ALTERNATIVE MOLECULAR METHODS FOR IMPROVED DETECTION OF MENINGOCOCCAL CARRIAGE AND MEASUREMENT OF BACTERIAL DENSITY.

Running title: Improved diagnosis of meningococcal carriage

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Abstract

Background: Conventional methods for detecting pharyngeal carriage of *Neisseria meningitidis* (*Nm*) are complex. There is a need for simpler methods with improved performance. We have investigated two alternative approaches.

Methods: Three pharyngeal swabs were collected from 999 pupils aged 10 to 18 years in The Gambia. Carriage of *Nm* was investigated using three different methods: (i) plating on Thayer-Martin selective medium and testing by conventional microbiological methods followed by PCR testing, (ii) seeding in Todd Hewitt broth (THB) and, after overnight culture, testing by PCR, (iii) compression of the swab on filter paper and, after DNA concentration, testing by PCR.

Results: PCR after culture in THB was more than twice as sensitive as conventional methods in detecting Nm (13.2% versus 5.7%; p<0.0001). PCR after DNA extraction from filter paper had a similar sensitivity to that of conventional methods (4.9% vs 5.7%, p=0.33). Capsular genogroups detected by broth culture were W (21), B (12), Y (8), E (3), and X (2), and 68 meningococci had the capsule null intergenic region. The distribution of genogroups and of capsule null organisms was similar with each of the three methods. Carriage density in samples extracted from filter paper ranged from 1 to 25,000 DNA copies.

Conclusions: PCR of overnight broth culture doubled the yield of *Nm* carriage isolates compared with conventional methods. This approach could improve the efficiency of carriage studies. Collection on filter paper followed by quantitative PCR could be useful for density measurement and for carriage studies in areas with limited resources.

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Introduction

Infection with *Neisseria meningitidis (Nm)* is usually characterized by asymptomatic, or minimally symptomatic, carriage of bacteria in the pharynx; meningococcal septicemia and/or meningitis are rare events occurring in as few as 1:100 to 1:1000 colonized individuals. For this reason, selective pressure induced by antimicrobials or by naturally-acquired or vaccine-induced immunity is exerted primarily on bacteria carried by asymptomatic carriers. Therefore, the epidemiology of meningococcal infections cannot be understood fully without considering carriers (1, 2).

Carriage of *Nm* is relatively uncommon and thus determining carriage prevalence requires large-scale surveys (3), which are usually conducted using microbiological techniques developed several decades ago. Detection of meningococcal carriage involves three main steps that could be improved, namely sample collection, transport to the laboratory and identification of meningococci. Most studies have focused on the optimization of methods for collecting pharyngeal swabs; these have shown that a per-oral swab taken behind the uvula is the site most likely to yield a positive result, and that taking two sequential swabs increases sensitivity (4-6). Until recently, little has been done to improve methods of transport (7, 8), and we are aware of only two studies aimed at improving identification of meningococci by using molecular methods (9, 10). PCR testing on DNA extracted directly from a swab has been employed in some studies, but a recent investigation in UK adolescents showed that this method was less sensitive than conventional culture followed by PCR (11). We have developed two new methods for detecting meningococcal carriage; molecular analysis of extracted DNA obtained either after overnight culture in Todd-Hewitt broth (THB) or from a filter paper on which a swab had

been pressed, and compared the results obtained with these methods with those obtained by conventional microbiology confirmed by PCR in a study of meningococcal carriage in 999 Gambian schoolchildren.

Materials and methods

Study design and participants

Following sensitization of the community, and with permission from the educational authorities, a cross-sectional carriage survey was undertaken from July 1st - 30th 2013, the start of the rainy season, in children attending middle or high schools in the peri-urban area of Fajikunda, The Gambia, West Africa. Healthy school attenders aged between 10 to 18 years were recruited sequentially until 1,000 had been enrolled. No children or parents invited to join the study refused to participate. Written, informed consent was obtained from 18-year old students. Assent and written consent from a parent or guardian was obtained from those aged 10 to 17 years. A questionnaire which investigated potential risk factors for meningococcal carriage was administered to all participants. Three pharyngeal swabs were then collected from each student on the same occasion. One was streaked directly onto a Thayer-Martin selective agar plate, a second was placed in THB and a third smeared on a filter paper strip. Children were randomized prior to collection of the samples to one of three groups, which indicated the order in which the three samples were to be collected (figure 1), to ensure that first, second and third swabs had an equal chance of being tested by each of the three laboratory methods.

Journal of Clinical Microbiology

The study was approved by the Scientific Coordinating Committee of the Medical Research Council (MRC) Unit, The Gambia, by the Gambian Government/MRC Joint Ethics Committee and by the Ethical Committee of the London School of Hygiene & Tropical Medicine.

Conventional microbiology

The conventional methods employed to identify *Nm* by the MenAfriCar consortium have been described in detail elsewhere (12) and are reported briefly here. Swabs were streaked directly onto a modified selective Thayer-Martin agar plate in the field, and plates held in a 5% CO_2 jar until transported to the laboratory within six hours of collection. After 24 hours of sub-culture on chocolate agar plates, an oxidase test and a Gram stain were performed. All oxidase positive, Gram negative diplococci (OPGNDC) were tested for β-galactosidase activity with ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and for γ-glutamyl-transferase (GGT) and butyrate esterase activity (Tributyrin). ONPG negative, GGT positive, Tributyrin negative bacteria were then serogrouped by slide agglutination, initially with serogroup A and W antisera and then, if negative, with X and Y antisera. DNA was extracted from all OPGNDC isolates using Qiagen extraction of a bacterial suspension boiled for 20 minutes and then tested with the multiplex real-time PCR (rt-PCR) described below. In the conventional microbiology group, OPGNDC isolates which were ONPG negative, GGT positive and tributyrin negative and also PCR positive (see below) were considered to be *Nm.*

Broth culture

Prior to the field study, the ability of two broth cultures to support the growth of *Nm* was tested using aliquots spiked with serial dilutions of a serogroup A reference strain (ATCC® 13077™), starting with a dilution of approximately 1200 X 10^8 CFU per ml. The first medium

tested was Mueller-Hinton broth (MHB - Oxoid, Basingstoke, UK) supplemented with VCNT (Oxoid SR0091E) containing vancomycin (3mg/L), colistin (7.5mg/L), nystatin (1250U/L) and trimethoprin (5mg/L)]. The second medium investigated was Todd-Hewitt broth (THB - Oxoid, Basingstoke, UK) supplemented with 0.5% yeast, rabbit serum (B&K Universal Ltd, Grimston, East Yorkshire, UK), to facilitate pre-incubation before arriving at the laboratory, and the same antibiotic combination described above. DNA was extracted from broth cultures using the Qiagen method according to the manufacturers' instructions (with elution in 200 µL). The Todd-Hewitt medium supported bacterial growth at higher dilutions than the Mueller-Hinton broth (see Table 1) and this medium was used in the field study.

Filter paper cards

The ability of filter paper strips (Whatman FTA MiniCard® WB 120055) to preserve *Nm* DNA prior to DNA extraction was explored in the laboratory using serial dilutions of a suspension of a serogroup A meningococcal reference strain (ATCC® 13077™); samples spiked with different dilutions of bacterial suspension were spotted onto filter paper strips and held at room temperature (18°C to 23°C) for 48 hours prior to DNA extractions using Qiagen. Next, elution was done twice with a volume of 25 µl, a lower volume than the usual 200 µl in order to concentrate DNA. In the laboratory, it was possible to detect approximately one *porA* gene copy of *Nm* by rt-PCR. In the field, a swab was smeared directly onto a MiniCard which was held at room temperature for several weeks prior to extraction. In the field, a swab was smeared directly onto a MiniCard which was held at room temperature for 6-12 months prior to extraction as it is well known that DNA collected on FTA cards can be preserved for years at room temperature. Using a punch, a small segment of the filter paper (approximately 6 mm diameter) was obtained from the centre of the smear and DNA

extracted as described above. A second sample was obtained as close as possible to the centre and tested using the same procedure as it was considered that bacteria might have been concentrated off-center when the swab was compressed onto the MiniCard.

Multiplex rt- PCR for Nm detection

Detection of *Nm* was undertaken by targeting both *porA* and *sodC* genes and the capsule null intergenic region (*cnl*) simultaneously. Genogrouping of all *porA* and/or *sodC* positive samples, considered as *Nm,* was done according to the method developed by Wang *et al* (13) for genogroups A, W and Y, followed by B, C and X. All samples of *Nm* that could not be characterized in this way underwent an H, E, Z multiplex PCR (see table S1). The cycling conditions were the same for all tests: 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C, 50 cycles of 15 s at 95°C and 1 min at 60°C. The ABI 7500 fast cycler was used to perform the reaction and the results analysed using the 7500 fast software. Samples were kept at 4°C after amplification. For both the examination of THB samples and PCR confirmation of isolates obtained by conventional microbiological methods, a stringent Ct threshold of 25 was used for both *porA* and *sodC* genes to select true positives. This threshold was shown to be optimal by comparison with positive controls diluted at different concentrations and plotted on standard curves. For genogrouping multiplex PCR, the conventional Ct of 35 was used as the threshold for positivity and samples between 36 and 40 were retested after 10-fold dilution as per Wang's method (13). For filter paper testing, using positive controls at different dilutions plotted on standard curves, better results were obtained with the *porA* monoplex rt-PCR than with the multiplex *porA/sodC/cnl* during our preliminary evaluation and, therefore, we used only *porA* monoplex rt-PCR during the field study with a Ct threshold of 35 as the criterion of positivity.

Measurement of bacterial density

For calculation of bacterial density, reference strain Nm W ATCC® 35559™ was harvested from an overnight culture and diluted in phosphate buffered saline to reach a 4.0 McFarland concentration. Serial dilutions were made and DNA extraction done using Qiagen kits according to the manufacturers' recommendations to build standard curves for DNA quantification through rt-PCR. Standard curves of DNA measurements were made using a NanodropTM spectrophotometer (ThermoScientific, USA) and PicoGreen® dsDNA quantitation assay (Lifetechnologies, France) techniques. The average of both DNA measurements was used when values were discrepant. Genome copies were estimated using the formula: mass = number of DNA base pairs per genome X 1 mole/6.022 140 X 10^{23} X 660 g per mole, where 6.022 140 X 10^{23} is the Avogadro's number (molecules per mole) and 660 g/mole is the average molecular weight of a double-stranded DNA molecule. Extracted bacterial DNA was then subject to PCR as described above.

Statistical analyses

The study was designed to have 80% power to detect an increase in carriage prevalence from an estimated carriage prevalence of 5% in the conventional microbiology group to at least 7% from paired samples employing one of the two novel approaches to carriage detection; this required a sample size of 870. Data were analyzed using Stata v12.0. Sensitivity was compared between methods using the exact McNemar's significance probability. Differences in carriage prevalence by group (e.g. age, sex) were investigated using chi-squared tests and logistic regression.

Results

Journal of Clinical Microbiology

One thousand students were recruited into the study; one child was excluded because of a missing questionnaire leaving 999 for analysis. The majority (n=859) were aged 10-13 years, the remainder (n=138) were 14- 18 years old; age was not recorded for two children. More male than female students were enrolled (589 vs 410). Pharyngeal carriage of *Nm* was detected in 143 students by one or other method (overall carriage prevalence 14.3%). Carriage prevalence was similar in males (80/589 [15.3%]) and females (63/410 [13.5%]) (p=0.428). Carriage was not associated with age within the limited age range investigated (p=0.519).

One hundred and eleven oxidase positive, gram negative diplococci were isolated using the conventional microbiology approach; 49 were characterized as *Nm* by biochemical methods (ONPG, GGT and Tributyrin tests). This diagnosis was confirmed by multiplex PCR in all but three samples. An additional 21 OPGNDC isolates had a weak reaction with one of the three biochemical tests and were tested by rt-PCR. Eleven were positive for *Nm* on rt-PCR testing making a total of 57 *Nm* detected by conventional microbiology followed by rt-PCR (carriage prevalence 5.7%). Several false positive reactions were seen using sero-agglutination. For example, 13 *Nm A* were identified by sero-agglutination which were not confirmed by *Nm A* specific genogrouping rt-PCR. Genogrouping of the 57 *Nm* isolates identified the following genogroups in decreasing order of prevalence: *cnl* (21), W (11), B (5), Y (3), E (2), C (2) and dual infection *cnl*/W (2) and *cnl*/Y (1). Ten samples could not be classified through genogrouping PCR (NG). All samples positive by *sodC* and *porA* negative were *cnl* or NG.

Overnight culture in THB followed by rt-PCR detected 132 carriers of *Nm* (carriage prevalence 13.2%), a marked increase in sensitivity compared to the conventional method

206 (p<0.0001). Sixty-eight of the 132 *Nm* isolates (51.5%) possessed only the *cnl* intergenic
207 region (see figure 2). Genogroups detected following broth culture were W (21), B (12), Y
208 (8), E (3) and X (2). Four additional samples (not classed as meningococci in the primary
209 analyses) had doubtful results, with a Ct between 25 and 30. No genogroups A, C, H or Z *Nm*
210 were detected. Eleven strains grown in THB were non groupable (NG) and 7 dual carriers
211 were identified. Overall, genogrouping results matched well between methods, but there
212 were a few discrepancies between the results obtained with THB culture compared to the
213 conventional method: two genogroups C and two E were not confirmed in THB. The
214 superiority of the THB culture over conventional microbiology in detecting both *cnl* strains
215 and different genogroups of meningococci is demonstrated in figure 2.

216 Forty-nine *Nm* carriers (carriage prevalence 4.9%) were identified using filter paper
217 minicards, a similar prevalence to the conventional method (p=0.33). Among these, 18
218 carried the *cnl* gene with Ct values lower than or equal to 35. Genogrouping rt-PCR could
219 not be performed on the other samples due to the low volume of elution used to
220 concentrate the DNA for *Nm* detection and the fact that repeated tests were done to
221 verify positive tests. The density of carriage, as reflected in the number of DNA copies
222 obtained from the filter paper, varied between 1 and 25,000 DNA copies with a median
223 value of 59 copies (figure 3). There was no association between subject's age and carriage
224 density. Additional testing with a second punch taken away from the centre of the smear
225 allowed detection of one additional carrier. Median density from 13 second punch
226 samples (2.0 [95% CI 1.0.10.8]) was lower than that found in 48 first punch samples (58.5
227 [95% CI 35.0, 226.0]), emphasising the importance of taking the punch from the centre of

Journal of Clinical Microbiology

the smear. Only two of 49 subjects had > 10,000 copies of *porA* DNA and only seven had > 1,000 copies.

The overlap in detection of carriage between the different methods is shown in the Venn diagram (figure 4). Most of carriers detected by conventional microbiology (80.7%) were also detected by broth culture and 47.4% were detected on filter paper. Forty-seven of the 49 carriers detected by filter paper were also detected through THB culture and 27 by conventional microbiology. Using both *porA* and/or *sodC* genes to define positivity, and assuming detection by any method to be a true positive, the sensitivity of conventional microbiology was 39.6%, that of the filter paper method 34% (using a *porA* monoplex rt-PCR) and that of THB culture 91.6%.

Discussion

Detection of meningococcal carriage in children attending schools in Fajikunda, The Gambia was enhanced more than two fold compared to conventional culture by culture overnight in a selective medium and subsequent detection of meningococcal DNA. The genogroup distribution of carriers detected using broth culture was similar to that seen using conventional culture, suggesting that overnight culture did not have a major selective effect, although genogrouping of a few strains did not match between methods. The enhanced sensitivity of the broth culture approach probably reflects the fact that many carriers have only a low density of bacteria on the surface of the pharyngeal mucosa, and that liquid broth provides *Nm* with a better environment for immediate growth than solid Thayer-Martin medium. The broth culture technique is cheap and easily implementable, with

Journal of Clinical Microbiology

collected samples cultured overnight, boiled in PBS and then stored at -20°C for later rt-PCR analysis at a convenient time.

A limitation of this study is that the preservative medium made of skimmed milk, (tryptone), glucose and glycerin [S(T)GG], commonly used for transport in pneumococcal carriage studies, was not evaluated. However, in a qualitative and semi-quantitative comparison of preservation in STGG with conventional direct plating in the field, O'Brien *et al*. demonstrated that direct plating was slightly superior to STGG for recovery of *S. pneumoniae* (14). In a recent study of 601 students in Portugal (10), a similar prevalence of meningococcal carriage was obtained from culture and by direct *sodC* rt-PCR on samples collected in STGG (13.3% vs 14.5% respectively) (Adam Finn, personal communication). Our study demonstrated that broth can be used both as a transport and culture medium but more studies are needed to define which medium is optimal. The use of rabbit serum is a limitation of our method as this reagent has to be shipped frozen, a challenge for resource-limited countries. Further studies are needed to define whether the efficiency of detection is similar without this reagent and whether the efficacy of broths for short-term culture of *Nm* can be improved further.

Collection of blood spots on filter paper strips with subsequent DNA extraction and molecular analysis is a technique used widely in study of other infectious diseases including malaria and HIV (15) but, as far as we are aware the filter paper technique has not been for collection of meningococcal DNA although it has been used for detection of meningococcal antigen in cerebrospinal fluid samples sent to a central laboratory (17). We tested filter paper eluates on which a swab had been pressed by *porA* monoplex rt-PCR only rather than by *porA* and *sodC* as this gave us better results during the evaluation on spiked samples so

that the comparison of the results obtained with the filter paper technique and conventional microbiology reflect a comparison of two optimum techniques rather than a comparison of directly similar PCR methods. Employing the optimum techniques for each method, the filter paper approach had a similar sensitivity to that of the conventional method. However, it has the advantage that samples can be stored at room temperature for prolonged periods and readily transported to a central laboratory without degradation of DNA prior to analysis, avoiding the need for a cold chain or transport medium with temperature monitoring. Another advantage of the filter paper technique is that it does not involve any culture pre-amplification and so allows direct measurement of the density of bacteria present on the swab and hence an indication of the density of pharyngeal carriage in an individual. We found a wide range of bacterial density in the filter paper samples with a density distribution similar to that found in UK students (16). Since high density carriers are likely to be more infectious than individuals carrying only a few bacteria, determination of carriage density is likely to become an important end-point in future meningococcal vaccine trials. Strips were stored for up to six months at room temperature before testing and experience with malaria filter paper blood spots suggests that samples could be held for up to six years without loss of DNA (19), especially when short DNA fragments are targeted. The simplicity of the filter paper technique could make this a useful approach when carriage surveys are needed in areas with few facilities or when there is a need to define the groups with the highest bacterial densities; this warrants further investigation.

The increased yield of *Nm* detected using short term culture in THB needs confirmation, but suggests that meningococcal carriage studies to date have considerably underestimated the true level of pharyngeal carriage of *Nm*. Using this new technique, the size and cost of

Journal of Clinical Microbiology

carriage studies, for example those required to evaluate the impact of new meningococcal vaccines, that are being developed could be reduced.

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Conflict of interest statement

CLT reports receiving a consulting payment from GSK in 2013;

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Journal of Clinical Microbiology

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365 **Tables**

366 **Table 1.** Comparative efficacy of Todd-Hewitt and Mueller-Hinton broths in supporting the
367 growth of *N. meningitidis* in overnight culture. PCR positivity obtained with serial dilutions
368 of a serogroup A reference strain of *N. meningitidis* is shown.

369

Average Log_{10} number of genomes copies by spectrophotometry	Average Todd-Hewitt broth *porA* Ct	Average Mueller-Hinton broth *porA* Ct
7.47	11.28	14.38
6.48	13.83	16.73
5.57	18.08	22.45
4.41	20.10	24.03
3.57	16.38	21.02
2.71	20.60	25.85
1.85	25.09	29.51
0.90	27.95	33.33
Approximately 1 copy	35.21	Undetermined
No copy	Undetermined	Undetermined

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373 **Figure 1.** Summary of the methodology employed in the study.

374 **Figure 2**. Comparison of *N. meningitidis* positivity using *sodC* and *porA* and genogroup
375 characterization by real-time PCR on isolates after conventional microbiology versus Todd-
376 Hewitt Broth overnight culture.

377 **Figure 3**: Bacterial density distribution measured by copies of a fragment of the *porA* DNA
378 gene.

379 **Figure 4:** Venn diagram representing the positive swabs collected and tested by the three
380 methods (i) conventional microbiology and PCR after DNA extraction (ii) collection on filter
381 paper and direct PCR after DNA extraction (iii) Todd-Hewitt Broth overnight culture and
382 direct PCR after DNA extraction.

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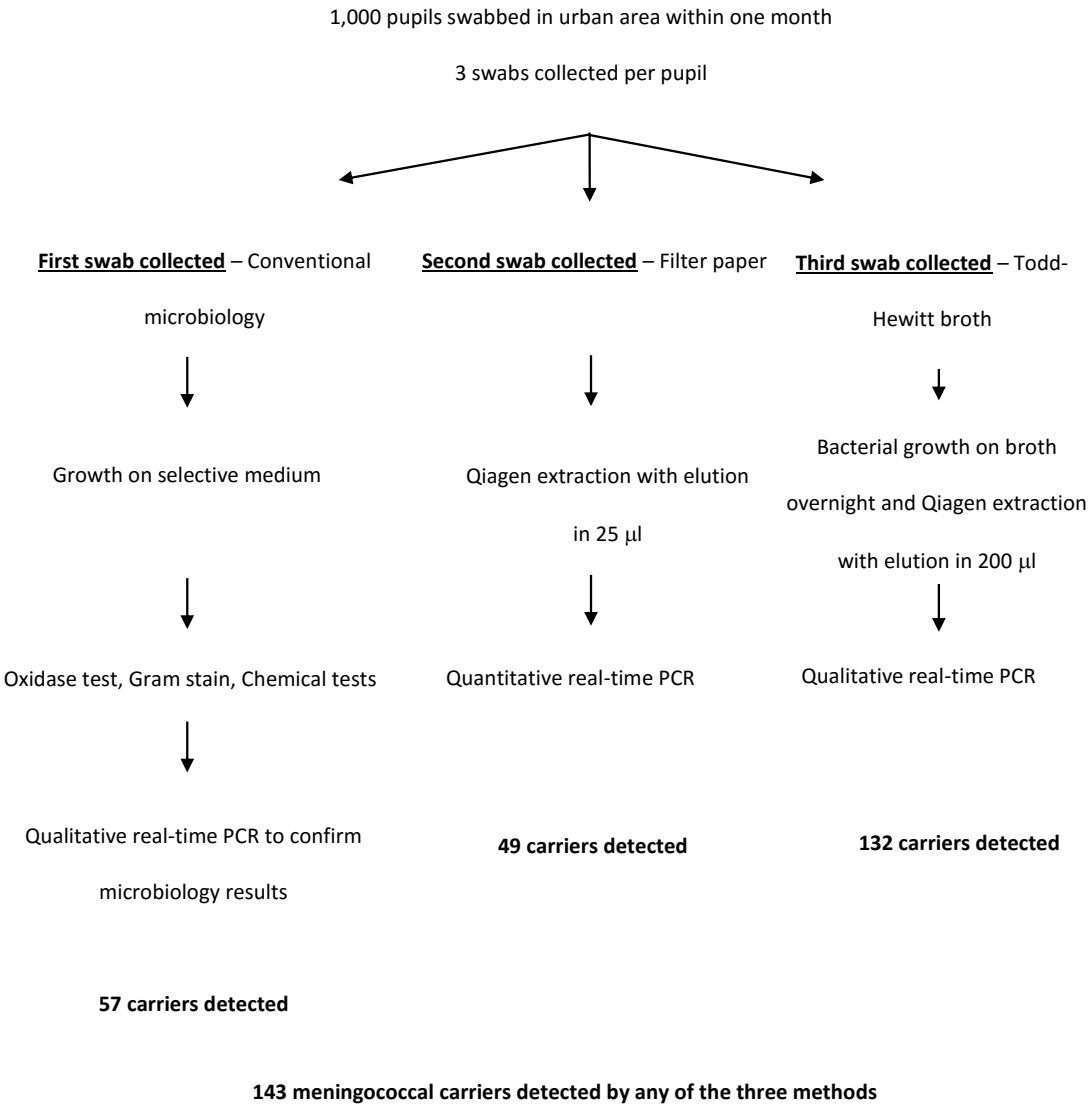

Figure 1. Summary of the methodology employed in the study.

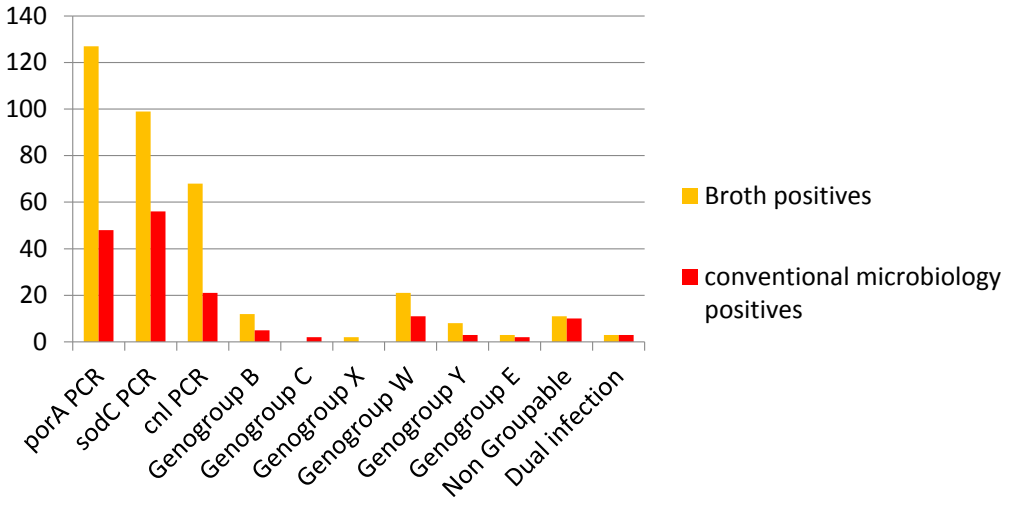

Figure 2. Comparison of *N. meningitidis* positivity using *sodC* and *porA* and genogroup characterization by real-time PCR on isolates after conventional microbiology versus Todd-Hewitt Broth overnight culture.

Journal of Clinical Microbiology

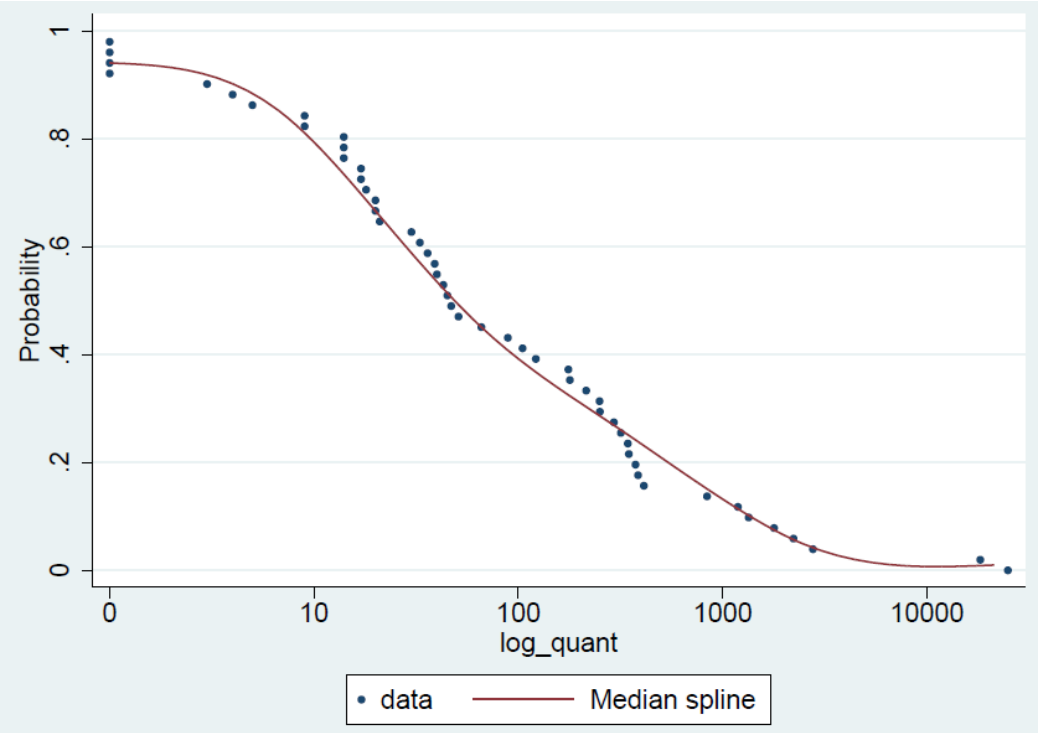

Figure 3: Bacterial density distribution measured by copies of a fragment of the *porA* DNA gene.

Journal of Clinical Microbiology

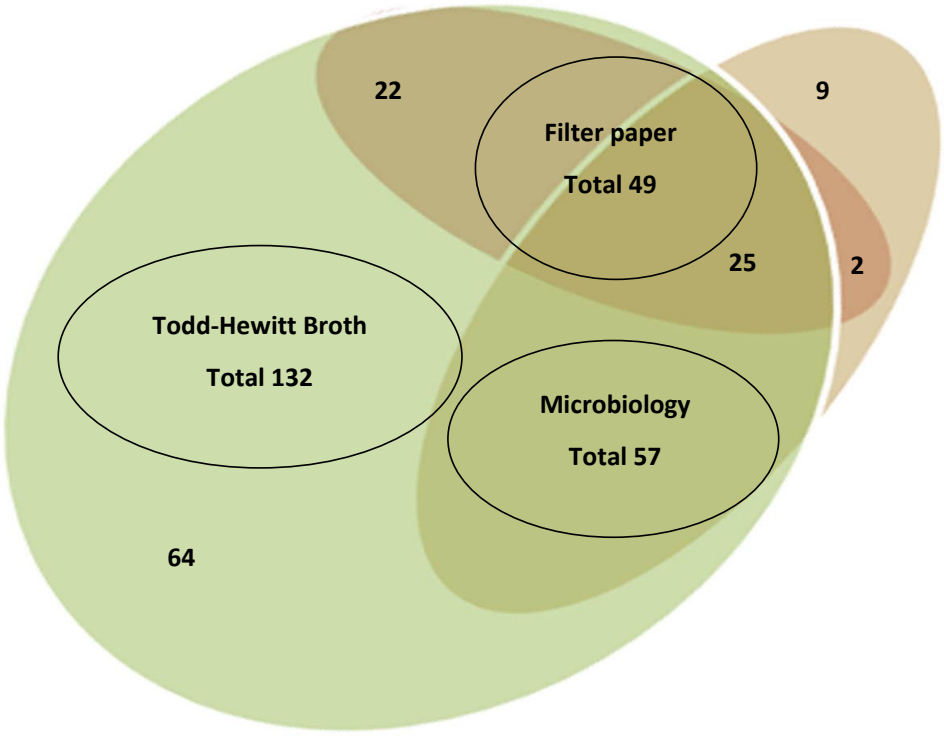

Figure 4: Venn diagram representing the positive swabs collected and tested by the three methods (i) conventional microbiology and PCR after DNA extraction (ii) filter papers direct PCR after DNA extraction (iii) Todd-Hewitt Broth overnight culture direct PCR after DNA extraction.