478 5. Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, Hurlburt DA, Butler JC,
479 Rudolph K, Parkinson A. 2007. Invasive pneumococcal disease caused by nonvaccine
480 serotypes among alaska native children with high levels of 7-valent pneumococcal
481 conjugate vaccine coverage. JAMA 297:1784-92.
482 6. Feikin DR, Kagucia EW, Loo JD, Link-Gelles R, Puhan MA, Cherian T, Levine OS,
483 Whitney CG, O'Brien KL, Moore MR, Serotype Replacement Study G. 2013. Serotype-
484 specific changes in invasive pneumococcal disease after pneumococcal conjugate

vaccine introduction: a pooled analysis of multiple surveillance sites. PLoS Med 10:e1001517.

7. Weinberger DM, Malley R, Lipsitch M. 2011. Serotype replacement in disease after pneumococcal vaccination. Lancet 378:1962-73.

8. Adegbola RA, DeAntonio R, Hill PC, Roca A, Usuf E, Hoet B, Greenwood BM. 2014. Carriage of Streptococcus pneumoniae and other respiratory bacterial pathogens in low and lower-middle income countries: a systematic review and meta-analysis. PLoS One 9:e103293.

9. da Gloria Carvalho M, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, Millar EV, O'Brien KL, Whitney CG, Cohen AL, Beall BW. 2010. Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol 48:1611-8.

10. Swarthout TD MT, Everett D, Bar Zeev N, Kamng'ona A, Chipasula T, Malisita K, Mwansambo C, Banda M, Hinds J, Gould KA, French N, Heyderman RS. 2016. Persistent vaccine-type carriage of Streptococcus pneumoniae four years after introducing PCV13 in a 3+0 schedule in Malawi, abstr International Symposium on Pneumococci and Pneumococcal Disease, Glasgow, Scotland, 2016.

11. Turner P, Hinds J, Turner C, Jankhot A, Gould K, Bentley SD, Nosten F, Goldblatt D. 2011. Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol 49:1784-9.

12. Austrian R. 1976. The quellung reaction, a neglected microbiologic technique. Mt Sinai J Med 43:699-709.

508 13. Satzke C, Dunne EM, Porter BD, Klugman KP, Mulholland EK, PneuCarriage project g.
509 2015. The PneuCarriage Project: A Multi-Centre Comparative Study to Identify the
510 Best Serotyping Methods for Examining Pneumococcal Carriage in Vaccine
511 Evaluation Studies. PLoS Med 12:e1001903; discussion e1001903.
512 14. Swarthout TD, Fronterre C, Lourenco J, Obolski U, Gori A, Bar-Zeev N, Everett D,
513 Kamng'ona AW, Mwalukomo TS, Mataya AA, Mwansambo C, Banda M, Gupta S,
514 Diggle P, French N, Heyderman RS. 2020. High residual carriage of vaccine-serotype
515 Streptococcus pneumoniae after introduction of pneumococcal conjugate vaccine in
516 Malawi. Nat Commun 11:2222.
517 15. Satzke C, Turner P, Virolainen-Julkunen A, Adrian PV, Antonio M, Hare KM, Henao-
518 Restrepo AM, Leach AJ, Klugman KP, Porter BD, Sa-Leao R, Scott JA, Nohynek H,
519 O'Brien KL, Group WHOPCW. 2013. Standard method for detecting upper respiratory
520 carriage of Streptococcus pneumoniae: updated recommendations from the World
521 Health Organization Pneumococcal Carriage Working Group. Vaccine 32:165-79.
522 16. Newton R, Hinds J, Wernisch L. 2011. Empirical Bayesian models for analysing
523 molecular serotyping microarrays. BMC Bioinformatics 12:88.
524 17. Hinds J GK, Witney AA, Baldry SJ, Lambertsen L, Hannage WP, Antonio M, Turner P,
525 Newton R, Wernisch L, Aanensen DM, Bentley S. 2009. Molecular serotyping of
526 Streptococcus pneumoniae: a microarray-based tool with enhanced utility for isolate
527 typing, novel serotype discovery, non-typeable investigation, multiple carriage
528 detection, and direct analysis of nasopharyngeal swabs, abstr 9th European Meeting
529 on the Molecular Biology of the Pneumococcus (Europneumo), Bern, Switzerland
530 18. Kapatai G, Sheppard CL, Al-Shahib A, Litt DJ, Underwood AP, Harrison TG, Fry NK.
531 2016. Whole genome sequencing of Streptococcus pneumoniae: development,

evaluation and verification of targets for serogroup and serotype prediction using an automated pipeline. PeerJ 4:e2477.

19. O'Brien KL, Nohynek H, World Health Organization Pneumococcal Vaccine Trials Carraige Working G. 2003. Report from a WHO working group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae. Pediatr Infect Dis J 22:133-40.

20. Gratten M, Montgomery J, Gerega G, Gratten H, Siwi H, Poli A, Koki G. 1989. Multiple colonization of the upper respiratory tract of Papua New Guinea children with Haemophilus influenzae and Streptococcus pneumoniae. Southeast Asian J Trop Med Public Health 20:501-9.

21. Hill PC, Cheung YB, Akisanya A, Sankareh K, Lahai G, Greenwood BM, Adegbola RA. 2008. Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian infants: a longitudinal study. Clin Infect Dis 46:807-14.

22. Ortika BD, Habib M, Dunne EM, Porter BD, Satzke C. 2013. Production of latex agglutination reagents for pneumococcal serotyping. BMC Res Notes 6:49.

23. Epping L, van Tonder AJ, Gladstone RA, The Global Pneumococcal Sequencing C, Bentley SD, Page AJ, Keane JA. 2018. SeroBA: rapid high-throughput serotyping of Streptococcus pneumoniae from whole genome sequence data. Microb Genom 4.

24. Carvalho Mda G, Pimenta FC, Moura I, Roundtree A, Gertz RE, Jr., Li Z, Jagero G, Bigogo G, Junghae M, Conklin L, Feikin DR, Breiman RF, Whitney CG, Beall BW. 2013. Non-pneumococcal mitis-group streptococci confound detection of pneumococcal capsular serotype-specific loci in upper respiratory tract. PeerJ 1:e97.

25. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. 2014. The importance of the viable but non-culturable state in human bacterial pathogens. Front Microbiol 5:258.

556 26. Swarthout TD, Gori A, Bar-Zeev N, Kamng'ona AW, Mwalukomo TS, Bonomali F,
557 Nyirenda R, Brown C, Msefula J, Everett D, Mwansambo C, Gould K, Hinds J,
558 Heyderman RS, French N. 2020. Study data and dictionary - evaluation of
559 pneumococcal serotyping methods, Blantyre, Malawi, 2015-2017
560 doi:10.6084/m9.figshare.12807872.

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562

563 **Figure legends**

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

569

570 **Figure 2** Laboratory procedures. Nasopharyngeal swabs (NPS) were inoculated into
571 STGG and subsequently plated on a growth agar of sheep blood and gentamycin.
572 Bacteria growth (from single-colony picks) from samples culture-confirmed for
573 Streptococcus pneumoniae were used for latex serotyping. Remaining pure-growth
574 isolates, retained at -80°C in sterile STGG, were later grown for DNA extraction and
575 WGS. Aliquots of original samples (NPS-STGG) that were culture-confirmed for
576 *Streptococcus pneumoniae* were assessed by microarray. NPS=nasopharyngeal
577 swabs, STGG=skim-milk-tryptone-glucose-glycerol, WGS=whole-genome
578 sequencing, Spn=Streptococcus pneumoniae, SBG=sheep blood and gentamycin,
579 SSI= Statens Serum Institute, 13v=13-valent, NSP-STGG=NPS inoculated into
580 STGG.

581

582 **Figure 3** Concordance between assays. Concordance between two assays was
583 defined as both assays identifying pneumococcal carriage as VT or both as NVT.
584 Latex and PneumoCaT reported one result per sample, both using the same pure-
585 growth culture. Microarray, using an aliquot of the original NPS-STGG, differentiated
586 individual serotypes in multiple serotype carriage, when present. When comparing
587 the three assays, concordance was based on serotype if latex reported VT carriage.

Journal of Clinical Microbiology

If latex reported NVT, this was considered concordant to any NVT reported by PneumoCaT and microarray, as long as PneumoCaT and microarray reported the same NVT.

Figure 4 Proportion of individual VT serotypes contributing to total VT carriage. The proportion of individual VT serotypes detected is not significantly different when comparing microarray to latex. Refer to Table S1 in the supplemental material for the reported frequency of each vaccine serotype (VT) detected by microarray and latex. VT=vaccine serotype.

Notes

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618

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620 grants from GlaxoSmithKline Biologicals and from Takeda Pharmaceuticals outside
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622 behalf of St George's, University of London or BUGS Bioscience that are sponsored
623 or funded by vaccine manufacturers, including Pfizer, GlaxoSmithKline and Sanofi
624 Pasteur. Dr. Jason Hinds is also co-founder and shareholder of BUGS Bioscience, a
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626 competing interests.

627

628 **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%

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

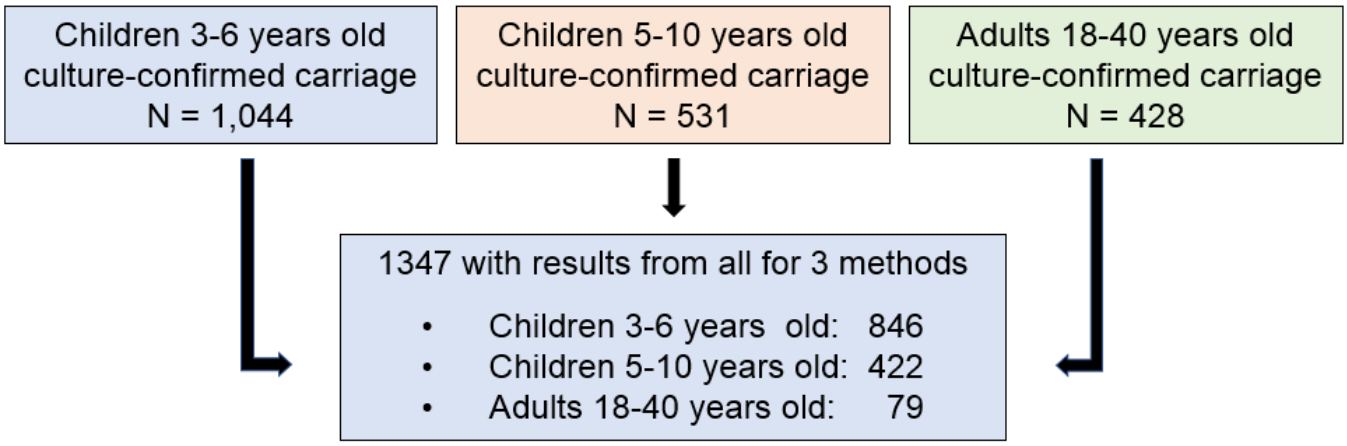
631 **Table 2** Key comparative parameters of serotyping methods.
632

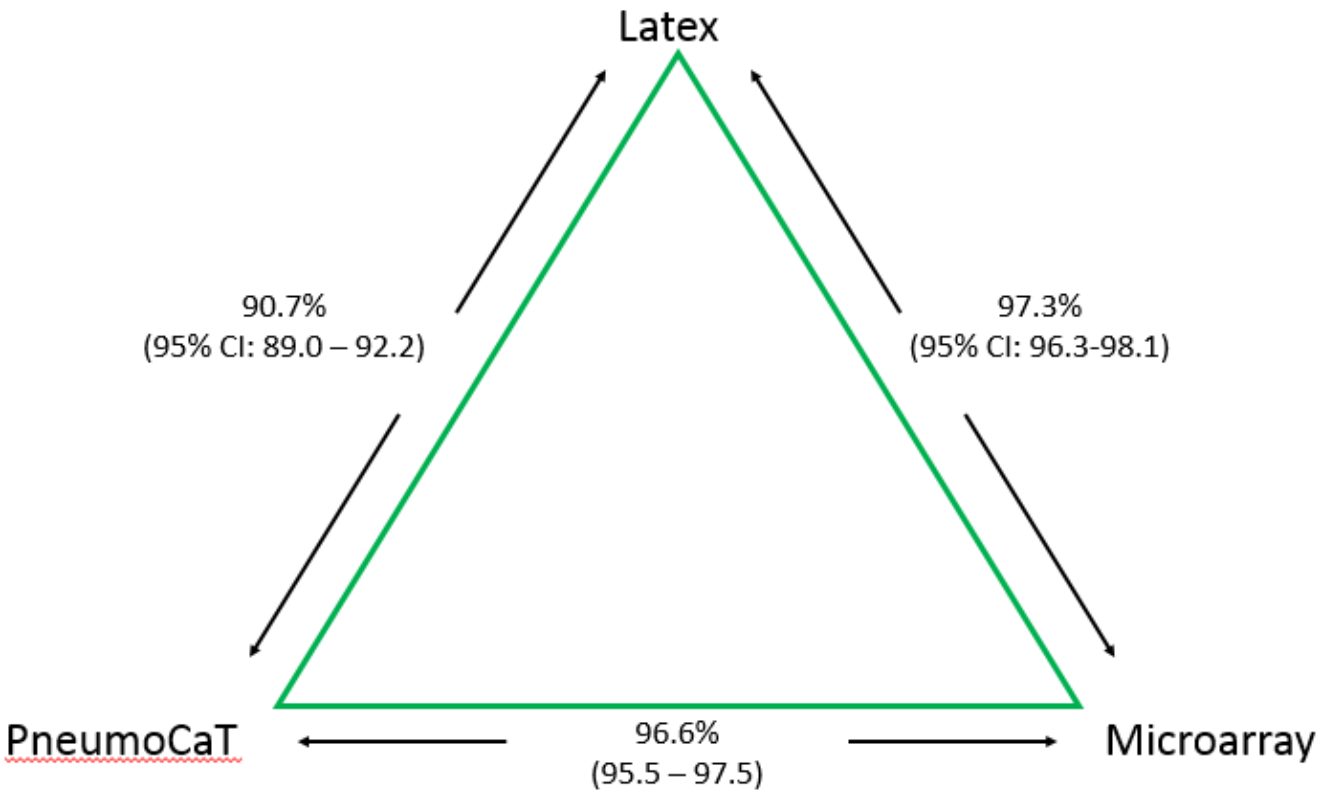
	Latex (phenotypic)	Microarray (genomic)	PneumoCaT (genomic)
Assay implementation			
Sample used in assay	• Pure growth from single isolate	• Original sample in STGG	• Pure growth from single isolate
Cost estimate[1]	• Lowest of three assays	• Highest of three assays	• Middle of three assays
Implementation of assay[1]	• 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
Assay output and interpretation			
Serotypes reported	• Single	• Multiple, if present	• Single
NVT differentiation	• No[3]	• Yes	• Yes
Relative abundance of individual serotypes reported	• No	• Yes	• No
Additional outputs	• Isolates archived and available for further analyses	• AMR profile[2] • 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

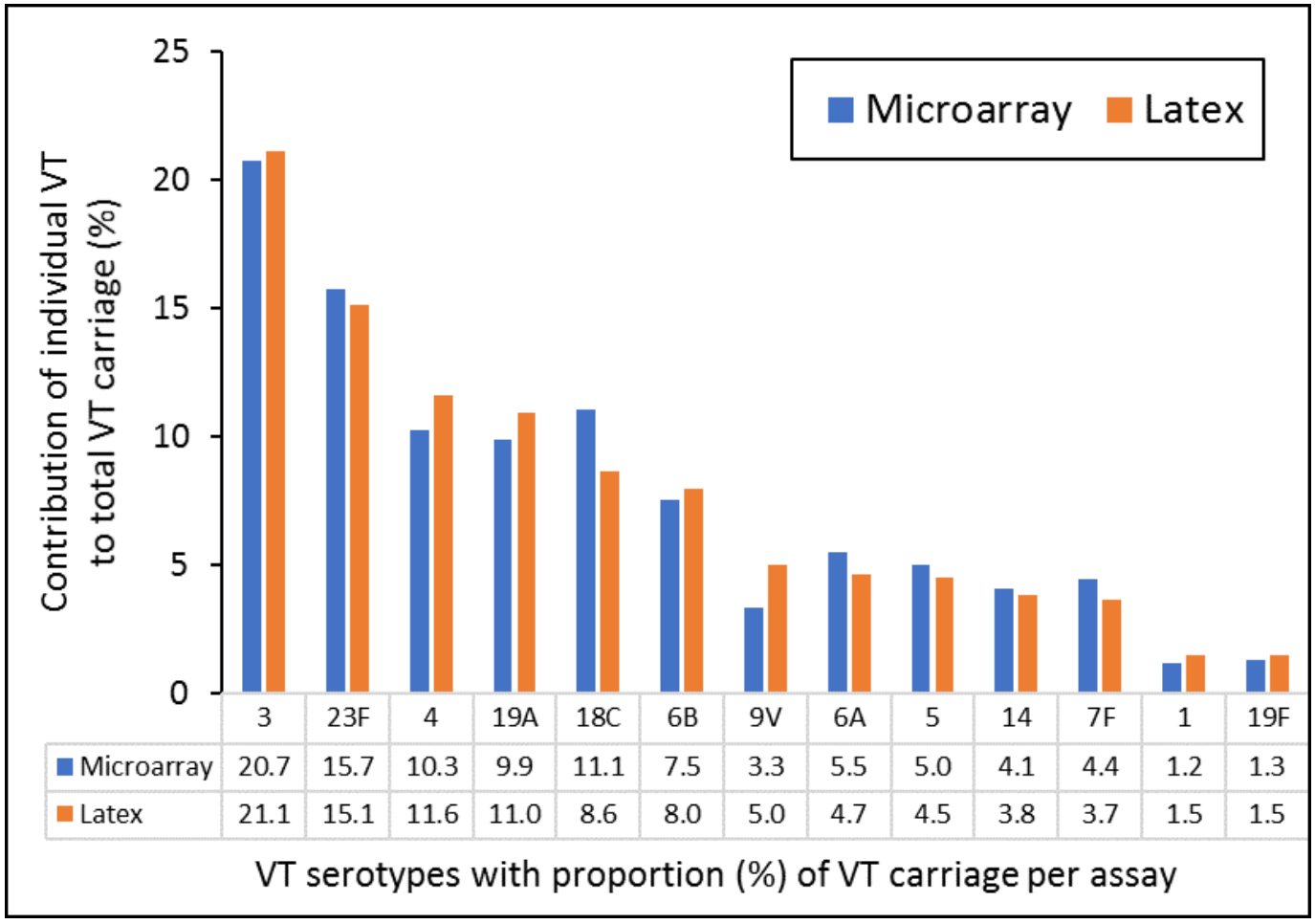
633 [1]Estimated costs and feasibility of implementation and maintenance are specific to the study requirements and laboratory capacity
634 (including no capacity for WGS or microarray) at the Malawi-Liverpool-Wellcome Trust Clinical Research Programme in Blantyre.
635 [2] AMR profile cannot be assigned to a single strain in a sample with multiple-serotype or multiple-pathogen carriage.

[3]NVT & NT reported as NVT. Use of both commercial products and production of in-house latex serotyping reagents can significantly increase the number of NVT serotypes that can be differentiated by latex.

638







	3	23F	4	19A	18C	6B	9V	6A	5	14	7F	1	19F
Microarray	20.7	15.7	10.3	9.9	11.1	7.5	3.3	5.5	5.0	4.1	4.4	1.2	1.3
Latex	21.1	15.1	11.6	11.0	8.6	8.0	5.0	4.7	4.5	3.8	3.7	1.5	1.5