Standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*: Updated recommendations from the World Health Organization Pneumococcal Carriage Working Group

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ABSTRACT

In 2003 the World Health Organization (WHO) convened a working group and published a set of standard methods for studies measuring nasopharyngeal carriage of *Streptococcus pneumoniae* (the pneumococcus). The working group recently reconvened under the auspices of the WHO and updated the consensus standard methods. These methods describe the collection, transport and storage of nasopharyngeal samples, as well as provide recommendations for the identification and serotyping of pneumococci using culture and non-culture based approaches. We outline the consensus position of the working group, the evidence supporting this position, areas worthy of future research, and the epidemiological role of carriage studies. Adherence to these methods will reduce variability in the conduct of pneumococcal carriage studies undertaken in the context of pneumococcal vaccine trials, implementation studies, and epidemiology studies more generally so variability in methodology does not confound the interpretation of study findings.

1. Introduction

Between 1998 and 2001 the World Health Organization (WHO[1]) convened the Pneumococcal Carriage Working Group. This group was charged with formulating a set of core methods for conducting studies of pneumococcal nasopharyngeal (NP) colonization primarily in the context of pneumococcal conjugate vaccine (PCV) efficacy trials [1]. The PCV efficacy trials led to PCV licensure and now widespread inclusion of PCV in routine immunization programs around the world. Numerous studies of PCV effect on NP colonization were published in the pre-licensure period and were available for consideration by regulators, although no indication was sought for this outcome. PCV impact studies have also included carriage components, thereby providing important lessons about the performance and impact of PCV on a population level [2–4]. Carriage studies have provided the key biological link to the indirect effect of PCV on pneumococcal disease [2], shown that there is no change in the invasiveness of pneumococcal strains since PCV implementation [2,3], anticipated the impact of PCV on cross-reacting serotypes [2,5,6], contributed to the identification of new pneumococcal serotypes [7,8], and have been central to our understanding of antimicrobial resistance evolution and impact [9,10]. The variability in results from pneumococcal carriage studies across diverse epidemiologic settings can be understood to derive from biologic effects rather than methodological differences, in large part because many of the standard pneumococcal carriage methods have been widely adopted.

In the decade since last convening the working group there have been many key accomplishments including sequencing of 90 pneumococcal capsular loci [11], the advent of molecular detection and quantification of pneumococci in NP specimens and serotype-specific detection including improved detection of multiple serotype colonization. There have been significant advances in molecular typing, and in modeling and statistical methods for longitudinal studies of carriage dynamics.

In light of these advances, and the importance of carriage studies, WHO invited an ad hoc group of experts, some of whom participated in the previous working group, to evaluate the state of knowledge, revise the core methods where appropriate, and outline the important scientific questions for the future. In developing this update, the authors reviewed newly published literature pertinent to each aspect of the consensus method, sought unpublished data on relevant issues and wrote a set of draft recommendations. This document was circulated to the working group and formed the basis of a review meeting in Geneva, 29–30th March 2012. The resultant consensus methods were then circulated for final approval. Our recommendations, outlined in detail below, provide researchers with a set of methods that we believe are a minimum set of requirements for pneumococcal carriage studies.

2. Site of sample

It is possible to detect microbial colonization of the upper respiratory tract by sampling the nose, nasopharynx or the oropharynx. We considered the choice between the nasopharynx and oropharynx for detecting pneumococcal carriage (the sensitivity of nasal sampling is covered in Section 3). We have identified nine studies (including one unpublished) that have compared the sensitivity of sampling the nasopharynx and oropharynx of children (Table 1), and five studies for adults (Table 2). It was not possible to extract paired information from all studies, so we compared the sensitivity of NP or oropharyngeal (OP) swabs alone in the detection of pneumococcal carriage against a gold standard of detection by either method when both were sampled in an individual. We restricted our review to studies published from 1975 onwards, as prior to this, swabs were often collected with rigid wooden applicators, which were assumed to be less effective when sampling via the nose than when passed via the mouth.

In children, the additional yield provided by sampling the oropharynx as well as the nasopharynx is relatively small, as the sensitivity of sampling the nasopharynx alone is >90% in seven of nine studies and <80% in only one small study (Table 1). In adults, the advantage to the NP route is not so marked and an ideal strategy involves sampling by both routes (Table 2). Data relating to detection of *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and respiratory viruses from different sites are described in the Supplementary Material (including Supplementary Table 1).

2.1. Recommendation

For detecting pneumococci in infants and children, we recommend sampling the nasopharynx only. Sampling the oropharynx marginally increases sensitivity but substantially increases the resources required, and may not be acceptable to the study population. For adults, both NP and OP samples should be collected, however if only one sample is possible, collecting from the nasopharynx is more sensitive than from the oropharynx for pneumococci.

2.2. Future research

All studies reviewed here used culture to detect respiratory bacteria. Therefore molecular testing of paired NP/OP samples is needed to establish if the recommendations for anatomic site of sampling apply also to studies using molecular detection of pneumococci.

3. NP and nasal sample collection

Conventional teaching is that nasal specimens are less sensitive than NP samples for detecting pneumococci. We identified only three studies directly comparing NP and nasal sampling methods for detecting pneumococci in children (Supplementary Table 2). Rapola et al. [12] found that pneumococcal isolation rates from NP aspirates, NP swabs and nasal swabs did not differ. The same conclusion was reached by Carville et al. [13] for NP aspirates and nasal swabs, and Van den Bergh et al. [14] for NP swabs and nasal swabs. However, in two of these studies children had respiratory symptoms, either acute respiratory infection [12] or rhinorrhea [14], conditions that are known to enhance pneumococcal carriage and possibly affect the sensitivity of detection from nasal specimens. As such, there is currently insufficient evidence to conclude that nasal swabbing is as effective as NP swabbing for the detection of pneumococcal carriage in healthy children. A fourth comparative study [15] found that NP washes performed better than NP swabs, but concluded that the additional gain was not sufficiently large to offset the discomfort and reduced acceptability to study subjects.

Lieberman et al. [16] and Gritzfeld et al. [17] found no difference between NP swabs and NP or nasal washes for the detection of pneumococci in adults with respiratory infection (Supplementary Table 2). The adults found nasal washes more comfortable than NP swabbing, but nasal washes were not recommended for children because of the level of participant cooperation required [17].

There are potential disadvantages of nasal/NP aspirates and washes for pneumococcal detection; the methods are difficult to standardize, and frequent washes in an individual hypothetically may disrupt the flora or affect immune responses. Given that nasal

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[1] Abbreviations: CI, confidence interval; NP, nasopharyngeal; OP, oropharyngeal; OTU, operational taxonomic unit; PCV, pneumococcal conjugate vaccine; STGG, Skim milk tryptone-glucose-glycerol; ULT, ultra-low temperature, ≤−70 °C; WHO, World Health Organization.

Table 1
Sensitivity of sampling the nasopharynx or oropharynx for detecting pneumococcal carriage in infants and children.

Study [ref]	Year	Study details	No. positive samples (NP)[a]	No. positive samples (OP)[a]	No. positive samples (NP or OP)	Sensitivity[b] of NP samples (95% CI)	Sensitivity[b] of OP samples (95% CI)
Hendley et al. [1]	1975	27 healthy American children	8	13	14	57% (29, 82)	93% (66, 100)
Converse and Dillon [2]	1977	Longitudinal study of 100 healthy American infants, 132 paired swabs	55	51	58	95% (86, 99)	88% (77, 95)
Gray et al. [3]	1980	Longitudinal study of 82 healthy American children aged <2 years	456	394	476	96% (94, 97)	83% (79, 86)
Capeding et al. [4]	1995	Longitudinal study of 296 healthy Filipino infants aged 6–65 weeks	607[c]	222	639	95% (93, 97)	35% (31, 39)
Rapola et al. [5]	1997	96 Finnish children aged <7 years with acute respiratory infection	29	19	32[d]	91% (75, 98)	59% (41, 76)
Greenberg et al. [6]	2004	216 healthy Israeli children aged <5 years	144	36	147	98% (94, 100)	24% (18, 32)
Taylor et al. [7]	2006	47 Canadian children with Cystic Fibrosis	12[e]	0	12	100% (74, 100)	0% (0, 26)
Katz et al. [8]	2007	125 healthy Russian children	63	39	75	84% (74, 91)	52% (40, 64)
Hare et al. (unpublished data)	2013	120 Australian Aboriginal children with bronchiectasis	43	7	44	98% (93, 100)	16% (5, 27)

References: [1] Hendley JO, Sande MA, Stewart PM, Gwaltney JM, Jr. Spread of *Streptococcus pneumoniae* in families. I. Carriage rates and distribution of types. J Infect Dis. 1975;132:55-61. [2] Converse GM, 3rd, Dillon HC, Jr. Epidemiological studies of *Streptococcus pneumoniae* in infants: methods of isolating pneumococci. J Clin Microbiol. 1977;5:293-6. [3] Gray BM, Converse GM, 3rd, Dillon HC, Jr. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. J Infect Dis. 1980;142:923-33. [4] Capeding MR, Nohynek H, Sombrero LT, Pascual LG, Sunico ES, Esparar GA, et al. Evaluation of sampling sites for detection of upper respiratory tract carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae* among healthy Filipino infants. J Clin Microbiol. 1995;33:3077-9. [5] Rapola S, Salo E, Kiiski P, Leinonen M, Takala AK. Comparison of four different sampling methods for detecting pharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae* in children. J Clin Microbiol. 1997;35:1077-9. [6] Greenberg D, Broides A, Blancovich I, Peled N, Givon-Lavi N, Dagan R. Relative importance of nasopharyngeal versus oropharyngeal sampling for isolation of *Streptococcus pneumoniae* and *Haemophilus influenzae* from healthy and sick individuals varies with age. J Clin Microbiol. 2004;42:4604-9. [7] Taylor L, Corey M, Matlow A, Sweezey NB, Ratjen F. Comparison of throat swabs and nasopharyngeal suction specimens in non-sputum-producing patients with cystic fibrosis. Pediatr Pulmonol. 2006;41:839-43. [8] Katz A, Leibovitz E, Timchenko VN, Greenberg D, Porat N, Peled N, et al. Antibiotic susceptibility, serotype distribution and vaccine coverage of nasopharyngeal and oropharyngeal *Streptococcus pneumoniae* in a day-care centre in St. Petersburg, Russia. Scand J Infect Dis. 2007;39:293-8.

[a] The nasopharynx and oropharynx were sampled by swab unless otherwise indicated.
[b] Sensitivities were estimated against a gold standard of 'positive in either sample' using the data presented in the published findings.
[c] This study used nasal swabs taken from the nostrils with a cotton-tipped wooden applicator.
[d] This study included positive results from nasal swabs and NP aspirates within the gold standard.
[e] This study used NP suction.

or NP washing is generally less well tolerated by children, a single NP swab is preferred for the detection of pneumococcal carriage but washes/aspirates are an acceptable method [15].

NP swabbing techniques may vary across studies unless the investigators adhere closely to the standard method, summarized here. Hold the infant or young child's head securely. Tip their head backwards slightly and pass the swab directly backwards, parallel to the base of the NP passage. The swab should move without resistance until reaching the nasopharynx, located about one-half to two-thirds the distance from the nostril to ear lobe (Fig. 1).

If resistance occurs, remove the swab and attempt again to take the sample entering through the same or the other nostril. Failure to obtain a satisfactory specimen is often due to the swab not being fully passed into the nasopharynx. Once the swab is in location, rotate the swab 180°, or leave in place for 5 s to saturate the swab tip; remove the swab slowly. All swabs should be processed; however, to assist with interpreting the results, investigators should record whether the procedure was acceptable or suboptimal. Recording if secretions are present on the swab [18] and whether the swab was potentially contaminated (e.g. touched

Table 2
Sensitivity of nasopharyngeal (NP) or oropharyngeal (OP) sampling for detecting pneumococcal carriage in adults.

Study [ref]	Year	Study details	No. positive samples (NP)[a]	No. positive samples (OP)[a]	No. positive samples (NP or OP)	Sensitivity[b] of NP samples (95% CI)	Sensitivity[b] of OP samples (95% CI)
Hendley et al. [1]	1975	24 healthy American adults	0	9	9	0% (0, 34)	100% (66,100)
Greenberg et al. [2]	2004	216 Israeli mothers of young children	19	18	33	58% (39, 75)	55% (36, 72)
Watt et al. [3]	2004	1994 Native American adults	222	115	304	73% (68, 78)	38% (32, 44)
Lieberman et al. [4]	2006	300 Israeli adults with respiratory infection	29	13	36	81% (64, 92)	36% (21, 54)
Levine et al. [5]	2012	742 Israeli army recruits	31	27	49	63% (48, 77)	55% (40, 69)

References: [1] Hendley JO, Sande MA, Stewart PM, Gwaltney JM, Jr. Spread of *Streptococcus pneumoniae* in families. I. Carriage rates and distribution of types. J Infect Dis. 1975;132:55-61. [2] Greenberg D, Broides A, Blancovich I, Peled N, Givon-Lavi N, Dagan R. Relative importance of nasopharyngeal versus oropharyngeal sampling for isolation of *Streptococcus pneumoniae* and *Haemophilus influenzae* from healthy and sick individuals varies with age. J Clin Microbiol. 2004;42:4604-9. [3] Watt JP, O'Brien KL, Katz S, Bronsdon MA, Elliott J, Dallas J, et al. Nasopharyngeal versus oropharyngeal sampling for detection of pneumococcal carriage in adults. J Clin Microbiol. 2004;42:4974-6. [4] Lieberman D, Shleyfer E, Castel H, Terry A, Harman-Boehm I, Delgado J, et al. Nasopharyngeal versus oropharyngeal sampling for isolation of potential respiratory pathogens in adults. J Clin Microbiol. 2006;44:525-8. [5] Levine H, Zarka S, Dagan R, Sela T, Rozhavski V, Cohen DI, et al. Transmission of *Streptococcus pneumoniae* in adults may occur through saliva. Epidemiol Infect. 2012;140:561-5.

[a] The nasopharynx and oropharynx were sampled by swab unless otherwise indicated.
[b] Sensitivities were estimated against a gold standard of 'positive in either sample' using the data presented in the published findings.

by the investigator or dropped on the ground) may also be helpful in interpretation.

Because NP specimen collection (by swab or by wash) requires training, demands adherence to the methodology, and is unpleasant for the study subject, and because sometimes even nasal swabs are not well tolerated, alternate methods have been assessed. Leach et al. [19] found that in an Australian population with a high pneumococcal burden, nose blowing into a paper tissue, followed by swabbing and culture of the material on the tissue, was an effective alternative to nasal swabbing when nasal secretions were present. The sensitivity of detecting pneumococcus from nose blowing samples (compared with nasal swabs, and when secretions were visible at the time of sampling) was 97% in Aboriginal children aged 3–7 years and 94% in children aged less than 4 years who were attending urban child care centers. For children without visible secretions, direct NP or nasal sampling was required [19]. Recently, Van den Bergh et al. [14] found that the proportion of pneumococcal-positive cultures was similar when sampling secretions from a tissue (tissue swab 65%, whole tissue 74%), or taking NP and nasal swabs (both 64%) in 66 Dutch children aged 0–4 years with rhinorrhea. Data relating to detection of *H. influenzae, M. catarrhalis, S. aureus* and respiratory viruses by various sampling methods are described in the Supplementary Material (including Supplementary Table 3).
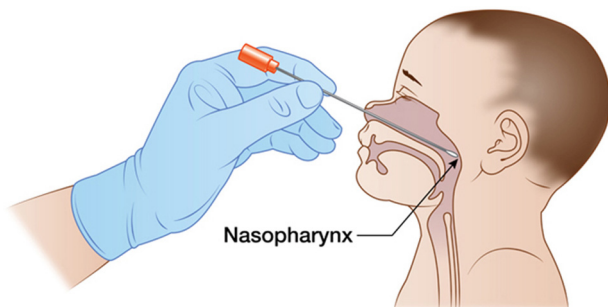

Fig. 1. Collecting a nasopharyngeal swab.

3.1. Recommendation

We recommend the NP swab approach for collection of the sample. NP aspirates or washes are also acceptable methods of specimen collection as they have sensitivity for pneumococcal detection equal to, or greater than, that of NP swabs, but may be less tolerated by participants. In the event that NP sampling cannot be implemented, nasal swabs or swabbing visible secretions from nose blowing into a tissue are better than collecting no specimens. However, any deviation from the recommended NP swab should be clearly reported to allow accurate comparisons across studies.

3.2. Future research

All data presented are from studies using culture to detect pneumococci. Specimen collection comparison studies should be undertaken using molecular methods for pneumococcal detection. Direct comparisons of NP and nasal sampling methods in healthy children are also needed.

4. Number of NP specimens

A single NP swab is unlikely to represent the colonizing bacteria of the upper respiratory tract with complete sensitivity, as these bacteria may not reside uniformly across the mucosal surface, and there is inherent variability in the mucosal surfaces touched by each sample swab. The insensitivity of a single swab has been demonstrated by studies that have sampled the upper respiratory tract twice at the same visit, usually by taking one swab from the nasopharynx and another from the oropharynx (Tables 1 and 2). This prompts two questions: what is the sensitivity of a single NP swab and could this sensitivity be optimized by increasing the number of swabs collected?

The sensitivity of a single swab has been estimated using NP wash as a gold standard among healthy Kenyan children [15]. NP swabs had sensitivity of 85% (95% CI 73–95%) when both a swab and wash were collected in immediate sequence. In all children with a negative NP wash, the NP swab was also negative. Furthermore, two NP swabs (one swab passed into each nostril a few minutes apart) were found to be only marginally superior to a single NP

swab. Taking the combined positive results of the two swabs as a reference gold standard, the sensitivity of a single swab was 95% (95% CIs 88–98%). There was no evidence of a systematic advantage to swabbing either the right or left nostril [15].

4.1. Recommendation

Increasing the number of NP swabs taken at the same time-point does not increase the sensitivity appreciably, but increases the discomfort to the subject. Therefore, we recommend collecting a single NP swab to detect pneumococcal carriage.

4.2. Future research

The study cited for this recommendation used culture-based detection and was confined to a single setting. Additional studies of multiple swabs would contribute meaningfully to the evidence for this recommendation if conducted among children in low prevalence settings, among adults, and/or including molecular methods of detection.

5. Swab material

Ideally, NP swabs used for colonization studies should (1) be safe for use with minimal irritation or side effects, (2) be efficient at extracting micro-organisms from the nasopharynx onto the swab, (3) have no effect on the viability of the isolated pneumococci or any other pathogens (viral or bacterial) to be assayed, (4) allow easy elution of organisms from the swab and (5) be compatible with all intended assays. For example, calcium alginate inhibits some real-time PCR assays resulting in a reduced sensitivity of detection of *Bordetella pertussis* [20], and natural fibers (e.g. cotton, rayon, or calcium alginate) often contain nucleic acids, which may be detected in whole microbiome sequencing studies (D. Bogaert, unpublished data) or may include inhibitors to pneumococcal growth (e.g. cotton).

Materials that have been widely used in pneumococcal NP clinical studies include calcium alginate, rayon, Dacron and nylon flocked swabs. There are no clinical studies comparing the performance of these materials head-to-head, so any distinctions, if they exist, are inferred from studies of spiked samples and cross study clinical comparisons. Rayon, Dacron and calcium alginate swabs were compared for their ability to culture pneumococci directly from the swab or from the surrounding skim milk tryptone-glucose-glycerol (STGG) medium [21]. Rayon was shown to be superior for culture from both the STGG medium and the swab, followed by calcium alginate and then Dacron. By contrast, Dube et al. found Dacron was superior to rayon in efficiency of pneumococcal elution from the swab into STGG (eluting approximately 44% vs. 8% of the inoculum respectively), and that nylon flocked swabs (eluting 100% of the inoculum) were the most efficient [22]. Collectively these data, along with the generally comparable recovery rates from studies using any of the rayon, calcium alginate or Dacron swabs, suggest that in practice, the majority of swab material currently used in NP studies will collect sufficient bacteria to be detected, and possible differences in the swab materials will most likely appear only in samples with very low yields of organisms.

Recently, flocked nylon swabs have been introduced into clinical practice, on the premise that the protruding nylon fibres improve the recovery of target organisms from the sampled surface, and allow for the rapid elution of collected material into the transport medium. There are no large published clinical studies comparing flocked swabs and other swab types for the recovery of pneumococci from the nasopharynx, although a study with spiked and paired NP samples suggests that flocked swabs are superior to both Dacron and rayon [22], and clinical evidence from other types of sampling (i.e. sampling for viral pathogen detection) indicates that flocked swabs are equivalent or superior to Dacron or rayon swabs in proportion of positive specimens, and the quantity of organism recovered [23–27]. Flocked swabs have been used in a variety of large pneumococcal NP studies with high rates of colonization measured, supporting their use [28,29]. Since flocked swabs are made from inert nylon material, they are unlikely to interfere with any culture or molecular assay. These swabs may also result in higher yields of organisms which would improve the sensitivity of detection, in particular from samples with low density of carriage and minor serotypes. Note that collecting dual swabs (where two swabs are twisted together and inserted into one nostril) can be useful for comparison studies. Unfortunately the flocked swabs that are currently on the market cannot be twisted together.

5.1. Recommendation

NP swabs made from calcium alginate, rayon, Dacron or nylon materials are suitable for culture based carriage studies to determine the circulating serotypes in a population. For molecular analyses, synthetic materials such as nylon or Dacron are preferred as they are least likely to inhibit amplification of DNA. Flocked nylon swabs are superior for the detection of other pathogens such as respiratory viruses.

5.2. Future research

Clinical and laboratory studies to compare nylon flocked swabs, Dacron, rayon and calcium alginate in samples with low pathogen concentrations, would be of value. Studies that include molecular assays and a broad range of pathogen types would be optimal. Production of flocked swabs that can be divided in two may be useful for comparison studies.

6. Swab transport and storage

STGG medium was previously recommended as a swab transport and storage medium [1] because it is non-proprietary, is easily made with commonly available ingredients, is inexpensive and had been successfully used by many groups investigating carriage of pneumococci and other upper respiratory tract bacterial organisms. Interestingly, a recent study investigated NP carriage in 574 Nepalese children using two intertwined rayon swabs. They found that the carriage prevalence was 41% with a NP swab that had been stored in silica desiccant sachets for up to 2 weeks, compared with 59% with a NP swab that had been placed in STGG and processed within 8 h. There was 79% agreement between the two methods. As such, silica desiccant sachets may be useful when there is delayed or limited access to microbiological facilities, although it likely results in an underestimate of the carriage rate and may alter the serotype and/or genotype distribution (David Murdoch, personal communication).

Therefore, although no systematic comparisons have been conducted, consensus is that STGG remains the medium of choice for transport and storage of NP swabs for the present time.

6.1. Sample collection medium

The STGG medium has been adapted from Gibson and Khoury [30] and Gherna [31], and should be produced as described by O'Brien et al. [32]. In brief, mix 2.0 g of skim milk powder, 3.0 g of tryptone soy broth powder, 0.5 g of glucose, and 10 ml of glycerol and dissolve in 100 ml of distilled water. The STGG medium should

be autoclaved before use: dispense 1.0 ml of STGG medium into 1.5 ml screw-capped vials and autoclave for 10 min at 121 °C. STGG vials can be stored frozen at −20 °C (or colder) or refrigerated until use. A standard volume of 1.0 ml is preferred to allow for comparisons across studies in quantification of pneumococci. The volume of STGG should be reported for all studies. Allow tubes of STGG medium to reach room temperature before use. Usually the milk solids pellet in the bottom of the tube is resuspended by vortexing for 10–20 s, although there is no evidence that this is necessary and in practice this is not always done. Consensus is that STGG medium should be used within 6 months of preparation whether stored frozen or refrigerated. A quality control test for sterility of the STGG medium must be performed on each batch. The ability of STGG medium to support recovery of viable pneumococci should also be checked.

6.2. Inoculation and transport

Immediately following sample collection the NP swab is aseptically placed into the room-temperature STGG, inserting it to the bottom of the STGG medium, raising it slightly and cutting off the shaft with sterile scissors (to enable lid closure), leaving the swab in the STGG media. The closed tube is then placed in a cool box or on wet ice and transported to the laboratory within 8 h. Once in the laboratory, the specimen is vortexed at high speed for 10–20 s to disperse organisms from the swab tip, and immediately processed and stored as described below.

6.3. Processing and storage

To prevent sample loss in the event of freezer failure, we recommend dividing the vortexed specimen into two aliquots, one of ~0.2–0.3 ml, and the second comprised of the remainder of the STGG containing the swab. The two aliquots should preferably be stored in separate freezers.

Several studies have investigated the impact of frozen storage (at −20 °C and ULT (ultra low temperature, −70 °C or colder)) on the recovery of upper respiratory tract bacterial pathogens including pneumococci in STGG medium over time [15,30,32–37]. These studies have shown minimal or no significant effects of ULT freezing. For example, Abdullahi et al. [15] reported that recovery of pneumococci by culture from fresh and frozen (ULT for two months) NP swab samples in STGG was indistinguishable, although there were differences in the serotype distribution recovered. This could be, at least in part, attributed to the differential capacity of pneumococcal serotypes to survive the freezing process. Kwambana et al. [35] investigated the difference between NP swabs stored in STGG and analyzed within hours of collection, and those analyzed after 30 days of storage at ULT. 16S rRNA gene-based terminal restriction fragment length polymorphism and clone analysis showed that the mean number of operational taxonomic units (OTUs), a measure of overall microbial diversity, decreased after frozen storage, although the changes to the relative abundance of most species was minimal.

Long-term ULT storage has been evaluated with clinical [34] or laboratory-prepared samples (T. Kaijalainen, unpublished data) finding no demonstrable changes in semi-quantitative viability of pneumococcus over a 12 year period.

Our previous recommendations stated that STGG swabs could be held at -20 °C for up to six weeks [1]. This recommendation was based on a relatively limited evidence base [32,33] and consensus practice. However, a recent publication found that the numbers of culturable pneumococci declined within 24 h at −20 °C [37],

suggesting that this temperature may only be suitable for very short periods.

6.4. Recommendation

STGG is recommended as the primary transport and storage medium. Specimen swabs should be transported on wet ice or colder conditions during transport and handling, and be frozen at ULT as soon as possible after collection. Storage at −20 °C is acceptable if the specimen will be tested in the short term (within days) but is not recommended for longer term storage. Investigators should consider dividing the original STGG specimen into two or more aliquots and storing these in separate freezers.

6.5. Future research

Efficacy of newer transport media to maintain microorganism viability at room temperature, cold or ULT storage of NP swabs could be evaluated in field settings. Future research should assess the recovery of pneumococci after storage of different aliquots of NP material in STGG medium in different storage conditions, and the impact of long-term frozen storage of STGG samples on the recovery of pneumococci for low-density specimens, particularly to establish guidelines around −20 °C storage. Finally, an assessment of limits of the duration of storage of STGG medium prior to use, at various temperatures but especially frozen, would assist sites with limited ability to produce STGG themselves.

7. Culture for pneumococci

An ideal culture medium should prevent growth of non-pneumococcal species without inhibiting growth of the pneumococci itself. To this end, defibrinated blood agar (from a non-human source such as sheep, horse or goat) supplemented with 5 µg/ml gentamicin has been the most widely used selective medium to culture pneumococci from NP samples [38–40]. For culture of pediatric NP and throat swabs, this medium has been shown to result in a similar yield of pneumococci to anaerobically incubated blood agar plates [41]. The concentration of gentamicin in agar has been shown to have a significant effect on isolation of pneumococci [42]. There are similar yields of pneumococci when culturing respiratory tract specimens on blood agar supplemented with 2.5–5 µg/ml gentamicin compared with culture on plain blood agar or by mouse inoculation [43–45]. Alternative supplements used to improve the isolation of pneumococci by culture include combinations of colistin and nalidixic acid (CNA) or colistin and oxolinic acid (COBA) [46]. Unlike blood agar-gentamicin and COBA, blood-CNA agar does not suppress the growth of staphylococci.

7.1. Recommendation

Blood agar, either Columbia or trypticase soy agar base with sheep, horse, or goat blood, supplemented with 5 µg/ml gentamicin is considered the core primary isolation media. Blood-CNA or COBA agars are acceptable alternatives, whereas human blood agar should never be used [45,47]. Thoroughly mix a fresh or fully-thawed NP swab-STGG specimen using a vortex and inoculate 10 µl onto a selective plate and streak into all four plate quadrants with sterile loops. Some investigators may choose to use larger volumes of STGG medium (e.g. 50 µl or 100 µl). As this will affect the sensitivity of detection, the volume used should be noted when reporting. Incubate the pneumococcal plate(s) overnight at 35–37 °C in a CO_2 enriched atmosphere, either by using a candle jar or 5–10% CO_2 incubator. Plates with no growth should be re-incubated for another 24 h before being discarded

as negative. If required, record the semi-quantitative growth of alpha-hemolytic colonies [1]. Single colonies are then picked and subcultured for analysis, including identification as described below.

7.2. Future research

Culture of NP specimens, by scraping or drilling into the frozen STGG media using a sterile microbiological loop, might permit prolongation of specimen integrity. This technique has been used successfully in the sub-culture of pneumococcal isolates stored in STGG, but requires quantitative validation for use with NP samples.

8. Culture-based broth enrichment of nasopharyngeal samples

Several investigators have applied a culture enrichment step to samples in order to enhance the sensitivity of pneumococcal identification and serotyping methods. For example, Kaltoft et al. [48] demonstrated that a serum broth (beef infusion supplemented with horse serum and blood) improved the ability of traditional methods to detect multiple serotypes. Similarly, Carvalho et al. [49] found that an enrichment step in Todd Hewitt broth supplemented with yeast extract and rabbit serum increased the proportion of specimens with pneumococcus identified, as well as increasing the detection of multiple serotypes by culture and molecular methods.

However, there are some remaining concerns with broth culture-amplification. The pneumococci may be overgrown by other species, and not all pneumococcal strains or serotypes grow at the same rate in vitro [50–52]. Moreover, broth culture enrichment may reduce detection of co-colonization of other species [53], or may not be appropriate for all sample types. In addition, some media components (such as animal serum) may be difficult to access in developing countries.

8.1. Recommendation

There is insufficient evidence to make a recommendation regarding inclusion of a broth culture-based enrichment step for the detection of pneumococci. Quantification of pneumococcal load should not be determined using samples that have undergone broth enrichment.

8.2. Future research

Whole-genome amplification methods may overcome limitations of low amounts of DNA. It would be useful to optimize broth culture-amplification (e.g. by including a selective agent), and to test the effects of broth-culture amplification on culture and molecular-based identification and serotyping methods.

9. Picking pneumococcal colonies for identification and serotyping

These recommendations establish the minimum set of criteria to determine the presence of pneumococci, and the dominant pneumococcal serotype, in order to ascertain the prevalence of pneumococcal carriage and the serotypes present in the overall population under study. Given this objective, there are two main issues to consider: how many colonies to pick, and how to select them. Detecting multiple serotype carriage is important for some epidemiologic questions, but serotyping a few colonies is an insensitive method to detect the true prevalence of multiple serotype carriage [54–56]. For colony selection, the truly random approach (e.g. where the STGG medium is diluted and spread on agar plates to obtain single colonies, then all the colonies are numbered and selected using a list of random numbers) may be optimal statistically, but is considered impractical for routine use. Choosing colonies based on morphology is more efficient [54], but leads to a bias towards detecting those that are morphologically distinct such as serotype 3 or nontypeable (NT) pneumococci [57].

9.1. Recommendation

Select one colony from the selective plate. If more than one morphology is present, this colony should be from the predominant morphology. We also suggest that one colony from each morphology be selected; however they should be recorded and reported separately (e.g. subdominant 1, subdominant 2 in order of prevalence). This allows for collection of information regarding possible multiple serotype carriage, albeit in a biased fashion. If there is only one morphology present, and it is later identified as non-pneumococcus, return to the primary culture plate and repeat colony selection at least once to verify that pneumococci are not present.

10. Culture-based identification of pneumococci

Traditionally, identification of pneumococci has focused on isolates cultured from normally sterile sites that tend to display a classical phenotype, in particular being optochin susceptible and bile soluble. These identification criteria are generally satisfactory for clinical application and are widely applied in diagnostic microbiology. However, alternative pneumococcal forms are frequently cultured from NP specimens [58,59]. These non-classical forms may give test results normally expected for other members of the viridans group of streptococci [60,61] and some other viridans group streptococci have been reported to give test results normally associated with pneumococci [62–64]. For example, the original description of *Streptococcus pseudopneumoniae* was optochin susceptible when grown in ambient air conditions, and resistant when incubated in 5% CO_2 atmosphere [62]. However, recent studies have found that these phenotypic characteristics are not universal for *S. pseudopneumoniae* [65]. These issues create difficulties for identification and differentiation between pneumococci and other oral streptococci in carriage studies.

10.1. Recommendation

Although optochin susceptibility and bile solubility are still considered key tests, we recommend extending the criteria for presumptive identification of pneumococci to encompass non-classical forms of pneumococci (Fig. 2). Further testing by a reference laboratory may be needed if the research question requires a more definitive identification than this algorithm provides. We now recommend that all α-hemolytic colonies growing on selective media are potentially analyzable, rather than just those with 'typical pneumococcal colony morphology' [66], and reiterate that the optochin test culture plate is incubated in 5% CO_2 atmosphere, rather than ambient air.

10.2. Future research

Further work is needed to more clearly differentiate pneumococci, particularly the non-classical forms, from other oral microbes. As a clearer understanding of how to fully define the species is achieved, a revised pragmatic definition of pneumococci will be needed for use in carriage studies.
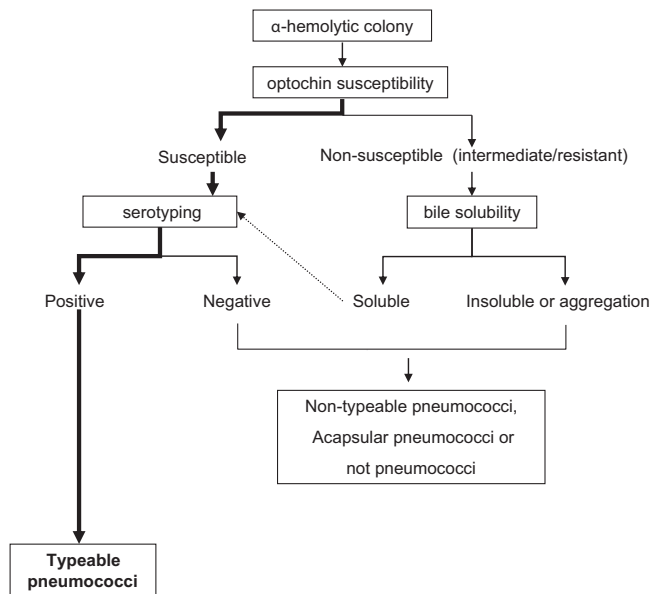
Fig. 2. Identification algorithm for carriage isolates of *Streptococcus pneumoniae* (the pneumococcus).

11. Non-culture based identification

Non-culture based techniques have some advantages in detecting pneumococci from NP samples: they do not require viable organisms, preserve the original composition of the NP sample and, depending on the methods used, provide a detailed characterization and quantification of the pneumococci within a sample.

The detection of pneumococci in a NP swab by a non-culture method is complicated by the intrinsic complexity of the sample, the low numbers of pneumococci in the sample and by difficulties in interpreting the epidemiological relevance when pneumococcal genetic material is detected in culture-negative samples. The sample is a representation of the NP microbiome, which contains numerous bacterial species [67] and may include close relatives of pneumococci such as *S. pseudopneumoniae*, *Streptococcus mitis* and other streptococcal species that also inhabit this niche [68].

The ideal method for non-culture identification in NP swabs should unequivocally detect the pneumococcus with high sensitivity and specificity; it should also be rapid, easy to perform, inexpensive, and deployable on a large scale. In the last decade, several non-culture methods aiming to detect pneumococci in biological samples have been developed including PCR-based strategies targeting specific DNA markers such as *rpoA* [69], *sodA* [70], *tuf* [71], *recA* [72], *piaA* [73], *Spn9802* [74], *ply* [75], a 181-bp pneumococcal-specific fragment [76], 16S-rDNA [77], *psaA* [78], and *lytA* [79–81]. For many of these methods specificity problems have been detected [64,65,82,83]. For others, there has been insufficient validation against diverse collections of close relatives of pneumococci. In addition, there is an increasing body of more sophisticated methods that, although promising, may not be easily applied in routine analysis of NP samples [84–87]. While there is currently no gold standard method for non-culture identification of pneumococci from NP swabs [63,88,89], the *lytA* real-time PCR assay described by Carvalho et al. [81] is widely used and appears to be species-specific. However, given the capacity of pneumococci to exchange genes with other oral streptococci [88,90] a multilocus approach such as used in multilocus sequence typing (MLST), microarray or whole genome-sequencing may prove valuable [64,91,92].

11.1. Recommendation

Culture should remain the gold standard for detection of pneumococci in NP swab samples. Investigators may wish to complement culture detection with a non-culture technique; the method we currently recommend is *lytA* real-time PCR [81].

11.2. Future research

A systematic laboratory validation of non-culture methods against large collections of nasopharyngeal and non-classical isolates is needed to guide future recommendations. Studies that are designed to determine the clinical relevance of pneumococcal culture-negative but DNA-positive samples are needed.

12. Serotyping

12.1. Quellung

The current standard method for serotyping of pneumococcal isolates is the capsular reaction/swelling test (Quellung reaction or Neufeld test) [1]. The traditional method described by Lund [93], Austrian [94] and the Statens Serum Institut [95] using ×100 magnification with oil immersion, is still widely used in Europe and North America. In Australia and Papua New Guinea, the 'dry' method using ×40 magnification without oil [96] has been in use since at least the 1970s (M. Gratten, personal communication). The 'dry' method is quicker and simpler than the 'wet' method using oil immersion [97] but there is no evidence to suggest that one method is superior to the other. Phase contrast microscopy improves the visibility of the capsule, however it is not essential in conducting the Quellung reaction.

Since publication of our previous recommendation, 11 European reference laboratories participated in the validation of pneumococcal serotyping [98]. A high degree of agreement was found between the Quellung test and other serotyping methods, including latex agglutination and gel diffusion. Specifically, there was no significant difference in the percentage of mistypings (39 out of 735 serotypings) by the Quellung method (5.2%, six laboratories) compared to the non-Quellung methods (5.7%, five laboratories) [98]. An inter-laboratory quality control program conducted in four laboratories over ten years found a serotyping concordance of 95.8% using Quellung [99]. Although costly and time-consuming, the Quellung reaction may be preferred in laboratories with suitably experienced staff and a comprehensive set of antisera.

12.2. Latex agglutination

Compared with Quellung, latex agglutination is less expensive, easier to learn, and does not require a microscope. It may therefore be more suitable for settings with limited budgets and training capacity. Commercial reagents are available; alternatively latex reagents can be produced and validated in-house. In the latter case antibodies from commercial antisera are passively bound onto latex particles under aseptic conditions [100,101]. Latex reagents produced in-house must undergo careful quality control. Reagents are stored at 4°C. As the long-term viability of these reagents is unknown, they should be quality control tested at least annually. Reactions should be conducted using reagents at room-temperature, on a glass surface, using a consistent inoculum of fresh, low passage pneumococci.

12.3. New serotyping methods

Recently, a variety of new serotyping methods have been developed including phenotypic methods that rely on antigen detection,

Table 3
Key advantages and disadvantages of selected serotyping methods.

Method	Key advantages	Key disadvantages	Example references
Phenotypic detection			
Quellung	Gold standard High sensitivity and specificity	Requires experience to interpret Typing sera are expensive	[1,2]
Latex agglutination	High sensitivity and specificity Relatively simple to interpret	Commercial latex reagents are expensive In-house latex reagents require extensive QC Lab procedures to prevent mis-interpretation of results needed	[3–5]
Dot blot	Cost effective – uses highly diluted typing sera	Lack of specificity through cross-reactions Interpretation is subjective Requires significant optimization for each serotype	[6]
Microbead assays e.g. Flow cytometry or Luminex	High throughput Sensitivity and specificity similar to Gold standard methods Can be designed to detect capsular polysaccharide, or PCR products	Expensive capital equipment May need expensive polyclonal typing serum and serotype specific monoclonal antibodies Technically demanding, particularly in assay optimisation and set-up	[7–12]
Genotypic detection			
Multiplex PCR	Highly sensitive (although less than individual PCRs) Detection of non-viable organisms Can be coupled with different detection methods (e.g. hybridisation, bead-based or mass-spectrometry) Technically straightforward Widely used	Not quantitative Risk of amplicon contamination Closely related serotypes cannot be discriminated and are detected as a group	[11,13–19]
Real-time PCR	Extremely sensitive Detection of non-viable organisms Semi-quantitative	Closely related serotypes cannot be discriminated and are detected as a group	[20,21], Paranhos-Baccalà et al., unpublished data
Microarray	Large number of serotypes detected Array can include targets for all serotypes, including virulence factors and antimicrobial resistance markers May be able to measure relative abundance Detection of non-viable organisms	Expensive reagents and equipment required Operator needs a reasonably high level of technical expertise, particularly for interpretation of unusual findings Can be difficult to distinguish closely related serotypes, although has capacity to include multiple targets for each serotype.	[3,22,23]
Single PCR with Sequencing	Only one primer set used Detection of non-viable organisms	May be difficult to fully discriminate between all serotypes	[24,25]

References: [1] Lund E. Laboratory diagnosis of Pneumococcus infections. Bull World Health Organ. 1960;23:5-13. [2] Hare KM, Smith-Vaughan H, Binks M, Park IH, Nahm MH, Leach AJ. "Dodgy 6As": differentiating pneumococcal serotype 6C from 6A by use of the Quellung reaction. J Clin Microbiol. 2009;47:1981-2. [3] Turner P, Hinds J, Turner C, Jankhot A, Gould K, Bentley SD, et al. Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol. 2011;49:1784-9. [4] Slotved HC, Kaltoft M, Skovsted IC, Kerrn MB, Espersen F. Simple, rapid latex agglutination test for serotyping of pneumococci (Pneumotest-Latex). J Clin Microbiol. 2004;42:2518-22. [5] Ortika BD, Habib M, Dunne EM, Porter BD, Satzke C. Production of latex agglutination reagents for pneumococcal serotyping. BMC Res Notes. 2013;6:49. [6] Fenoll A, Jado I, Vicioso D, Casal J. Dot blot assay for the serotyping of pneumococci. J Clin Microbiol. 1997;35:764-6. [7] Sheppard CL, Harrison TG, Smith MD, George RC. Development of a sensitive, multiplexed immunoassay using xMAP beads for detection of serotype-specific *Streptococcus pneumoniae* antigen in urine samples. J Med Microbiol. 2011;60:49-55. [8] Ceyhan M, Yildirim I, Sheppard CL, George RC. Pneumococcal serotypes causing pediatric meningitis in Turkey: application of a new technology in the investigation of cases negative by conventional culture. Eur J Clin Microbiol Infect Dis. 2010;29:289-93. [9] Findlow H, Laher G, Balmer P, Broughton C, Carrol ED, Borrow R. Competitive inhibition flow analysis assay for the non-culture-based detection and serotyping of pneumococcal capsular polysaccharide. Clin Vaccine Immunol. 2009;16:222-9. [10] Lal G, Balmer P, Stanford E, Martin S, Warrington R, Borrow R. Development and validation of a nonaplex assay for the simultaneous quantitation of antibodies to nine *Streptococcus pneumoniae* serotypes. J Immunol Methods. 2005;296:135-47. [11] Park MK, Briles DE, Nahm MH. A latex bead-based flow cytometric immunoassay capable of simultaneous typing of multiple pneumococcal serotypes (Multibead assay). Clin Diagn Lab Immunol. 2000;7:486-9. [12] Yu J, Carvalho M da G, Beall B, Nahm MH. A rapid pneumococcal serotyping system based on monoclonal antibodies and PCR. J Med Microbiol. 2008;57:171-8. [13] Zhou F, Kong F, Tong Z, Gilbert GL. Identification of less-common *Streptococcus pneumoniae* serotypes by a multiplex PCR-based reverse line blot hybridization assay. J Clin Microbiol. 2007;45:3411-5. [14] Kong F, Brown M, Sabananthan A, Zeng X, Gilbert GL. Multiplex PCR-based reverse line blot hybridization assay to identify 23 *Streptococcus pneumoniae* polysaccharide vaccine serotypes. J Clin Microbiol. 2006;44:1887-91. [15] Yu J, Lin J, Kim KH, Benjamin WH, Jr., Nahm MH. Development of an automated and multiplexed serotyping assay for *Streptococcus pneumoniae*. Clin Vaccine Immunol. 2011;18:1900-7. [16] Pai R, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. J Clin Microbiol. 2006;44:124-31. [17] Saha SK, Darmstadt GL, Baqui AH, Hossain B, Islam M, Foster D, et al. Identification of serotype in culture negative pneumococcal meningitis using sequential multiplex PCR: implication for surveillance and vaccine design. PLoS ONE. 2008;3:e3576. [18] Rivera-Olivero IA, Blommaart M, Bogaert D, Hermans PW, de Waard JH. Multiplex PCR reveals a high rate of nasopharyngeal pneumococcal 7-valent conjugate vaccine serotypes co-colonizing indigenous Warao children in Venezuela. J Med Microbiol. 2009;58:584-7. [19] Massire C, Gertz RE, Jr., Svoboda P, Levert K, Reed MS, Pohl J, et al. Concurrent serotyping and genotyping of pneumococci by use of PCR and electrospray ionization mass spectrometry. J Clin Microbiol. 2012;50:2018-25. [20] Azzari C, Moriondo M, Indolfi G, Cortimiglia M, Canessa C, Becciolini L, et al. Realtime PCR is more sensitive than multiplex PCR for diagnosis and serotyping in children with culture negative pneumococcal invasive disease. PLoS ONE. 2010;5:e9282. [21] Pimenta FC, Roundtree A, Soysal A, Bakir M, du Plessis M, Wolter N, et al. Sequential triplex real-time PCR assay for detecting 21 pneumococcal capsular serotypes that account for a high global disease burden. J Clin Microbiol. 2012. [22] Tomita Y, Okamoto A, Yamada K, Yagi T, Hasegawa Y, Ohta M. A new microarray system to detect *Streptococcus pneumoniae* serotypes. J Biomed Biotechnol. 2011:352736. [23] Wang Q, Wang M, Kong F, Gilbert GL, Cao B, Wang L, et al. Development of a DNA microarray to identify the *Streptococcus pneumoniae* serotypes contained in the 23-valent pneumococcal polysaccharide vaccine and closely related serotypes. J Microbiol Methods. 2007;68:128-36. [24] Leung MH, Bryson K, Freystatter K, Pichon B, Edwards G, Charalambous BM, et al. Sequetyping: serotyping *Streptococcus pneumoniae* by a single PCR sequencing strategy. J Clin Microbiol. 2012;50:2419-27. [25] Elberse KE, van de Pol I, Witteveen S, van der Heide HG, Schot CS, van Dijk A, et al. Population structure of invasive *Streptococcus pneumoniae* in The Netherlands in the pre-vaccination era assessed by MLVA and capsular sequence typing. PLoS ONE. 2011;6:e20390.

and those that are genotype based. Several of these new methods are summarized in Table 3. Examples of genotypic methods include microarray [102–105], single or multiplex real-time PCR ([106,107], Paranhos-Baccalà et al., unpublished data), single-plex PCR combined with sequencing [108,109] and multiplex PCR [110–112]. Multiplex PCR products are usually detected by gel electrophoresis, but may also be detected by mass-spectrometry [113], DNA hybridization [114,115] or automated fluorescent capillary electrophoresis [116] for example. Phenotypic methods include the dot blot assay [117,118], latex agglutination (see Section above) and bead-based assays on a flow-cytometry or Luminex-based platform [119–124].

In general, methods that involve antibody-antigen reactions are prone to cross reactivity although this is reduced where a significant amount of bound antibody is required for serotype identification, such as capsule swelling reactions (Quellung), or in methods that involve quantitative detection of bound antibody (flow-cytometry or Luminex) [123,125]. Improvements to these methods can be made through the absorption of non-specific reactive antibodies [117] and the use of monoclonal antibodies [124]. In the case of genotype detection, the primary limitations are the sequence diversity of the capsular loci, which can lead to target mismatches, and the inability to discriminate between closely related serotypes. The continued production of new sequence data should result in better target selection and primer/probe design that can produce results with similar sensitivity and specificity to the gold standard methods.

For pure pneumococcal cultures, many methods are valid, and the most appropriate one will depend on the study setting. As such, we do not recommend a particular method over another, except to note that the particular method's performance should be rigorously validated against the Quellung test.

Serotyping pneumococci directly from the NP sample is more challenging. As mentioned in Section 11, pneumococci may be present in low numbers (leading to low sensitivity), and/or as a small proportion of the NP cells (i.e. compared with cells from other organisms or the host), leading to low specificity. Divergent homologues of pneumococcal capsular genes also have been found in non-pneumococcal species [126]. Furthermore, the clinical relevance of identifying serotype-specific DNA in a culture-negative sample is not known.

12.4. Recommendation

Serotyping of pure pneumococcal isolates using Quellung by the wet or dry method is considered the core method. Latex agglutination serotyping may also be used. Many new serotyping methods are being developed, and although some may be valid there is currently insufficient evidence to provide recommendations. Serotyping directly from the NP specimen is insufficiently developed to recommend as a core method.

12.5. Future research

Assessment of the assay and clinical performance of new serotyping methods, particularly when testing directly from the NP sample is needed.

13. Multiple serotype carriage

Carriage of multiple pneumococcal serotypes is relatively common, particularly in areas where the carriage rate and disease burden are high [54,112,127,128]. Multiple carriage usually involves carriage of a major serotype, together with one or more minor serotype populations. Although it is clear that standard serotyping methods underestimate multiple carriage [49,55], the

clinical and public health relevance of multiple carriage is less well established. Theoretically, detection of minor serotypes may help to predict the shift in serotype distribution through serotype replacement following pneumococcal vaccination, particularly in high burden settings [129], and allow a better understanding of how epidemic serotypes emerge in some populations. Recent data using new serotyping methods suggest that the impact of vaccination on multiple serotype carriage may be complex [87,130].

To some extent, our understanding is limited by the methods used to detect and characterize multiple carriage. Ideally, a new method should detect multiple serotypes directly from the specimen (i.e., without a culture step which may alter the relative proportions of various strains) without false positive reactions, and be quantitative, affordable, practical and capable of detecting all known serotypes. Although many potential methods have recently been developed they have not been sufficiently validated. The PneuCarriage project has compared 20 serotyping methods from 15 research groups, including their ability to detect multiple serotype carriage, using a well-characterized reference bank of samples (Satzke et al., manuscript in preparation). This project will provide further information on suitable methods for detecting multiple serotype carriage with high sensitivity and specificity.

13.1. Recommendation

Current methods routinely underestimate the prevalence of multiple serotype carriage. Although many new techniques are in development, there is insufficient evidence to make a recommendation. For studies where multiple carriage is relevant, we recommend retaining the original STGG specimens for future assessment when optimal methods are defined.

13.2. Future research

A thorough comparison of methods to detect NP carriage of multiple pneumococcal serotypes from pneumococcal cultures and directly from specimens is needed. The clinical and public health importance of multiple serotype carriage needs to be determined.

14. Storage and recovery of isolates

Several storage methods, such as lyophilization, or ULT storage on commercially available chemically-treated beads, are appropriate for long-term storage of pure pneumococcal isolates. However, our recommendations for storage of pneumococcal isolates in STGG media are consistent with the 2003 methods [1], but with some minor amendments to reflect the breadth of consensus practice.

14.1. Recommendation

The storage of at least one tube of each pneumococcal isolate is recommended. To do this inoculate (using a swab or loop) a fresh, overnight, pure lawn culture into suitable media, such as STGG, under aseptic conditions. After ensuring the growth is homogenized, for example by a short vortex step, freeze at ULT. Short-term storage (<12 months) of these high-titer stocks at −20 °C in a non-defrosting freezer is acceptable, although survival will decrease over this time [33,37].

To recover the isolate, a small amount of frozen material can be scraped from the surface of the STGG medium, or the entire volume thawed and an aliquot taken. The scraping or aliquot is then usually inoculated onto solid medium to check for purity of the isolate. Recovery of isolates should be undertaken aseptically, with a view to minimizing temperature fluctuations of the stored isolate by, for example, keeping tubes on dry-ice (or if necessary, and for short

periods, wet ice) when handling them, and only processing a few tubes at a time.

14.2. Future research

Investigation of the effect of vortexing and frozen storage on recovery, identification (e.g. optochin susceptibility) and serotyping (e.g. production of capsule) is needed. The performance of simpler storage media could be validated.

15. Shipping of isolates

There are many methods available for shipping of pneumococcal isolates. These include using STGG, silica gel desiccant sachets (stable for a fortnight at room-temperature or a month at 4 °C [66,131]), Dorset media, Amies transport media, chocolate or similar agar slopes, or lyophilization. There is no evidence base for preferring one method over another.

15.1. Recommendation

Any of the methods outlined above, or others that are shown to be equally as effective are acceptable.

15.2. Future research

Comparison of effectiveness of different transport methods could be undertaken, although it is likely that many would prove satisfactory.

16. Epidemiological role of carriage studies

In previous sections we have provided a core methodology to perform pneumococcal NP carriage studies. We now consider the role of these carriage studies, especially in the context of pneumococcal disease control.

Significant attention is being directed to whether and how NP studies of pneumococcal ecology in communities can be used to infer or predict disease impact. As the understanding of the quantitative relationship between colonization and disease matures, the role of NP colonization outcomes as a tool for evaluating the global rollout of PCV and other pneumococcal vaccines could become more central. The gold standard for such assessments has to date been population-based surveillance of invasive pneumococcal disease (IPD) as exemplified by the Active Bacterial Core Surveillance of the Centers for Disease control in the USA [132]. This requires a significant clinical and diagnostic microbiology infrastructure, not present in many developing countries. Further, the collection of IPD isolates requires a clinical environment in which the great majority of suspected cases of meningitis receive a lumbar puncture, and a sufficient number of blood cultures are taken to recognize an impact of PCV, given that blood culture will detect only 2–3% of pediatric pneumonias prevented by PCV [133]. An alternate to IPD surveillance is syndromic surveillance for changes in pneumonia hospitalization or death following PCV introduction. These types of studies have relied on large networks of electronic surveillance [134] not available in developing countries, and can measure only the aggregate effect of a reduction in vaccine type disease and replacement. While such an approach based on just one or a few hospitals may be possible, this depends on the care-seeking behavior of those most at risk for serious morbidity and mortality [135]; in many settings those are the very children with least access to the health facility study sites. There are also considerable variations in the numbers of cases over time depending on comorbidities such as the severity of the influenza season [134] and the impact

of antiretroviral rollout if HIV is a significant risk for pneumonia hospitalization in the community [136]. Thus these studies are not likely to be a primary strategy to detect the impact of PCVs and when undertaken are at risk of being confounded by changes in pneumonia burden or mortality trends unrelated to pneumococcal disease (e.g. respiratory viral epidemics, malaria).

The assessment of carriage of vaccine type and non-vaccine type pneumococci is a direct, pathogen-specific measure of PCV impact that is an indicator of the success or failure of a PCV rollout program [129]. Cross sectional studies of carriage in the target age group of PCV, as well as in older children and adults, will give a measure of herd protection. Detection of important serotypes in developing countries (such as type 1) may still be done in carriage studies if the subjects are carefully chosen, by including the detection of carriage in subjects with pneumonia on arrival at health care facilities. Detection of such rarely carried types in pneumonia patients may reflect an etiological role of those types in pneumonia [137]. Carriage studies focused on young children with respiratory illness will identify the group at risk for pneumococcal disease but also provide access to older siblings who are often transmitters of the pathogen, and mothers who may be key to measurement of herd protection in adults. Cross sectional studies may detect changes in the distribution of vaccine type carriage as soon as a year post PCV introduction if sample size is sufficient, with detection of profound changes in distribution and herd protection, if present, by 3–4 years post PCV [138]. While carriage studies will not likely be a direct measure of reduction in disease burden due to PCV, they offer a direct measure of program effectiveness and the nature of replacing pathogens, including an assessment of the impact of PCV on the NP microbiome.

There are emerging data suggesting that quantitative detection of carriage using microbiological methods, but also more easily by quantitative PCR, may be diagnostic of pneumonia in adults [139]. These methods may also reflect co-infection with respiratory viruses in children [140] which may be a significant risk for pneumonia hospitalization [141]. The antimicrobial susceptibility profile of carried pneumococci may be used to inform treatment algorithms for pneumococcal disease in developing countries [142]. Quantitative molecular methods may increase the sensitivity of detection of pneumococcal carriage, and may also detect more easily than culture an impact of PCV on density of carriage. The detection of serotypes in carriage can be used together with the global distribution of those types in IPD [143] to develop an invasiveness index that may be predictive of the likelihood of invasive disease replacement due to emerging types detected in carriage. There are advances in work linking the NP and IPD post-PCV impact results, thereby providing a means to predict IPD impact using NP carriage [147].

Carriage studies are also important for the assessment of the serotype-specific basic reproductive number (R_0) of the pneumococcus in developing countries; whole genome sequencing of carriage strains pre- and post-PCV introduction in developing countries may give insight into the evolution of this pathogen in response to PCV. The human is the natural reservoir of the pneumococcus and more studies are needed on a human challenge model [144].

The pathway for licensure of novel pneumococcal vaccines such as those using pneumococcal proteins as conjugates, proteins given with existing formulations of PCV, protein alone or killed whole cell vaccine will depend in large part on proof-of-principle for impact on pneumonia or ability to induce herd protection by the demonstration of an impact on carriage. We speculate that carriage studies will likely be central to the further development and licensure of these novel vaccines [145].

There are few data on the sensitivity of culture to detect pneumococcal carriage. Demonstration of carriage may increasingly be

performed using molecular techniques such as quantitative PCR, microarray, or mass spectrometry based methods. The expression profile of pneumococci in carriage may differ from pneumococci invading the host, as may the host proteomic response to carriage or disease.

It is likely that future carriage studies will increasingly use molecular methods to detect carriage including analysis of gene expression, density of carriage and impact on the microbiome. Carriage detection should be an essential part of assessing novel pneumococcal vaccines, and measuring the impact and safety of PCV or other pneumococcal vaccines on human populations.

17. Conclusions

These WHO core methods provide an update on the options available and recommended approaches for studies of pneumococcal carriage. The consistent application of these methods in studies will provide the best opportunity to ensure that any observed differences in colonization are not confounded by differences in the specimen collection, handling or laboratory methods. A recent assessment of adherence to the core methods in published NP studies indicates that some but not all of the recommendations are being fully adopted [146]. As evidenced in this update, for some aspects of the recommended method there are few appropriately designed comparative studies to make definitive statements on preference. In these situations, best practice is to some degree a matter of expert opinion, field experience and a reflection of imperfect data. For study sites that have ongoing NP colonization studies, investigators may decide that consistency in methods over time is more important than modifying their methods now to those recommended here. In such cases a bridging study comparing the results of NP colonization using existing and the core methods would help to clarify the degree to which study findings are modified by the chosen methods. Notwithstanding these limitations, the application of these core methods allows researchers around the world to have confidence in carriage study results, and allows them to contribute to our understanding of the pneumococcus and its control through vaccines.

Conflicts of interest

C.S. received the Robert Austrian award funded by Pfizer; P.A. works in a department which holds research grants from GlaxoSmithKline on evaluation of pneumococcal conjugate vaccines; M.A. works in a department which holds a research grant from PATH on evaluation of GlaxoSmithKline's combined pneumococcal proteins and conjugates vaccine trial; K.H. received partial funding from GlaxoSmithKline and Pfizer to attend ISPPD7 and ISPPD8 respectively; A.L. has research grant, conference travel and accommodation support from Pfizer and GlaxoSmithKline, and received the Medical Journal of Australia/Pfizer award; K.K. has research grant support from Pfizer and has served on pneumococcal external expert committees convened by Pfizer, Merck, Aventis-pasteur, and GlaxoSmithKline; R.S.L. has received research grant support and speaking fees from Pfizer; J.A.S. has received research grant support from GlaxoSmithKline and travel and accommodation support to attend a meeting convened by Merck; H.N. has served on pneumococcal vaccination external expert committees convened by GlaxoSmithKline, Pfizer, and Sanofi Pasteur, and works in a department which holds a major research grant from GlaxoSmithKline on phase IV evaluation of a pneumococcal conjugate vaccine; K.O.B. has research grant support from Pfizer and GlaxoSmithKline, and has served on pneumococcal external expert committees convened by Merck, Aventis-pasteur, and GlaxoSmithKline; P.T., A.V.J., A.M.H.R. and B.P. have no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.08.062.

References

[1] O'Brien KL, Nohynek H, World Health Organization Pneumococcal Vaccine Trials Carriage Working Group Report from a WHO Working Group: standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*. Pediatr Infect Dis J 2003;22:e1–11. Erratum in: Pediatr Infect Dis J. 2011;30(2):185.

[2] Scott JR, Millar EV, Lipsitch M, Moulton LH, Weatherholtz R, Perilla MJ, et al. Impact of more than a decade of pneumococcal conjugate vaccine use on carriage and invasive potential in Native American communities. J Infect Dis 2012;205:280–8.

[3] Flasche S, Van Hoek AJ, Sheasby E, Waight P, Andrews N, Sheppard C, et al. Effect of pneumococcal conjugate vaccination on serotype-specific carriage and invasive disease in England: a cross-sectional study. PLoS Med 2011;8:e1001017.

[4] Park SY, Moore MR, Bruden DL, Hyde TB, Reasonover AL, Harker-Jones M, et al. Impact of conjugate vaccine on transmission of antimicrobial-resistant *Streptococcus pneumoniae* among Alaskan children. Pediatr Infect Dis J 2008;27:335–40.

[5] Roca A, Hill PC, Townend J, Egere U, Antonio M, Bojang A, et al. Effects of community-wide vaccination with PCV-7 on pneumococcal nasopharyngeal carriage in the Gambia: a cluster-randomized trial. PLoS Med 2011;8:e1001107.

[6] Huang SS, Platt R, Rifas-Shiman SL, Pelton SI, Goldmann D, Finkelstein JA. Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. Pediatrics 2005;116:e408–13.

[7] Jin P, Kong F, Xiao M, Oftadeh S, Zhou F, Liu C, et al. First report of putative *Streptococcus pneumoniae* serotype 6D among nasopharyngeal isolates from Fijian children. J Infect Dis 2009;200:1375–80.

[8] Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH. Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. J Clin Microbiol 2007;45:1225–33.

[9] Cohen R, Levy C, de La Rocque F, Gelbert N, Wollner A, Fritzell B, et al. Impact of pneumococcal conjugate vaccine and of reduction of antibiotic use on nasopharyngeal carriage of nonsusceptible pneumococci in children with acute otitis media. Pediatr Infect Dis J 2006;25:1001–7.

[10] Moore MR, Hyde TB, Hennessy TW, Parks DJ, Reasonover AL, Harker-Jones M, et al. Impact of a conjugate vaccine on community-wide carriage of nonsusceptible *Streptococcus pneumoniae* in Alaska. J Infect Dis 2004;190:2031–8.

[11] Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, Collins M, et al. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet 2006;2:e31.

[12] Rapola S, Salo E, Kiiski P, Leinonen M, Takala AK. Comparison of four different sampling methods for detecting pharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae* in children. J Clin Microbiol 1997;35:1077–9.

[13] Carville KS, Bowman JM, Lehmann D, Riley TV. Comparison between nasal swabs and nasopharyngeal aspirates for, and effect of time in transit on, isolation of *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae*, and *Moraxella catarrhalis*. J Clin Microbiol 2007;45:244–5.

[14] van den Bergh MR, Bogaert D, Dun L, Vons J, Chu ML, Trzcinski K, et al. Alternative sampling methods for detecting bacterial pathogens in children with upper respiratory tract infections. J Clin Microbiol 2012;50:4134–7.

[15] Abdullahi O, Wanjiru E, Musyimi R, Glass N, Scott JA. Validation of nasopharyngeal sampling and culture techniques for detection of *Streptococcus pneumoniae* in children in Kenya. J Clin Microbiol 2007;45:3408–10.

[16] Lieberman D, Shleyfer E, Castel H, Terry A, Harman-Boehm I, Delgado J, et al. Nasopharyngeal versus oropharyngeal sampling for isolation of potential respiratory pathogens in adults. J Clin Microbiol 2006;44:525–8.

[17] Gritzfeld JF, Roberts P, Roche L, El Batrawy S, Gordon SB. Comparison between nasopharyngeal swab and nasal wash, using culture and PCR, in the detection of potential respiratory pathogens. BMC Res Notes 2011;4:122.

[18] Stubbs E, Hare K, Wilson C, Morris P, Leach AJ. *Streptococcus pneumoniae* and noncapsular *Haemophilus influenzae* nasal carriage and hand contamination in children: a comparison of two populations at risk of otitis media. Pediatr Infect Dis J 2005;24:423–8.

[19] Leach AJ, Stubbs E, Hare K, Beissbarth J, Morris PS. Comparison of nasal swabs with nose blowing for community-based pneumococcal surveillance of healthy children. J Clin Microbiol 2008;46:2081–2.

[20] Cloud JL, Hymas W, Carroll KC. Impact of nasopharyngeal swab types on detection of *Bordetella pertussis* by PCR and culture. J Clin Microbiol 2002;40:3838–40.

[21] Rubin LG, Rizvi A, Baer A. Effect of swab composition and use of swabs versus swab-containing skim milk-tryptone-glucose-glycerol (STGG) on culture- or PCR-based detection of *Streptococcus pneumoniae* in simulated and clinical respiratory specimens in STGG transport medium. J Clin Microbiol 2008;46:2635–40.

[22] Dube FS, Kaba M, Whittaker E, Zar HJ, Nicol MP. Detection of *Streptococcus pneumoniae* from Different Types of Nasopharyngeal Swabs in Children. PLoS ONE 2013;8:e68097.

[23] De Silva S, Wood G, Quek T, Parrott C, Bennett CM. Comparison of flocked and rayon swabs for detection of nasal carriage of *Staphylococcus aureus* among pathology staff members. J Clin Microbiol 2010;48:2963–4.

[24] Jang D, Gilchrist J, Portillo E, Smieja M, Toor R, Chernesky M. Comparison of dacron and nylon-flocked self-collected vaginal swabs and urine for the detection of *Trichomonas vaginalis* using analyte-specific reagents in a transcription-mediated amplification assay. Sex Transm Infect 2012;88:160–2.

[25] Hernes SS, Quarsten H, Hagen E, Lyngroth AL, Pripp AH, Bjorvatn B, et al. Swabbing for respiratory viral infections in older patients: a comparison of rayon and nylon flocked swabs. Eur J Clin Microbiol Infect Dis 2011;30:159–65.

[26] Verhoeven P, Grattard F, Carricajo A, Pozzetto B, Berthelot P. Better detection of *Staphylococcus aureus* nasal carriage by use of nylon flocked swabs. J Clin Microbiol 2010;48:4242–4.

[27] DeByle C, Bulkow L, Miernyk K, Chikoyak L, Hummel KB, Hennessy T, et al. Comparison of nasopharyngeal flocked swabs and nasopharyngeal wash collection methods for respiratory virus detection in hospitalized children using real-time polymerase chain reaction. J Virol Methods 2012;185:89–93.

[28] Miernyk K, Debyle C, Harker-Jones M, Hummel KB, Hennessy T, Wenger J, et al. Serotyping of *Streptococcus pneumoniae* isolates from nasopharyngeal samples: use of an algorithm combining microbiologic, serologic, and sequential multiplex PCR techniques. J Clin Microbiol 2011;49:3209–14.

[29] Sharma D, Baughman W, Holst A, Thomas S, Jackson D, Carvalho M da G, et al. Pneumococcal Carriage and Invasive Disease in Children Before Introduction of the 13-valent Conjugate Vaccine: Comparison With the Era Before 7-valent Conjugate Vaccine. Pediatr Infect Dis J 2013;32:196.

[30] Gibson LF, Khoury JT. Storage and survival of bacteria by ultra-freeze. Lett Appl Microbiol 1986;3:127–9.

[31] Gherna RL. Preservation. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, editors. Manual of methods for general bacteriology. Washington, DC: American Society for Microbiology; 1981. p. 208–17.

[32] O'Brien KL, Bronsdon MA, Dagan R, Yagupsky P, Janco J, Elliott J, et al. Evaluation of a medium (STGG) for transport and optimal recovery of *Streptococcus pneumoniae* from nasopharyngeal secretions collected during field studies. J Clin Microbiol 2001;39:1021–4.

[33] Kaijalainen T, Ruokokoski E, Ukkonen P, Herva E. Survival of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* frozen in skim milk-tryptone-glucose-glycerol medium. J Clin Microbiol 2004;42:412–4.

[34] Hare KM, Smith-Vaughan HC, Leach AJ. Viability of respiratory pathogens cultured from nasopharyngeal swabs stored for up to 12 years at -70°C in skim milk tryptone glucose glycerol broth. J Microbiol Methods 2011;86:364–7.

[35] Kwambana BA, Mohammed NI, Jeffries D, Barer M, Adegbola RA, Antonio M. Differential effects of frozen storage on the molecular detection of bacterial taxa that inhabit the nasopharynx. BMC Clin Pathol 2011;11:2.

[36] Charalambous BM, Batt SL, Peek AC, Mwerinde H, Sam N, Gillespie SH. Quantitative validation of media for transportation and storage of *Streptococcus pneumoniae*. J Clin Microbiol 2003;41:5551–6.

[37] Mason CK, Goldsmith CE, Moore JE, McCarron P, Leggett P, Montgomery J, et al. Optimisation of storage conditions for maintaining culturability of penicillin-susceptible and penicillin-resistant isolates of *Streptococcus pneumoniae* in transport medium. Br J Biomed Sci 2010;67:1–4.

[38] Lloyd-Evans N, O'Dempsey TJ, Baldeh I, Secka O, Demba E, Todd JE, et al. Nasopharyngeal carriage of pneumococci in Gambian children and in their families. Pediatr Infect Dis J 1996;15:866–71.

[39] Syrjanen RK, Kilpi TM, Kaijalainen TH, Herva EE, Takala AK. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Finnish children younger than 2 years old. J Infect Dis 2001;184:451–9.

[40] Granat SM, Mia Z, Ollgren J, Herva E, Das M, Piirainen L, et al. Longitudinal study on pneumococcal carriage during the first year of life in Bangladesh. Pediatr Infect Dis J 2007;26:319–24.

[41] Robins-Browne RM, Kharsany AB, Ramsaroop UG. Detection of pneumococci in the upper respiratory tract: comparison of media and culture techniques. J Clin Microbiol 1982;16:1–3.

[42] Nichols T, Freeman R. A new selective medium for *Streptococcus pneumoniae*. J Clin Pathol 1980;33:770–3.

[43] Dilworth JA, Stewart P, Gwaltney Jr JM, Hendley JO, Sande MA. Methods to improve detection of pneumococci in respiratory secretions. J Clin Microbiol 1975;2:453–5.

[44] Hendley JO, Sande MA, Stewart PM, Gwaltney Jr JM. Spread of *Streptococcus pneumoniae* in families, I. Carriage rates and distribution of types. J Infect Dis 1975;132:55–61.

[45] Gratten M, Battistutta D, Torzillo P, Dixon J, Manning K. Comparison of goat and horse blood as culture medium supplements for isolation and identification of *Haemophilus influenzae* and *Streptococcus pneumoniae* from upper respiratory tract secretions. J Clin Microbiol 1994;32:2871–2.

[46] Petts DN. Colistin-oxolinic acid-blood agar: a new selective medium for streptococci. J Clin Microbiol 1984;19:4–7.

[47] Russell FM, Biribo SS, Selvaraj G, Oppedisano F, Warren S, Seduadua A, et al. As a bacterial culture medium, citrated sheep blood agar is a practical alternative to citrated human blood agar in laboratories of developing countries. J Clin Microbiol 2006;44:3346–51.

[48] Kaltoft MS, Skov Sorensen UB, Slotved HC, Konradsen HB. An easy method for detection of nasopharyngeal carriage of multiple *Streptococcus pneumoniae* serotypes. J Microbiol Methods 2008;75:540–4.

[49] Carvalho M da G, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, Millar EV, et al. Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol 2010;48:1611–8.

[50] Battig P, Hathaway LJ, Hofer S, Muhlemann K. Serotype-specific invasiveness and colonization prevalence in *Streptococcus pneumoniae* correlate with the lag phase during *in vitro* growth. Microbes Infect 2006;8:2612–7.

[51] Hathaway LJ, Brugger SD, Morand B, Bangert M, Rotzetter JU, Hauser C, et al. Capsule type of *Streptococcus pneumoniae* determines growth phenotype. PLoS Pathog 2012;8:e1002574.

[52] Slotved HC, Satzke C. *In vitro* growth of pneumococcal isolates representing 23 different serotypes. BMC Res Notes 2013;6:208.

[53] Gould KA, Pond MJ, Baldrey SJ, Turner P, Bentley SD, Hinds J. Evaluation and optimization of methods to facilitate direct analysis of nasopharyngeal swabs by microarray-based molecular serotyping. In: 7th International Symposium on Pneumococci and Pneumococcal Diseases. 2010.

[54] Hare KM, Morris P, Smith-Vaughan H, Leach AJ. Random colony selection versus colony morphology for detection of multiple pneumococcal serotypes in nasopharyngeal swabs. Pediatr Infect Dis J 2008;27:178–80.

[55] Turner P, Hinds J, Turner C, Jankhot A, Gould K, Bentley SD, et al. Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol 2011;49:1784–9.

[56] Huebner RE, Dagan R, Porath N, Wasas AD, Klugman KP. Lack of utility of serotyping multiple colonies for detection of simultaneous nasopharyngeal carriage of different pneumococcal serotypes. Pediatr Infect Dis J 2000;19:1017–20.

[57] Valente C, de Lencastre H, Sa-Leao R. Selection of distinctive colony morphologies for detection of multiple carriage of *Streptococcus pneumoniae*. Pediatr Infect Dis J 2013;32:703–4.

[58] Marsh R, Smith-Vaughan H, Hare KM, Binks M, Kong F, Warning J, et al. The nonserotypeable pneumococcus: phenotypic dynamics in the era of anticapsular vaccines. J Clin Microbiol 2010;48:831–5.

[59] Simoes AS, Valente C, de Lencastre H, Sa-Leao R. Rapid identification of non-capsulated *Streptococcus pneumoniae* in nasopharyngeal samples allowing detection of co-colonization and reevaluation of prevalence. Diagn Microbiol Infect Dis 2011;71:208–16.

[60] Mundy LS, Janoff EN, Schwebke KE, Shanholtzer CJ, Willard KE. Ambiguity in the identification of *Streptococcus pneumoniae*. Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. Am J Clin Pathol 1998;109:55–61.

[61] Whatmore AM, Efstratiou A, Pickerill AP, Broughton K, Woodard G, Sturgeon D, et al. Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: characterization of "Atypical" pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor-encoding genes. Infect Immun 2000;68:1374–82.

[62] Arbique JC, Poyart C, Trieu-Cuot P, Quesne G, Carvalho M da G, et al. Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. J Clin Microbiol 2004;42:4686–96.

[63] Ikryannikova LN, Lapin KN, Malakhova MV, Filimonova AV, Ilina EN, Dubovickaya VA, et al. Misidentification of alpha-hemolytic streptococci by routine tests in clinical practice. Infect Genet Evol 2011;11:1709–15.

[64] Simoes AS, Sa-Leao R, Eleveld MJ, Tavares DA, Carrico JA, Bootsma HJ, et al. Highly penicillin-resistant multidrug-resistant pneumococcus-like strains colonizing children in Oeiras, Portugal: genomic characteristics and implications for surveillance. J Clin Microbiol 2010;48:238–46.

[65] Rolo DA, Domenech SS, Fenoll A, Linares A, de Lencastre JH, et al. Disease isolates of *Streptococcus pseudopneumoniae* and non-typeable *S. pneumoniae* presumptively identified as atypical *S. pneumoniae* in Spain. PLoS ONE 2013;8:e57047.

[66] Castillo D, Harcourt B, Hatcher C, Jackson M, Katz L, Mair R. Storage and Shipping of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. In: Mayer L, editor. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. 2nd ed. World Health Organization; 2011. p. 265–82.

[67] Bogaert D, Keijser B, Huse S, Rossen J, Veenhoven R, van Gils E, et al. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. PLoS ONE 2011;6:e17035.

[68] Kilian M, Poulsen K, Blomqvist T, Havarstein LS, Bek-Thomsen M, Tettelin H, et al. Evolution of *Streptococcus pneumoniae* and its close commensal relatives. PLoS ONE 2008;3:e2683.

[69] Park HK, Yoon JW, Shin JW, Kim JY, Kim W. *rpoA* is a useful gene for identification and classification of *Streptococcus pneumoniae* from the closely related viridans group streptococci. FEMS Microbiol Lett 2010;305:58–64.

[70] Hoshino T, Fujiwara T, Kilian M. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. J Clin Microbiol 2005;43:6073–85.

[71] Picard FJ, Ke D, Boudreau DK, Boissinot M, Huletsky A, Richard D, et al. Use of *tuf* sequences for genus-specific PCR detection and phylogenetic analysis of 28 streptococcal species. J Clin Microbiol 2004;42:3686–95.

[72] Zbinden A, Kohler N, Bloemberg GV. *recA*-based PCR assay for accurate differentiation of *Streptococcus pneumoniae* from other viridans streptococci. J Clin Microbiol 2011;49:523–7.

[73] Whalan RH, Funnell SG, Bowler LD, Hudson MJ, Robinson A, Dowson CG. Distribution and genetic diversity of the ABC transporter lipoproteins PiuA and PiaA within *Streptococcus pneumoniae* and related streptococci. J Bacteriol 2006;188:1031–8.

[74] Suzuki N, Yuyama M, Maeda S, Ogawa H, Mashiko K, Kiyoura Y. Genotypic identification of presumptive *Streptococcus pneumoniae* by PCR using four genes highly specific for *S. pneumoniae*. J Med Microbiol 2006;55:709–14.

[75] Toikka P, Nikkari S, Ruuskanen O, Leinonen M, Mertsola J. Pneumolysin PCR-based diagnosis of invasive pneumococcal infection in children. J Clin Microbiol 1999;37:633–7.

[76] Prere MF, Fayet OA. A specific polymerase chain reaction test for the identification of *Streptococcus pneumoniae*. Diagn Microbiol Infect Dis 2011;70:45–53.

[77] El Aila NA, Emler S, Kaijalainen T, De Baere T, Saerens B, Alkan E, et al. The development of a 16S rRNA gene based PCR for the identification of *Streptococcus pneumoniae* and comparison with four other species specific PCR assays. BMC Infect Dis 2010;10:104.

[78] Morrison KE, Lake D, Crook J, Carlone GM, Ades E, Facklam R, et al. Confirmation of *psaA* in all 90 serotypes of *Streptococcus pneumoniae* by PCR and potential of this assay for identification and diagnosis. J Clin Microbiol 2000;38:434–7.

[79] Messmer TO, Sampson JS, Stinson A, Wong B, Carlone GM, Facklam RR. Comparison of four polymerase chain reaction assays for specificity in the identification of *Streptococcus pneumoniae*. Diagn Microbiol Infect Dis 2004;49:249–54.

[80] Llull D, Lopez R, Garcia E. Characteristic signatures of the *lytA* gene provide a basis for rapid and reliable diagnosis of *Streptococcus pneumoniae* infections. J Clin Microbiol 2006;44:1250–6.

[81] Carvalho M da G, Tondella ML, McCaustland K, Weidlich L, McGee L, et al. Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. J Clin Microbiol 2007;45:2460–6.

[82] Jado I, Fenoll A, Casal J, Perez A. Identification of the *psaA* gene, coding for pneumococcal surface adhesin A, in viridans group streptococci other than *Streptococcus pneumoniae*. Clin Diagn Lab Immunol 2001;8:895–8.

[83] Zhang Q, Ma Q, Su D, Li Q, Yao W, Wang C. Identification of horizontal gene transfer and recombination of *psaA* gene in streptococcus mitis group. Microbiol Immunol 2010;54:313–9.

[84] Brugger SD, Hathaway LJ, Muhlemann K. Detection of *Streptococcus pneumoniae* strain cocolonization in the nasopharynx. J Clin Microbiol 2009;47:1750–6.

[85] Bishop CJ, Aanensen DM, Jordan GE, Kilian M, Hanage WP, Spratt BG. Assigning strains to bacterial species via the internet. BMC Biol 2009;7:3.

[86] Scholz CF, Poulsen K, Kilian M. A novel molecular identification method for *Streptococcus pneumoniae* applicable to clinical microbiology and 16S rRNA-sequence based microbiome studies. J Clin Microbiol 2012;50:1968–73.

[87] Valente C, Hinds J, Pinto F, Brugger SD, Gould K, Muhlemann K, et al. Decrease in pneumococcal co-colonization following vaccination with the seven-valent pneumococcal conjugate vaccine. PLoS ONE 2012:e30235.

[88] Johnston C, Hinds J, Smith A, van der Linden M, Van Eldere J, Mitchell TJ. Detection of large numbers of pneumococcal virulence genes in streptococci of the mitis group. J Clin Microbiol 2010;48:2762–9.

[89] Wessels E, Schelfaut JJG, Bernards AT, Claas ECJ. Evaluation of several biochemical and molecular techniques for identification of *Streptococcus pneumoniae* and *Streptococcus pseudopneumoniae* and their detection in respiratory samples. J Clin Microbiol 2012;50:1171–7.

[90] Dowson CG, Barcus V, King S, Pickerill P, Whatmore A, Yeo M. Horizontal gene transfer and the evolution of resistance and virulence determinants in Streptococcus. Soc Appl Bacteriol Symp Ser 1997;26:42S–51S.

[91] Hanage WP, Fraser C, Spratt BG. Fuzzy species among recombinogenic bacteria. BMC Biol 2005;3:6.

[92] Sullivan CB, Diggle MA, Clarke SC. Multilocus sequence typing. Mol Biotechnol 2005;29:245–53.

[93] Lund E. Laboratory diagnosis of Pneumococcus infections. Bull World Health Organ 1960;23:5–13.

[94] Austrian R. The quellung reaction, a neglected microbiologic technique. Mt Sinai J Med 1976;43:699–709.

[95] Diagnostica: SSI. Pneumococcal antisera for in vitro diagnostic use. 3rd ed. Copenhagen, Denmark: Statens Serum Institut; 2011.

[96] Habib M, Porter B, Satzke C. Capsular serotyping of *Streptococcus pneumoniae* using the Quellung reaction. J Vis Exp 2013 (in press).

[97] Hare KM, Smith-Vaughan H, Binks M, Park IH, Nahm MH, Leach AJ. "Dodgy 6As": differentiating pneumococcal serotype 6C from 6A by use of the Quellung reaction. J Clin Microbiol 2009;47:1981–2.

[98] Konradsen HB. Validation of serotyping of *Streptococcus pneumoniae* in Europe. Vaccine 2005;23:1368–73.

[99] Reasonover A, Zulz T, Bruce MG, Bruden D, Jette L, Kaltoft M, et al. The International Circumpolar Surveillance interlaboratory quality control program for *Streptococcus pneumoniae*, 1999 to 2008. J Clin Microbiol 2011;49:138–43.

[100] Adegbola RA, Hill PC, Secka O, Ikumapayi UN, Lahai G, Greenwood BM, et al. Serotype and antimicrobial susceptibility patterns of isolates of *Streptococcus pneumoniae* causing invasive disease in The Gambia 1996-2003. Trop Med Int Health 2006;11:1128–35.

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

[102] Wang Q, Wang M, Kong F, Gilbert GL, Cao B, Wang L, et al. Development of a DNA microarray to identify the *Streptococcus pneumoniae* serotypes contained in the 23-valent pneumococcal polysaccharide vaccine and closely related serotypes. J Microbiol Methods 2007;68:128–36.

[103] Tomita Y, Okamoto A, Yamada K, Yagi T, Hasegawa Y, Ohta M. A new microarray system to detect *Streptococcus pneumoniae* serotypes. J Biomed Biotechnol 2011:352736.

[104] Hinds J, Gould K, Witney A, Lambertsen L, Antonio M, Aanensen D, et al. Molecular serotyping of *Streptococcus pneumoniae*: a microarray-based genomic tool for isolate typing, detection of multiple carriage and surveillance of serotype replacement in vaccine trials. In: 6th International Symposium on Pneumococci & Pneumococcal Diseases. Reykjavik, Iceland. 2008.

[105] Hinds J, Gould K, Witney A, Baldrey S, Lambertsen L, Hanage W, et al. Investigation of serotype diversity, non-typeable isolates and nasopharyngeal co-colonization by microarray-based molecular serotyping of *Streptococcus pneumoniae*. In: 7th International Symposium on Pneumococci and Pneumococcal Diseases. Tel Aviv, Israel. 2010.

[106] Azzari C, Moriondo M, Indolfi G, Cortimiglia M, Canessa C, Becciolini L, et al. Realtime PCR is more sensitive than multiplex PCR for diagnosis and serotyping in children with culture negative pneumococcal invasive disease. PLoS ONE 2010;5:e9282.

[107] Pimenta FC, Roundtree A, Soysal A, Bakir M, du Plessis M, Wolter N, et al. Sequential triplex real-time PCR assay for detecting 21 pneumococcal capsular serotypes that account for a high global disease burden. J Clin Microbiol 2012.

[108] Leung MH, Bryson K, Freystatter K, Pichon B, Edwards G, Charalambous BM, et al. Sequetyping: serotyping *Streptococcus pneumoniae* by a single PCR sequencing strategy. J Clin Microbiol 2012;50:2419–27.

[109] Elberse KE, van de Pol I, Witteveen S, van der Heide HG, Schot CS, van Dijk A, et al. Population structure of invasive *Streptococcus pneumoniae* in The Netherlands in the pre-vaccination era assessed by MLVA and capsular sequence typing. PLoS ONE 2011;6:e20390.

[110] Pai R, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. J Clin Microbiol 2006;44:124–31.

[111] Saha SK, Darmstadt GL, Baqui AH, Hossain B, Islam M, Foster D, et al. Identification of serotype in culture negative pneumococcal meningitis using sequential multiplex PCR: implication for surveillance and vaccine design. PLoS ONE 2008;3:e3576.

[112] Rivera-Olivero IA, Blommaart M, Bogaert D, Hermans PW, de Waard JH. Multiplex PCR reveals a high rate of nasopharyngeal pneumococcal 7-valent conjugate vaccine serotypes co-colonizing indigenous Warao children in Venezuela. J Med Microbiol 2009;58:584–7.

[113] Massire C, Gertz Jr RE, Svoboda P, Levert K, Reed MS, Pohl J, et al. Concurrent serotyping and genotyping of pneumococci by use of PCR and electrospray ionization mass spectrometry. J Clin Microbiol 2012;50:2018–25.

[114] Zhou F, Kong F, Tong Z, Gilbert GL. Identification of less-common *Streptococcus pneumoniae* serotypes by a multiplex PCR-based reverse line blot hybridization assay. J Clin Microbiol 2007;45:3411–5.

[115] Kong F, Brown M, Sabananthan A, Zeng X, Gilbert GL. Multiplex PCR-based reverse line blot hybridization assay to identify 23 *Streptococcus pneumoniae* polysaccharide vaccine serotypes. J Clin Microbiol 2006;44:1887–91.

[116] Selva L, del Amo E, Brotons P, Munoz-Almagro C. Rapid and easy identification of capsular serotypes of *Streptococcus pneumoniae* by use of fragment analysis by automated fluorescence-based capillary electrophoresis. J Clin Microbiol 2012;50:3451–7.

[117] Fenoll A, Jado I, Vicioso D, Casal J. Dot blot assay for the serotyping of pneumococci. J Clin Microbiol 1997;35:764–6.

[118] Bronsdon MA, O'Brien KL, Facklam RR, Whitney CG, Schwartz B, Carlone GM. Immunoblot method to detect *Streptococcus pneumoniae* and identify multiple serotypes from nasopharyngeal secretions. J Clin Microbiol 2004;42:1596–600.

[119] Sheppard CL, Harrison TG, Smith MD, George RC. Development of a sensitive, multiplexed immunoassay using xMAP beads for detection of serotype-specific *Streptococcus pneumoniae* antigen in urine samples. J Med Microbiol 2011;60:49–55.

[120] Ceyhan M, Yildirim I, Sheppard CL, George RC. Pneumococcal serotypes causing pediatric meningitis in Turkey: application of a new technology in the investigation of cases negative by conventional culture. Eur J Clin Microbiol Infect Dis 2010;29:289–93.

[121] Findlow H, Laher G, Balmer P, Broughton C, Carrol ED, Borrow R. Competitive inhibition flow analysis assay for the non-culture-based detection and serotyping of pneumococcal capsular polysaccharide. Clin Vaccine Immunol 2009;16:222–9.

[122] Lal G, Balmer P, Stanford E, Martin S, Warrington R, Borrow R. Development and validation of a nonaplex assay for the simultaneous quantitation of antibodies to nine *Streptococcus pneumoniae* serotypes. J Immunol Methods 2005;296:135–47.

[123] Park MK, Briles DE, Nahm MH. A latex bead-based flow cytometric immunoassay capable of simultaneous typing of multiple pneumococcal serotypes (Multibead assay). Clin Diagn Lab Immunol 2000;7:486–9.

[124] Yu J, Carvalho M da G, Beall B, Nahm MH. A rapid pneumococcal serotyping system based on monoclonal antibodies and PCR. J Med Microbiol 2008;57:171–8.

[125] Yu J, Lin J, Kim KH, Benjamin Jr WH, Nahm MH. Development of an automated and multiplexed serotyping assay for *Streptococcus pneumoniae*. Clin Vaccine Immunol 2011;18:1900–7.

[126] Carvalho M da G, Bigogo GM, Junghae M, Pimenta FC, Moura I, Roundtree A, et al. Potential nonpneumococcal confounding of PCR-based determination of serotype in carriage. J Clin Microbiol 2012;50:3146–7.

[127] Gratten M, Montgomery J, Gerega G, Gratten H, Siwi H, Poli A, et al. 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 1989;20:501–9.

[128] Donkor ES, Bishop CJ, Gould K, Hinds J, Antonio M, Wren B, et al. High levels of recombination among *Streptococcus pneumoniae* isolates from the Gambia. MBio 2011;2, e00040-11.

[129] Mulholland K, Satzke C. Serotype replacement after pneumococcal vaccination. Lancet 2012;379:1387, author reply 8–9.

[130] Brugger SD, Frey P, Aebi S, Hinds J, Muhlemann K. Multiple colonization with *S. pneumoniae* before and after introduction of the seven-valent conjugated pneumococcal polysaccharide vaccine. PLoS ONE 2010;5:e11638.

[131] Pell CL, Williams MJ, Dunne EM, Porter BD, Satzke C. Silica Desiccant Packets for Storage and Transport of *Streptococcus pneumoniae* and Other Clinically Relevant Species. PLoS ONE 2013;8:e72353.

[132] Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 2010;201:32–41.

[133] Madhi SA, Kuwanda L, Cutland C, Klugman KP. The impact of a 9-valent pneumococcal conjugate vaccine on the public health burden of pneumonia in HIV-infected and -uninfected children. Clin Infect Dis 2005;40:1511–8.

[134] Simonsen L, Taylor RJ, Young-Xu Y, Haber M, May L, Klugman KP. Impact of pneumococcal conjugate vaccination of infants on pneumonia and influenza hospitalization and mortality in all age groups in the United States. MBio 2011;2:e00309–310.

[135] Reed C, Madhi SA, Klugman KP, Kuwanda L, Ortiz JR, Finelli L, et al. Development of the Respiratory Index of Severity in Children (RISC) score

[136] Nunes MC, von Gottberg A, de Gouveia L, Cohen C, Moore DP, Klugman KP, et al. The impact of antiretroviral treatment on the burden of invasive pneumococcal disease in South African children: a time series analysis. AIDS 2011;25:453–62.

[137] Greenberg D, Givon-Lavi N, Newman N, Bar-Ziv J, Dagan R. Nasopharyngeal carriage of individual *Streptococcus pneumoniae* serotypes during pediatric pneumonia as a means to estimate serotype disease potential. Pediatr Infect Dis J 2011;30:227–33.

[138] Hanage WP, Finkelstein JA, Huang SS, Pelton SI, Stevenson AE, Kleinman K, et al. Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. Epidemics 2010;2:80–4.

[139] Albrich WC, Madhi SA, Adrian PV, van Niekerk N, Mareletsi T, Cutland C, et al. Use of a rapid test of pneumococcal colonization density to diagnose pneumococcal pneumonia. Clin Infect Dis 2012;54:601–9.

[140] Vu HT, Yoshida LM, Suzuki M, Nguyen HA, Nguyen CD, Nguyen AT, et al. Association between nasopharyngeal load of *Streptococcus pneumoniae*, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. Pediatr Infect Dis J 2011;30:11–8.

[141] Madhi SA, Klugman KP. A role for *Streptococcus pneumoniae* in virus-associated pneumonia. Nat Med 2004;10:811–3.

[142] Mthwalo M, Wasas A, Huebner R, Koornhof HJ, Klugman KP. Antibiotic resistance of nasopharyngeal isolates of *Streptococcus pneumoniae* from children in Lesotho. Bull World Health Organ 1998;76:641–50.

[143] Johnson HL, Deloria-Knoll M, Levine OS, Stoszek SK, Freimanis Hance L, Reithinger R, et al. Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project. PLoS Med 2010:7.

[144] Wright AK, Ferreira DM, Gritzfeld JF, Wright AD, Armitage K, Jambo KC, et al. Human nasal challenge with *Streptococcus pneumoniae* is immunising in the absence of carriage. PLoS Pathog 2012;8:e1002622.

[145] Auranen K, Rinta-Kokko H, Goldblatt D, Nohynek H, O'Brien KL, Satzke C, et al. Design questions for *Streptococcus pneumoniae* vaccine trials with a colonisation endpoint. Vaccine 2013.

[146] Gladstone RA, Jefferies JM, Faust SN, Clarke SC. Sampling methods for the study of pneumococcal carriage: a systematic review. Vaccine 2012;30:6738–44.

[147] Weinberger DM, Bruden DT, Grant LR, Lipsitch M, O'Brien KL, Pelton SI, Sanders EA, Feikin DR. Using pneumococcal carriage data to monitor post-vaccination changes in invasive disease. Am J Epidemiol 2013. Sep 7 [Epub ahead of print], PMID: 24013204.