In vitro and *in vivo* comparison of transport media for detecting nasopharyngeal carriage of *Streptococcus pneumoniae*

Running title: pneumococcus media comparison

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Abstract

Background.

As standard method for pneumococcal carriage studies the World Health Organization recommends nasopharyngeal swabs ~~to~~ be transported and stored in skim-milk, tryptone, glucose, ~~and~~ glycerol (STGG) broth. An enrichment broth used for transport in three carriage studies performed in Norway ~~could~~ may have a higher sensitivity than STGG. We therefore compared the media *in vitro* and *in vivo*.

Methods.

For the *in vitro* ~~part~~component, ~~suspensions were made of~~ three strains ~~belonging to~~ (serotypes 4, 19F and 3) were suspended in STGG and enrichment broth. ~~The r~~Recovery was compared using latex agglutination, ~~by~~ quantification of ~~the~~ bacterial loads by real-time PCR of the *lytA* gene, and ~~by~~ counting of ~~the colony forming units (CFU)~~colonies from ~~the~~ incubated plates. For the *in vivo* comparison, paired swabs were obtained from 100 children and transported in STGG or enrichment broth. Carriage was identified by latex agglutination and confirmed by Quellung reaction.

Results.

In vitro, the cycle threshold~~s~~ values obtained by PCR did not differ between the two media (p=0.853) and no clear difference in colony counts was apparent after incubation (p=0.593). *In vivo*, pneumococci were recovered in 46% of swabs transported in STGG and ~~in~~ 51% of those transported in enrichment broth (Kappa statistic 0.90, p=0.063).

Discussion.

~~Overall, no statistical difference in sensitivity was found between STGG and enrichment broth. Nevertheless,~~Although some serotype differences were observed and STGG appeared slightly less sensitive than enrichment broth for detection of nasopharyngeal carriage of pneumococci by culturing, no statistical differences in sensitivity were found.

~~Conclusions~~

We recommend the continued use of STGG for transport and storage of nasopharyngeal swabs in ~~future~~ pneumococcal carriage studies ~~because its~~for the benefit ~~in terms~~ of

comparability between studies and settings, including more resource-limited settings~~,~~ ~~outweighs the (non-statistical) decreased sensitivity~~.

Introduction

Monitoring carriage of *Streptococcus pneumoniae* (pneumococci) is important for determining changes after vaccine introduction in national immunisation programmes. To ~~be~~ enable ~~to~~ compareison of results ~~between~~ from different studies and countries, the World Health Organization Pneumococcal Carriage Working Group ~~has~~ published a set of standard methods for ~~such studies~~ measuring nasopharyngeal carriage of pneumococci [1]. ~~In this method, STGG (s~~Skim milk powder, tryptone soy broth, glucose and glycerol in distilled water (STGG [2]) ~~has beenis~~ the recommended ~~as~~ medium for transport and storage of nasopharyngeal specimens [1]. Studies using STGG in developed countries have generally revealed prevalences of pneumococcal carriage in children of around 30-50% ~~[3-10]~~[3,5,10]. In Norway, several carriage studies have been performed using enrichment broth ~~for transport and direct incubation~~ (beef infusion enriched with 5% horse serum and 3.3% defibrinated horse blood [Statens Serum Institute, Copenhagen, Denmark]) for transport and direct incubation [11]. ~~The c~~Carriage ~~-~~prevalence ~~found~~ in those studies was around 80% before and after introduction of the 7-valent pneumococcal conjugate vaccine (PCV), and 64% (62% in the reference abstract?) two years after switching to the 13-valent PCV [12]. Although different factors may contribute to this high prevalence, such as the percentage of children in day-care (>90% [13]) and the low use of antibiotics in Norway [14, 15], ~~the~~ results ~~may~~ suggest that enrichment broth ~~is~~ may be more sensitive for detection of carriage than STGG.

We therefore compared 1) ~~the~~ *in vitro* recovery ~~rate~~from serial dilutions in STGG and enrichment broth, and 2) *in vivo* detection of nasopharyngeal carriage of pneumococci from swabs that were transported and stored in STGG or in enrichment broth.

Materials and methods

~~The study consisted of two parts.~~ In the *in vitro* part of the study we compared recovery rates of pneumococci from serial dilutions that had been stored at different temperatures and media using I) culturing, II) a commercial latex agglutination kit and III) quantitative real-time PCR

(qPCR). In the *in vivo* ~~part~~ component we compared carriage in paired swabs taken from children attending day-care centres (DCC) and transported in STGG or enrichment broth. In this second part we used methods I and II for detection of pneumococci. For a schematic overview of the procedures, see Figure 1.

In vitro comparison

Three strains belonging to different serotypes were used as pneumococcal samples; two reference strains (ATCC 49619 - serotype 19F, and TIGR4 - serotype 4), and a strain belonging to serotype 3 obtained from a previous Norwegian carriage study [12]. Colonies from each serotype were suspended in Todd-Hewitt (TH) broth ~~(TH)~~ at a concentration of 0.5 McFarland in serial 1:10 dilutions of 10^{-2} to 10^{-5} (minimal concentration for which recovery of pneumococcal DNA was possible; data not shown). ~~To mimic what would happen in a carriage study when an initial volume of inoculum (swab) would be added to a tube with transport medium, we added t~~The same volume (100µL) of serotype dilution in TH broth was added to each set of transport medi~~a~~um (either 1ml of STGG or 3ml of broth) to ~~make~~ prepare the pneumococcal samples. The samples were ~~incubated~~ left for 3h on wet ice (STGG) or at room temperature (broth). Subsequently, ~~we plated~~ 100µL aliquots of the STGG ~~or~~ and broth samples were plated on Columbia horse blood agar plates. Furthermore, ~~we added~~ 100µL of the STGG samples were added to fresh broth. All was done in triplicate. Plates and tubes (broth sample made from the STGG samples and the initial broth samples) were incubated overnight at 35°C with 5% CO_2.

Pneumococci were identified by latex agglutination ~~kit~~ (Pneumotest-Latex kit; Statens Serum Institut, Denmark) from the incubated broths. Quantification of the bacterial loads was performed by qPCR (see below for details) and counting of the colony forming units (CFU) from the incubated plates.

DNA extraction and amplification by qPCR

~~Of~~ From each sample 200µL was boiled for 10 minutes and DNA was extracted by QIAamp DNA Mini QIAcube kit (Qiagen, Inc., Valencia, CA, US) according to the manufacturer's recommendations. A qPCR assay for the detection of the autolysin-encoding gene (*lytA*) was

then performed as described before by Carvalho et al. [16]. Briefly, 25µL reaction volume composed of TaqMan Fast Universal PCR Master Mix (2x), 200 nM of each primer and probe, 10x Exo IPC-mix, 50x Exo IPC DNA and 2 µL of DNA was run at 50°C for 2 minutes, denaturation at 95°C for 10 minutes, followed by 40 amplification cycles of 95°C for 15 seconds and 60°C for 1 minute. Samples were considered negative if cycle thresholds (Ct) exceeded 40. A positive (ATCC49619) and a non-template control (sterile water) were included in each run, along with extraction controls.

In vivo comparison

This comparison was performed as part of a larger carriage study (abstract ISPPD-0116, 2016). The study was conducted in accordance ~~to the~~with principles of the Declaration of Helsinki, and approved by the Regional Committee for Medical Research Ethics, South-Eastern Norway (reference number: 2014/2046). Parents/guardians of the children gave written informed consent before including their child in the study. The study design resembles the design used in previous Norwegian carriage studies [12].

Two flocked nylon nasopharyngeal swabs (E-swabsTM, Copan, Italy) taken from the same nostril were collected from 100 children aged 1-6 years according to standard procedures. The first swab was placed in 1 ml STGG which was subsequently stored in a cool box and the second swab was stored and transported in enrichment broth at room temperature. The specimens were processed within 4 hours after swabbing. The STGG samples were vortexed at high speed and frozen at -70°C, following the recommendations of WHO [1]. After being thawed, the STGG samples were vortexed before 200 µl was added to fresh broth and 20 µl was plated on gentamycin-blood-agar (GBA). All STGG samples were thawed and analysed following initial freezing within one month. The broth samples were re-inoculated in ~~the~~ fresh enrichment broth (200 µl?) and plated on GBA (20 µl?) within ~~the~~ 4h ~~after~~ of sampling. All broths and GBA plates were incubated overnight at 35°C, with 5% CO_2.

Pneumococci were identified by latex agglutination from incubated broths. Confirmation and factor typing were ~~done~~ performed by Quellung reaction. All morphologically different pneumococcal colonies per plate were typed. In cases where the latex agglutination was positive but no colonies were found on plates after incubation over-night, ~~we re-cultured the~~

samples were re-cultured by plating 100µL of the incubated broth and incubating this for another night before further analysis.

Data analysis

For the *in vitro* analysis, ~~we compared the~~ Ct values and CFU counts (after a logarithmic transformation; logCFU) ~~of~~ from the two media were compared by linear regression using dummy variables for each dilution per serotype. In ~~such~~ this way, the comparison could be run simultaneously for all serotypes combined. Furthermore, ~~we run~~ the ~~analysis~~ analyses were conducted separately per serotype. Agreement in the *in vivo* comparison was determined using the kappa statistic [17] and ~~was tested by~~ the exact McNemar's probability test for paired data. Data were analysed in Stata 14.0 and GraphPad Prism 5.

~~The *in vivo* part of the study was approved by the Regional Committee for Medical Research Ethics, South-Eastern Norway. Parents/guardians gave informed consent before sampling.~~

Results

In vitro results

The latex agglutination test was positive for both media for all serotype dilutions tested, with the exception of serotype 3 at a dilution of 10^{-5} in STGG, where no pneumococci were detected.

The quantification of DNA, by Ct value, is presented in Figure 2. The Ct values overall did not differ significantly between STGG and broth samples (p=0.853), though~~,~~ for serotype 4, the values were significantly lower for STGG samples compared to broth samples (p=0.007). After culturing, no clear overall difference was found in the logCFU (p=0.593) but significant differences were observed for serotype 4 (Figure 3B; p=0.008; more colonies on plates incubated with STGG samples) and serotype 3 (Figure 3C; p=0.001; more colonies on plates incubated with enrichment broth samples).

In vivo results

Forty-six percent of the swabs transported and stored in STGG and 51% of those transported in enrichment broth were positive for pneumococci. This resulted in a Kappa statistic for carriage of 0.90 for the paired swabs (Table 1), indicating a trend to a higher sensitivity after transportation in enrichment broth compared to STGG (p=0.0625). If ~~we exclude~~ re-culturing after overnight incubation was excluded, carriage was 44% for the samples transported and stored in STGG and 48% for those transported in enrichment broth (Kappa=0.92; p=0.125). For each child for whom both swabs were positive, the same serotype was obtained. In one child, carriage of two serotypes was found in the enrichment broth sample, while one serotype was found in the STGG sample. ~~Nevertheless~~However, the plate incubated with the enrichment broth sample had only one colony of the serotype that was missed in the STGG sample (serotype 3) and the agglutination test was negative for this serotype, indicating presence at a very low concentration.

Discussion

Overall, no statistical difference in sensitivity was found between STGG and enrichment broth. Nevertheless, some serotype differences were observed ~~and~~ as well as a trend towards higher sensitivity for detection of pneumococcal carriage after transportation in broth compared to STGG.

There are several possible reasons for these differences. ~~While *i*~~*In vitro*, a pure dilution of one serotype was used ~~as a swab mimic~~in place of a swab. However, *in vivo* the swab contained different respiratory bacteria and viruses, and ~~it~~ was covered with mucus and cellular debris. The presence of other bacteria places pneumococci in competition for nutrients needed for growth and reproduction. These nutrients~~, which~~ are available in higher concentration in enrichment broth than in STGG. ~~. The difference in availability of nutrients~~ which may explain the small difference in sensitivity observed between the *in vitro* and *in vivo* settings. Nevertheless, for serotype 3, the bacterial load quantified by CFU counts *in vitro* indicated a higher sensitivity of enrichment broth compared to STGG. The s~~S~~erotype 3 ~~was an~~ isolate was obtained from a previous carriage study. For serotype 19F and 4, reference strains were used. The origin of the isolates~~, i.e. from~~ (reference strains versus ~~isolated from a previous~~ carriage ~~study,~~isolate) may have induced different bacterial growth characteristics. ~~Another~~

~~point to take into consideration is that~~Further, the capsule structure differs between serotypes (serotype 3 being very mucoid). The low number of serotypes tested and the difference in origin between serotypes are limitations to the *in vitro* part of this study.

The carriage prevalence ~~of carriage~~ found in ~~the *in vivo* part of~~ this study is lower than observed ~~before~~ previously in Norway [12], and more similar to what has been seen in other developed country settings ~~[3–10]~~ [3,5,10]. ~~Because this study was performed four years after switching to PCV13, the prevalence cannot be directly compared to the earlier studies.~~ The methods used for swab collection (?), transport~~the~~ in enrichment broth ~~samples~~ and culturing (?) ~~was~~ were unchanged from former studies, ~~which indicates~~ indicating a real difference in prevalence that may have resulted from PCV13 vaccination (unpublished data ~~not yet published~~).

Advantages of molecular based techniques compared to culture techniques include the fact that viable organisms are not required, the original composition of the nasopharyngeal specimen is preserved, and ~~also that a~~ detailed quantification and characterization of the pneumococci within a sample are possible, depending on the methods used [1]. Furthermore, ~~its~~ the sensitivity of molecular methods for detection of multiple co-colonising serotypes has been shown to be higher than conventional methods [18]. Nevertheless, isolation of strains enables further characterization ~~like~~ such as antimicrobial susceptibility testing and sequence typing, ~~as well as traditional bacteriology,~~ and should not be replaced by molecular methods alone~~, despite its high sensitivity~~. The PneuCarriage Project concluded that microarray with a culture amplification step has the highest sensitivity for determining carriage [19].

Finally, STGG is cheap and easy to make and can be stored longer than enrichment broth, thus enabling comparability between studies and settings, including more resource-limited settings. Furthermore, STGG enables studies which investigate the microbiome which is less affected by this medium [20, 21], whereas enrichment broth may selectively stimulate growth of pneumococci.

~~Conclusions~~

~~Even though~~Although STGG appeared slightly less sensitive than enrichment broth for detection of nasopharyngeal carriage of pneumococci by culturing, we recommend the continued use of ~~this medium~~STGG for transport and storage of nasopharyngeal swabs in

future carriage studies. As STGG is cheap, easy to make and can be stored longer than broth, STGG enables comparability between studies and settings, including more resource-limited settings. Furthermore, STGG enables studies to investigate the microbiome as this is less affected by this medium [20, 21], while broth may selectively stimulate growth of pneumococci.

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Tables

Table 1: ~~2 × 2 contingency table on p~~P~~neumococcal carriage determined by culturing ~~of the~~ paired nasopharyngeal swabs stored in STGG or enrichment broth

	Broth positive	Broth negative	*Total*
STGG positive	46	0	*46*
STGG negative	5	49	*54*
Total	*51*	*49*	*100*

STGG: skim-milk, tryptone, glucose and glycerol

Figures

Figure 1: Schematic overview of the experimental designs.

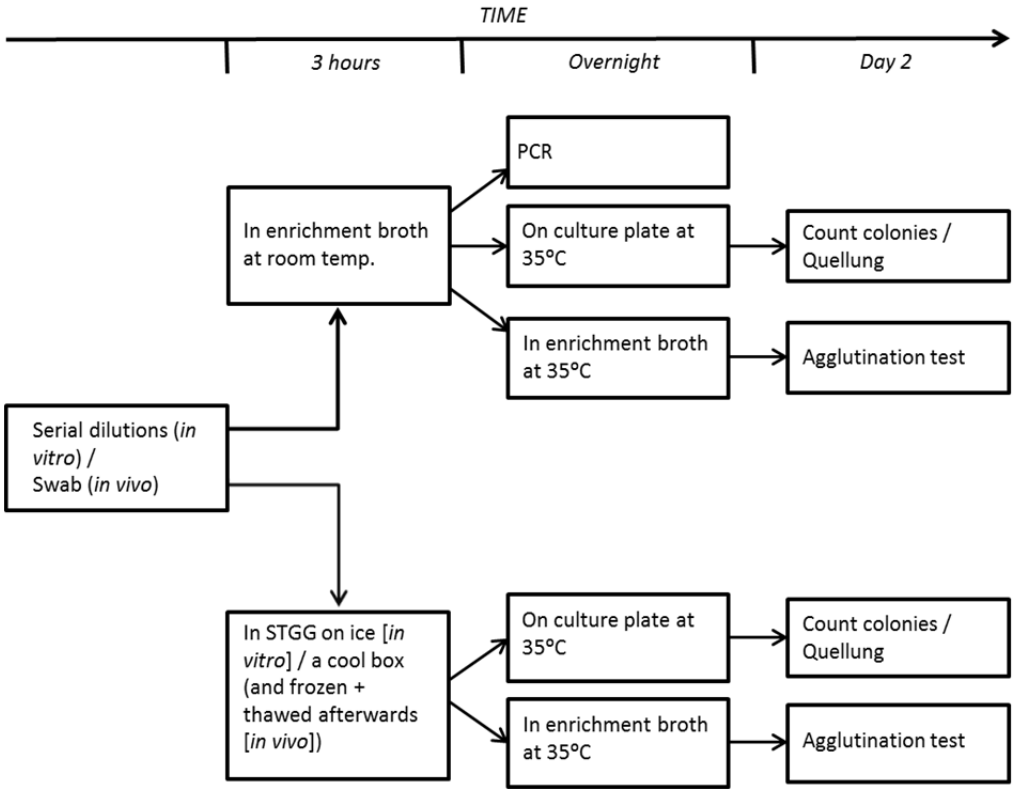


Figure 1 (suggested): Schematic overview of the experimental designs.

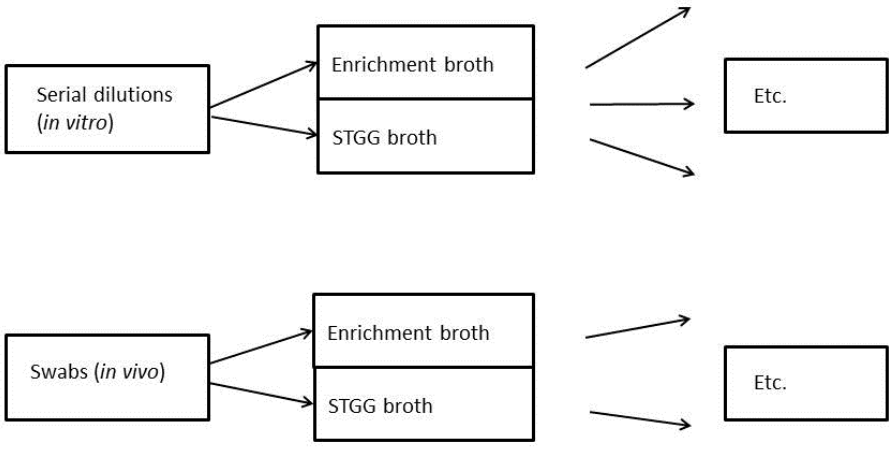
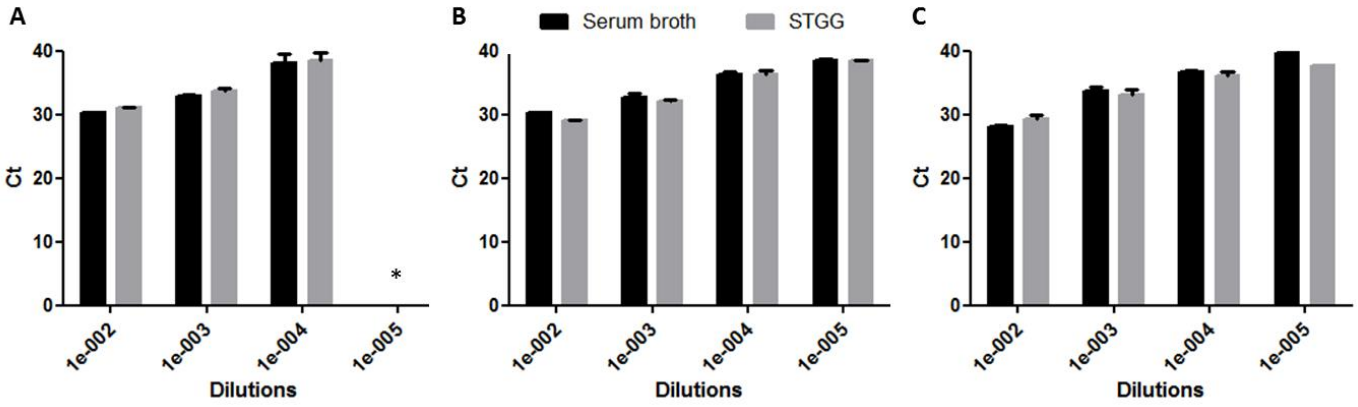
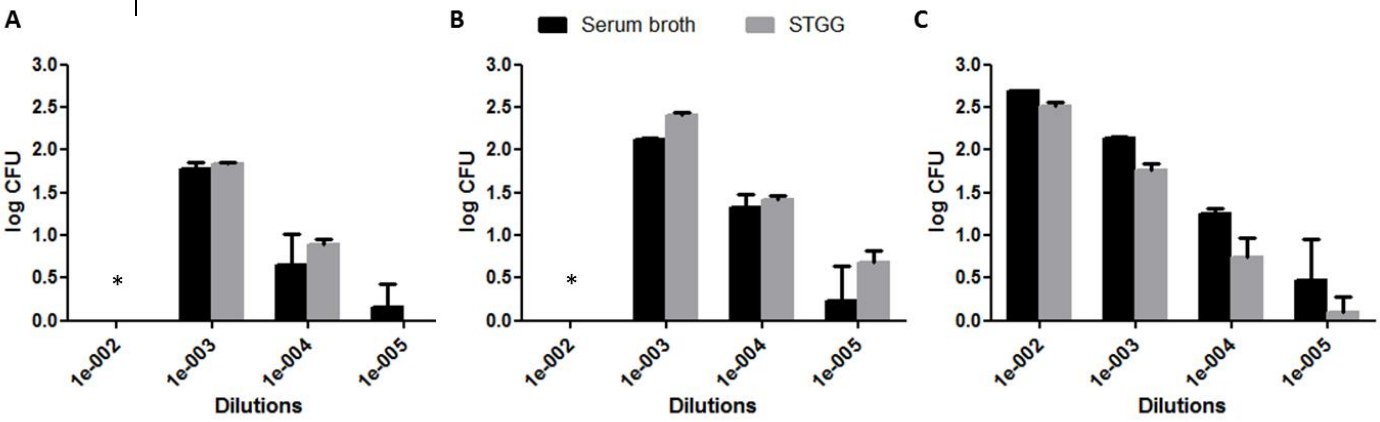


Figure 2: DNA quantification (Ct values) ~~of different serotypes~~ at different dilutions of ~~serum~~ enrichment broth ~~(black)~~ and STGG ~~(grey)~~. A: serotype 19F*; B: serotype 4; C: serotype 3.



* At a dilution of 10^{-5}, one of the triplicates ~~was~~had a cycle threshold (Ct) of 39.4, while the other two were above 40. This sample was therefore considered negative.

Figure 3: Quantification of ~~the~~ bacterial load (log counts of colony forming units [CFU] ~~counts~~) ~~of different serotypes~~ at different dilutions of ~~serum~~ enrichment broth and STGG. A: serotype 19F*; B: serotype 4*; C: serotype 3.



* ~~At a dilution of 10^{-2}, the CFU were~~ uncountable ~~(too many)~~.