Performance of the Biomark HD real-time qPCR System (Fluidigm) for the detection of nasopharyngeal bacterial pathogens and *Streptococcus pneumoniae* typing

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Traditional qPCR assays for pneumococcal detection and serotype characterization require large sample volume, is expensive and labor intensive. We aimed to develop a quantitative nanofluidic Fluidigm assay to overcome some of these shortcomings. A quantitative Fluidigm assay was established to detect 11 bacterial pathogens, 55 pneumococcal serotypes and 6 serotypes of *H. influenzae*. The Fluidigm assay results were compared to conventional qPCR and culture. All reactions in the Fluidigm assay effectively amplified their respective targets with high sensitivity and specificity compared to qPCR. There was excellent concordance between qPCR and Fluidigm for detection of carriage prevalence (kappa > 0.75) and density (Rho > 0.95). Fluidigm identified an additional 7 (4.2%) serotypes over those detected by qPCR. There was a modest concordance between culture and Fluidigm for the majority of reactions detecting *S. pneumoniae* serotypes/serogroups (kappa > 0.6), with Fluidigm identifying an additional 113 (39.1%) serotypes. Discordant results between the three methods were associated with a low carriage density. The Fluidigm assay was able to detect common pneumococcal serotypes, *H. influenzae* serotypes, and other common nasopharyngeal bacterial organisms simultaneously. Deployment of this assay in epidemiological studies could provide better insight into the effect of PCV immunization on the nasopharyngeal microbiota in the community.

The human nasopharynx is a common ecological niche for several respiratory pathogens including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *Haemophilus influenzae*.¹ There is a paucity of data and conflicting findings on the interactions between these bacteria in the human nasopharynx²–⁶. Furthermore, most nasopharyngeal colonization studies have used non-quantitate culture based methods, with a lack of quantitative data available to assist in understanding the dynamics of interaction of these bacteria in the nasopharynx⁷–⁹. Recently, molecular quantitative PCR-based methods have been developed, which has assisted our understanding of bacterial nasopharyngeal colonization and its relationship to disease⁹–¹². Serotyping of pneumococci by qPCR, however, has several shortcomings including large sample volumes needed to distribute across all reactions and it being labor intensive⁹,¹⁰.

Fluidigm is a nanofluidic automated real-time PCR system that relies on microfluidic technology in which dynamic arrays of integrated fluidic circuits (IFCs) are used. These IFC’s contain thousands of controlled valves and interconnected channels in which molecules of biological samples and reagents can be automatically mixed in a variety of patterns. The instrument uses an array of non-fluidic chips called dynamic arrays for qPCR, in which a typical chip format allows for 9,216 PCR reactions (96.96 chip format; 96 samples × 96 assays) in a single qPCR run (www.fluidigm.co.za)¹³. Other advantages of Fluidigm over standard qPCR include a larger number of reactions per plate, making it more cost-effective and less time-consuming. Further, IFCs not only reduce

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the reaction volume from 10 µL–20 µL down to 10 nL scale, but the technology allows for validations as well as increased throughput of qPCR reactions.

We aimed to develop a novel nanofluidic real-time PCR (Fluidigm) assay that simultaneously detected and quantified 11 bacterial pathogens in 96 different samples (92 samples and 4 controls) in a single run. The assay included serotyping for 55 pneumococcal serotypes (16 individual serotypes and 39 serotypes in 13 groups) and six *H. influenzae* (serotypes a-f) serotypes. We compared the results of the Fluidigm assay to that of traditional qPCR for detection of bacterial colonization and pneumococcal serotyping in a cohort of PCV-vaccinated African children. Our previous work had compared qPCR to standard culture methods for 46 pneumococcal serotypes (14 individual serotypes and 32 serotypes among 10 serogroups) and 6 bacterial pathogens in a different cohort of children. In these analyses, we expand on the latter by comparing the results from the Fluidigm assay to culture and qPCR.

Results

From the initial 407 nasopharyngeal swab samples collected at the two study visits, 335 (82.3%) were available for subsequent molecular nanofluidic qPCR (Fluidigm) analysis (Supplementary Fig. 1). Of these available samples, 83.4% were Black-African and 49.1% were male. Data from the two study visits were combined for the main analysis as findings from both 9 and 16 months of age were similar.

All reactions were effective in amplifying their respective targets with high sensitivity and specificity in the Biomark HD system (Fluidigm), with the efficiency of the reactions ranging from 89% to 105% (Supplementary Table 1). Within the linear dynamic range, the correlation coefficients ($r^2$) of the reactions were 0.99, except for reactions detecting *H. influenzae* E, IS481 (*B. pertussis* and *B. holmesii*) and *S. pneumoniae* serotype 19A, were $r^2$ was 0.98 (Supplementary Table 1). The limit of detection (LLD) for most of the reactions were equivalent ($>10$ copies, with exception to primer/probes that detected *H. influenzae* serotype E, IS481 (*B. pertussis* and *B. holmesii*) and pneumococcal serotypes/serogroups 5, 6A/B/C/D, 6C/D, 13 and 21, in which the LLD was 10 fold less ($>100$ copies). Further, no cross-reactivity occurred, with all primer and probes being specific for their respective target. Lastly, for all respective reactions, the intra- and inter-assay variation was $<0.1$, while the accuracy was within $\pm 0.1$ (Supplementary Table 1).

Detection of bacterial nasopharyngeal carriage by qPCR and Fluidigm. There was excellent concordance between qPCR and Fluidigm for the detection of *S. pneumoniae* ($\kappa = 0.98$), *H. influenzae* ($\kappa = 0.97$), *M. catarrhalis* ($\kappa = 0.98$), *S. aureus* ($\kappa = 0.98$) and *S. pyogenes* ($\kappa = 0.96$); and a high concordance between qPCR and Fluidigm for the detection of *N. meningitidis* ($\kappa = 0.75$; Fig. 1, Table 1). All additional bacteria detected by either qPCR or Fluidigm had estimated copy numbers of $<10^6$ colony forming units (CFU)/ml per swab (Fig. 2) and thus discordance between the methods was strongly associated with the density of carriage. The sensitivity and specificity of the Fluidigm method compared to conventional qPCR were high with all reactions having a sensitivity $>92$% and a specificity of $>99$% (Table 1).

| Bacterial Target | qPCR | Fluidigm | P-value | Concordance (kappa) | Sensitivity (%) | Specificity (%) |
|------------------|------|----------|---------|---------------------|----------------|----------------|
| *S. pneumoniae*  | 244(72.8) | 246(73.4) | 0.16 | 0.98 | 98.8 | 100 |
| *H. influenzae*  | 230(68.7) | 232(69.3) | 0.32 | 0.97 | 98.7 | 99 |
| *M. catarrhalis* | 189(56.4) | 192(57.3) | 0.08 | 0.98 | 98.4 | 100 |
| *S. aureus*      | 57(17) | 55(16.4) | 0.16 | 0.98 | 100 | 99.3 |
| *S. pyogenes*    | 11(3.3) | 12(3.6) | 0.32 | 0.96 | 91.7 | 100 |
| *N. meningitidis*| 3(0.9) | 5(1.5) | 0.16 | 0.75 | 91.7 | 100 |

Table 1. Concordance between qPCR and Fluidigm for the detection of common nasopharyngeal pathogens in PCV-vaccinated, HIV-uninfected children.
Detection of bacterial nasopharyngeal carriage by culture and Fluidigm. There was excellent concordance between culture and Fluidigm assay for detection of *S. aureus* (Kappa = 0.86), and a modest concordance for detection of *H. influenzae* (Kappa = 0.63) and *S. pneumoniae* (Kappa = 0.59). The Fluidigm assay was more sensitive than culture in detecting *S. pneumoniae* (73.4% vs. 60%; p < 0.001) and *H. influenzae* (69.3% vs. 54.6%, p < 0.001; Fig. 1, Supplementary Table 2). Discordance for bacterial detection between culture and Fluidigm methods were associated with carriage density, with the majority of Fluidigm-positive but culture-negative samples being <10^4 CFU/ml per swab, and the majority of culture-positive but Fluidigm-negative samples reported as “scant” growth (<5 colonies/plate) on culture.

Detection of pneumococcal serotype carriage by qPCR and Fluidigm. There was excellent concordance between qPCR and Fluidigm assays, with the majority of reactions detecting *S. pneumoniae* serotypes/serogroups (Kappa > 0.8) by both methods, with exception to serotype 1, for which the concordance was still high (Kappa = 0.75; Fig. 3, Table 2). Fluidigm was more sensitive in detecting serotypes/groups 1, 4, 10A, 18A/B/C, and 19B/F; while qPCR was more sensitive in detecting serotype 7C. In addition, the Fluidigm assay identified 7 (4.2%) additional serotypes compared to those identified by qPCR; and conversely, the qPCR method detected 2 (2.1%) additional serotypes not detected by Fluidigm. Discordant results between the methods were strongly associated with the density of carriage, with all additional pneumococcal serotypes/groups detected either by qPCR or Fluidigm having estimated copy numbers of <10^3 CFU/ml per swab (Fig. 4). The sensitivity and specificity of the Fluidigm compared to qPCR assay were high; i.e. sensitivity >80% and specificity >99.3%; Table 2.

Serotypes/serogroups 7A/F, 8, 22A/F, 25AF/38 and 35B were only tested by Fluidigm, with an overall detection prevalence of 0.3%, 0.9%, 0.6%, 2.7% and 0.3%, respectively.

Detection of pneumococcal serotype carriage by culture and Fluidigm. Generally, there was high concordance between culture and the Fluidigm assay for detecting *S. pneumoniae* serotypes/serogroups. Culture identified 21 (10.9%) serotypes/serogroups not detected by the Fluidigm assay, and conversely the Fluidigm assay identifying as additional 113 (39.1%) serotypes not identified by culture methods (Fig. 3, Supplementary Table 3). This included higher detection prevalence by the Fluidigm assay for serotypes/serogroups 1 (1.5% vs. 1%; p = 0.03), 5 (8.9% vs. 0%; p = 0.003), 16 (2.7% vs. 1.5%, p = 0.05), 18A/B/C (1.8% vs. 0.9%, p = 0.05), 19B/F (14.9% vs. 11%, p = 0.002), 23F (4.5% vs. 2.7%, p = 0.03), and 25A/25F/38 (3.6% vs. 0%, p = 0.005). The additional serotypes detected only by Fluidigm mostly had densities <10^4 CFU/ml per swab. Furthermore, the majority of
the additional serotypes detected by Fluidigm were carried concurrently with other pneumococcal serotypes, of 89% were a non-primary colonizing serotype (Supplementary Table 4). The sensitivity of the Fluidigm assay compared to culture were high for the majority of reactions; mainly having sensitivity >80%, with the exception of serotype 10A (60%) and serogroup 23A/B (71%). All Fluidigm reactions had high specificity (>95%) compared to culture (Supplementary Table 3).

Density of bacterial nasopharyngeal carriage as determined by qPCR and Fluidigm. There was an excellent correlation (Rho > 0.95) between qPCR and Fluidigm for measuring the density of colonization (Figs 2 and 4), with no significant differences observed between the two methods for all bacterial pathogens and pneumococcal serotypes (Table 3).

Discussion
A reliable molecular nanofluidic real-time PCR (Fluidigm) system was established to simultaneously detect 11 bacterial pathogens, 55 pneumococcal serotypes (16 individual serotypes and 39 serotypes in 13 groups) and 6 serotypes of H. influenzae (serotypes a-f) in a single run14. There was high concordance between qPCR and Fluidigm, including for identifying bacterial colonization, density of bacterial targets and pneumococcal

| Serotype | qPCR | Fluidigm | P-value | Concordance (kappa) | Sensitivity (%) | Specificity (%) |
|----------|------|----------|---------|---------------------|----------------|----------------|
| LytA     | 244(72.8) | 246(73.4) | 0.16    | 0.98               | 98.8           | 100            |
| 1        | 3(0.9)   | 5(1.5)   | 0.16    | 0.75               | 95             | 100            |
| 3        | 1(0.3)   | 1(0.3)   | —       | 1                   | 100            | 100            |
| 4        | 3(0.9)   | 4(1.2)   | 0.32    | 0.86               | 80             | 100            |
| 5        | 13(3.9)  | 13(3.9)  | —       | 1                   | 100            | 100            |
| 6A/B     | 31 (9.3) | 31 (9.3) | —       | 1                   | 100            | 100            |
| 6C/D     | 2(0.6)   | 2(0.6)   | —       | 1                   | 100            | 100            |
| 7C       | 8(2.4)   | 6(1.8)   | 0.16    | 0.85               | 100            | 99.3           |
| 9A/L/N/V | 8(2.4)   | 8(2.4)   | —       | 1                   | 100            | 100            |
| 10A      | 5(1.5)   | 6(1.8)   | 0.32    | 0.91               | 83.3           | 100            |
| 11A/B/C/D/F | 9(2.7) | 9(2.7)   | —       | 1                   | 100            | 100            |
| 12ABF/44/46 | 5(1.5) | 5(1.5)   | —       | 1                   | 100            | 100            |
| 13       | 6(1.8)   | 6(1.8)   | —       | 1                   | 100            | 100            |
| 14       | 7(2.1)   | 7(2.1)   | —       | 1                   | 100            | 100            |
| 15A/B/C/F | 14(4.2) | 14(4.2)  | —       | 1                   | 100            | 100            |
| 16F      | 9(2.7)   | 9(2.7)   | —       | 1                   | 100            | 100            |
| 17F      | 6(1.8)   | 6(1.8)   | —       | 1                   | 100            | 100            |
| 18A/B/C   | 6(1.8)   | 7(2.1)   | 0.32    | 0.93               | 85.7           | 100            |
| 19A      | 36(10.7) | 36(10.7) | —       | 1                   | 100            | 100            |
| 19B/F    | 48(14.3) | 50(14.9) | 0.08    | 0.96               | 94             | 100            |
| 20       | 1(0.3)   | 1(0.3)   | —       | 1                   | 100            | 100            |
| 21       | 5(1.5)   | 5(1.5)   | —       | 1                   | 100            | 100            |
| 23A/B    | 8(2.4)   | 8(2.4)   | —       | 1                   | 100            | 100            |
| 23F      | 15(4.5)  | 15(4.5)  | —       | 1                   | 100            | 100            |
| 34/17/17F | 13(3.9) | 13(3.9)  | —       | 1                   | 100            | 100            |

Table 2. Concordance between qPCR and Fluidigm for the detection of pneumococcal serotypes in PCV-vaccinated, HIV-uninfected children.

Figure 4. Density of colonization of pneumococcal serotypes as determined by qPCR and Fluidigm.
serotypes, with discordant results mainly associated with samples in which the carriage density was low (<10^2 CFU/ml per swab).

Discordance between the methods included the qPCR method detecting an additional 3 bacterial species and 2 pneumococcal serotypes above those detected by Fluidigm, and conversely the Fluidigm assay detecting an additional 11 bacterial species and 7 pneumococcal serotypes above those detected by qPCR. Although most of these differences could not be confirmed by culture, due to the additional pneumococcal serotypes detected being non-primary (second or third colonizing serotypes; Supplementary Fig. 3) colonizers, one additional serogroup 19B/F isolate detected by Fluidigm and one additional serotype 7C isolate detected by qPCR were concordant with culture results. These small differences (<5%) in the detection of targets between qPCR and Fluidigm methods are, however, expected based on Poisson's distribution that the assays had a 95% probability of detecting their respective targets with reasonable certainty14. This was further supported by the discordant readouts between the methods mainly being associated with samples where the density of carriage was very low (<10^2 CFU/ml).

Furthermore, the higher yield on the Fluidigm assay could be due to a pre-amplification step that selected for targets in this assay (and not the qPCR assay).

While culture remains the referent standard for bacterial carriage and pneumococcal serotype characterization studies, these assays lack quantitative data which impose limitations on our understanding of the association between nasopharyngeal carriage and its relation to disease15–19. Recently, several molecular qPCR methods have been developed to overcome the above shortcoming of conventional culture assays. Nevertheless, most molecular methods are still limited by the large sample volume required to distribute across all reactions, cost (approximately $ 1.85 per sample per reaction in duplex) and processing multiple qPCR reactions being labor intensive and time-consuming20. Further, there is a limited number of quantitative studies that have concurrently described colonization of a diverse number of bacterial pathogens and pneumococcal serotypes.

Our Fluidigm assay addressed some of the challenges posed by qPCR assays, including analyzing a larger number of reactions per plate making the assay more cost-effective (approximately $ 0.37 per sample per reaction), less time-consuming and less labor intensive (9216 reactions per one Fluidigm plate versus 96 reactions per...
one qPCR plate). Further, IFCs not only reduces the reaction volume from 10 µL–20 µL down to 10 nL scale, but the technology allows for validations as well as increased parallelism and throughput of qPCR reactions13.

Notably, the Fluidigm assay was more sensitive in detecting serotype 5 (3.9% vs. 0%) and serogroup 25AF/38 (3.6% vs. 0%) compared to culture (Fig. 3). Half of the isolates typed as serogroup 22AF/38 were not identified on culture, while the other half were commonly found as non-primary colonizers (Supplementary Fig. 2), which might explain them not being identified by culture methods. The majority of serotype 5 isolates (12/13; 92%) were commonly isolated as non-primary serotypes, raising the possibility of false positive readouts from the Fluidigm assay. These results were consistent with findings when we analyzed using the qPCR compared to culture assay10. This is of particular importance for serotype 5 since the primers used also detect S. mitis in Silico. Nevertheless, we recommend that results from serotype 5 and serogroup 22AF/38 are interpreted with caution and further investigation is warranted.

The Biomark HD system in a new instrument that has only been used to serotype pneumococcus by one other group; however, their assay differed in that they used Evagreen (a dsDNA-binding dye) chemistry instead of TaqMan probes to quantify bacterial carriage. Further, the study by Dhoubhadel et al. was designed to only detect 50 pneumococcal serotypes (17 individual serotypes and 33 serotypes in 12 groups) and did not include any additional bacterial pathogens10,11. The performance of our assay was, however, comparable to that described by Dhoubhadel et al.

Limitations of our study indicate that Fluidigm assay was unable to discriminate between all pneumococcal serotypes within some serogroups. Also, although the Fluidigm assay included additional pneumococcal serotypes and other bacterial targets compared to those investigated for by qPCR10,11, we did not test for all known pneumococcal serotypes. This should be considered for future studies. Further, the LytA gene was chosen to detect overall pneumococcal colonization; however, recent studies reported other isolates within the S. mitis group also harbor LytA22. No serotype was discernible for a small percentage (7.9%) of LytA positive samples detected by Fluidigm in our study, suggesting they were either non-typable or belonged to one of the serotypes not included in our assay. Alternatively, these could have been LytA positive non-pneumococcal species that were being identified. Future studies on pneumococcal colonization using qPCR or Fluidigm should consider including an alternative gene such as Xiscos, which is purportedly only present in Streptococcus pneumoniae23. As an initial step for gene expression on the Biomark HD Fluidigm system manufacturers recommends Specific target amplification (STA) for each sample to be done by combining all primers in a pool. Due to the high similarity between some pneumococcal primers, non-specific binding of primers to each other resulted (results not shown) in cross-specificity between some of the pneumococcal serotyping targets. This was addressed by separating primers into two separate pools (Supplementary Table 5), and then combing the STA products from the two assays. Careful monitoring and optimization of the Fluidigm assay are thus needed in future studies, especially if the assay is to be expanded with additional serotypes.

In conclusion, we established a Fluidigm assay that was highly sensitive and specific. Using the Fluidigm assay enabled simultaneous detection of nasopharyngeal colonization by common pneumococcal serotypes, H. influenzae serotypes, and other common nasopharyngeal bacterial pathogens; and provided quantitative data in a single run.

Material and Methods

Study population. Archived nasopharyngeal swab samples collected from a PCV7-vaccinated cohort of HIV-uninfected infants in Soweto, South Africa were retrospectively analyzed. The study cohort has been previously described24,25. Briefly, infants enrolled between April 2005 and June 2006 were 6–12 weeks old at enrolment and included participants who were both HIV-exposed-uninfected (HEU) and HIV-unexposed. These infants were scheduled to receive three doses of PCV7 (i.e. Prevnar®; Wyeth Vaccines, NJ, USA) at 6, 10 and 14 weeks of age25,26. PCV immunization was introduced into the public EPI program in May 2009 and thus, during the course of the study, PCV immunization of children in Soweto (birth cohort 28,000 per annum) was limited mainly to study-participants (approximately 600)27. As described, standard culture methods were used to culture samples for S. pneumoniae and the Quellung method was undertaken for serotyping28.

Nasopharyngeal (NP) swabs were collected at several time intervals, including at 9 and 16 months of age. Swabs were stored in skim milk-tryptone-glucose-glycerol (STGG) transport media at the Respiratory and Meningeal Pathogen Research Unit (RMPRU) in South Africa29. Samples were screened previously for S. pneumoniae, H. influenzae, S. aureus, M. catarrhalis, S pyogenes, and N meningitides by qPCR30, and pneumococcal serotyping was done by qPCR on all samples that tested positive for S. pneumoniae as described26. In this study, we now developed and evaluated a Fluidigm assay which screened for an additional 9 serotypes/serogroups (7A/F, 8, 22A/F, 25AF/38 and 35B) not included in our earlier qPCR assay, as well as investigated for presence of additional bacteria (Bordetella bronchiseptica, Bordetella parapertussis, Bordetella pertussis, Bordetella holmesii, and Neisseria lactamica). The Fluidigm assay was also designed to serotype H. influenzae. The additional serotypes included in the Fluidigm assay were chosen based on the most frequently isolated non-vaccine serotypes associated with colonization in South Africa as detected by culture methods, at the time of study design.

Bacterial and pneumococcal reference isolates. Control strains for the pneumococcal serotypes (1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 7A, 7F, 7C, 8, 9A, 9L, 9N, 9V, 10A, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 19A, 19B, 19F, 20, 21, 22A, 22F, 23A, 23B, 23F, 25A, 25F, 34, 35B and 37), H. influenzae serotypes (serotypes a-f), S. aureus, M. catarrhalis, N. meningitidis and S. pyogenes were obtained from the National Institute for Communicable Diseases (NICD). Additional isolates for B. bronchiseptica (ATCC® 4617), B. parapertussis (ATCC® 15311), B. pertussis (ATCC® 2397) and N. lactamica (ATCC® 23970) were purchased from Davies Diagnostics (South Africa), while an isolate for B. holmesii (ATCC® 51541)
was purchased from LGC standards South Africa. DNA from these strains were used to optimize the Fluidigm reactions and as positive controls.

**DNA extraction.** Total nucleic acid was automatically extracted from nasopharyngeal swab samples stored in STGG, using the NucliSens® easyMAG® extraction system (BioMérieux, Marcy l’Étoile, France), according to manufactures instructions. Similarly, total nucleic acid was also extracted from pneumococcal reference strains (positive control strains) grown in Todd-Hewitt broth supplemented with 5% yeast. *S. aureus*, *M. catarrhalis*, *S. pyogenes*, *N. meningitidis*, and *N. lactamica* reference strains were grown in Todd-Hewitt broth alone. *H. influenzae* serotypes a-f were grown in Brain-Heart infusion (BHI) broth and Bordetella species were grown in potato broth. Extracted DNA from samples and reference strains were stored at −20°C.

**Real-time qPCR multiplex assay.** Target DNA was previously pre-screened for *S. pneumoniae*, *M. catarrhalis*, *H. influenzae*, *S. aureus*, *N. meningitidis* and *S. pyogenes* as described10,11. All LytA positive samples (Cq < 35) were further molecularly serotyped for PCV7 serotypes/groups (4, 6A/B, 9A/L/N/V, 14, 18A/B/C, 19B/F and 23F) and non-vaccine serotypes/groups (1, 3, 4, 5, 6C/D, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/D/F, 16F, 17F, 19A, 20, 21, 23A/B and 34/37/17A) as described10.

**Fluidigm assay.** ABI primer express software package, version 3.0 (Applied Biosystems, ABI, Foster City, USA) was used to design oligonucleotide primers and FAM dye-labelled MGB probes for additional targets not included in the multiplex qPCR assay. Primer and probe sequences used in the Fluidigm assay are described in Table 4. GAPDH and BexA were included to confirm the efficiency of the DNA extraction and to confirm the presence of non-typable *H. influenzae*, respectively. The Fluidigm method was unable to discriminate between some pneumococcal serotypes within a particular serogroup due to the genotypic similarities between the capsular loci of certain serotypes (including serotypes: 6A/B, 6C/D, 7A/F 9A/L/N/V, 11A/B/C/D/F, 12A/B/F, 44/46, 15A/B/C/F, 18A/B/C, 19B/F, 22A/F, 23A/B, 25A/25F/38 and 34/37/17A); however, the method was able to identify serotypes 1, 3, 4, 5, 7C, 8, 10A, 13, 14, 16F, 17F, 19A, 20, 21, 23A/B and 34/37/17A) as described10.

**Pre-amplification of DNA for Fluidigm.** Specific target amplification (STA) was done per manufactures recommendations as the initial step (pre-amplification of DNA) for the Biomark HD system (Fluidigm). Briefly, two separate pools containing 24X TaqMan assays in each pool as described in Supplementary Table 5, were prepared by mixing forward and reverse primers and diluted with 10 mM Tris/HCl and 0.1 mM EDTA (pH 8) to give a final concentration of 200 nM of each primer. PCR reactions were carried out for each STA pool in 5 μl volumes each containing 2.5 μl TaqMan PreAmp Master Mix (Fluidigm, CA, USA), 1.25 μl of pooled assay and 1.25 μl of DNA. Reactions were amplified with the T100 Thermal Cycler (Bio-Rad Laboratories, CA, USA) was used to design oligonucleotide primers and FAM dye-labelled MGB probes for additional targets not included in the multiplex qPCR assay. Primer and probe sequences used in the Fluidigm assay are described in Table 4. GAPDH and BexA were included to confirm the efficiency of the DNA extraction and to confirm the presence of non-typable *H. influenzae*, respectively. The Fluidigm method was unable to discriminate between some pneumococcal serotypes within a particular serogroup due to the genotypic similarities between the capsular loci of certain serotypes (including serotypes: 6A/B, 6C/D, 7A/F 9A/L/N/V, 11A/B/C/D/F, 12A/B/F, 44/46, 15A/B/C/F, 18A/B/C, 19B/F, 22A/F, 23A/B, 25A/25F/38 and 34/37/17A); however, the method was able to identify serotypes 1, 3, 4, 5, 7C, 8, 10A, 13, 14, 16F, 17F, 19A, 20, 21, 23A/B and 34/37/17A) as described10.

**Real-time qPCR using the Biomark HD System (Fluidigm).** Fluidigm was carried out with 96.96 dynamic arrays (Fluidigm Corporation, CA, USA) according to manufactures instructions. 10X assay mixture and sample pre-mix were prepared, with each assay being prepared in duplicate (making up a total of 96 assays). Briefly, each 10X assays contained 2.5 ul 20X TaqMan gene expression assay (Fluidigm), prepared by mixing forward primer, reverse primers and probe, and 2.5 ul 2X Assay Loading Reagent (Fluidigm). The final concentration (at 10X) for each primer was 9 μM and 2 μM for the probe. Sample pre-mix was made by combining 2.5 ul TaqMan Universal PCR Master Mix (2X (Life Technologies, 0.25 ul 20X GE Sample Loading Reagent (Fluidigm PN 100–7610)) and 2.5 μl pre-amplified cDNA (diluted STA product containing a negligible concentration of carried-over STA primers) for each of the 96 sample inlets. IFC controller (Fluidigm) were used to prime 96.96 dynamic arrays IFC Chip (Fluidigm) with control line fluid. 5 μl of each assay and sample mix was then transferred into the appropriate inlets of the primed chip and loaded with the IFC controller. After loading, the chip was placed in the Biomark instrument for Fluidigm screening at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and annealing/extension at 60°C for 4 minutes). STA products from the two PCR reactions were combined and diluted 1:5 with 10 mM Tris/HCl and 0.1 mM EDTA (pH 8).

**Real-time qPCR methods.** Statistical analysis was performed with STATA Version 11.0 (Statacorp, Texas, USA). McNemar’s test was used to determine the sensitivities of culture/qPCR and Fluidigm. When analyzed for concordance, using kappa statistics, only common serogroups/types tested by both methods were included. Findings for non-typable (NT) pneumococci were not compared between culture and qPCR/Fluidigm, as the qPCR and Fluidigm methods were not optimized to detect all pneumococcal serotypes and thus some untested serotypes might be misidentified as NT pneumococcus. Paired student t-tests were used to compare the mean bacterial concentrations detected by qPCR and Fluidigm, following log_{10} transformation of data. Results were considered significant when the p-value was ≤ 0.05.

**Ethics approval.** Ethical consent was obtained from the Medical Human Research Ethics Committee (HREC) of the University of Witwatersrand for the initial enrolment of the cohort [Vaccinated cohort: HREC:
| Target Name                        | Primer/Probe Name | Forward Primer | Reverse Primer | Probe | Reference |
|-----------------------------------|-------------------|---------------|---------------|-------|-----------|
| Streptococcus pneumoniae          | 1                 | GTAATTGTATGTTGTTATCCT | TCGTGCAGCAGTGA | AGACGCACTGGAACCCCTCTTTAAGTAGGG | Maaroufi et al. 2019 |
| Haemophilus influenzae A          | 2                 | GCACTGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Haemophilus influenzae B          | 3                 | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Haemophilus influenzae C          | 4                 | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Haemophilus influenzae D          | 5                 | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Haemophilus influenzae E          | 6                 | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Moraxella catarrhalis             | 7                 | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Neisseria meningitidis            | 8                 | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Neisseria lactamica               | 9                 | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Bordetella pertussis and Bordetella holmesi | 10                | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Bordetella holmesi                | 11                | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Bordetella pertussis              | 12                | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Bordetella bronchi septica        | 13                | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serum type 1 | 14               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serum type 2 | 15               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serum type 3 | 16               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serum type 4 | 17               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serogroup 6A/B/C/D | 18               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serogroup 6C/D | 19               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serogroup 7A/F | 20               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serogroup 7C | 21               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serogroup 8 | 22               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serogroup 9A/L/N/V | 23               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Streptococcus pneumoniae serogroup 10A | 24               | GCCATGCTGACTTCATCTTACCAAA | TTCTCCTCCTATGCCATCAGAC | CTCATTCATGGAAGGAGGAGAAGGAGG | Maaroufi et al. 2019 |
| Continued                        |                  |               |               |       |           |
Table 4. Oligonucleotide primers and probe sequences for quantitative molecular Fluidigm detection of respiratory pathogen. Probe*: Minor Grove Binding (MGB) FAM labelled TaqMan probes.

| Target Name | Primer/Probe Name | Forward Primer | Reverse Primer | Probe | Reference |
|-------------|-------------------|----------------|----------------|-------|-----------|
| Streptococcus pneumoniae serogroup 12A/B/F/44/46 | 12A/12B/12F/44/46 | GATTTATCGGTGGCC TCTTCCTG | ATAGGGGAATAGCTTT CACGAA | AATTTGTAACGGGACGTGC | Azzari et al. 35 |
| Streptococcus pneumoniae serogroup 34/37/17A | 34/37/17A | GAGCATTGAAATGCTA GAGGAAAGAT | ATACGAGCTCATCA TACCTGACATCCT | TGTCATCTGGATGCA ACTTGATGGTTC | Azzari et al. 35 |
| Streptococcus pneumoniae serogroup 15A/B/C/F | 15A/B/C/F | TCTAATCTGGATGATGATT TATTTGCT | CTTACTACTCTAATGCA AATGATGATGATGGA | TCTCGGGTTTTGCTCTCGT | Azzari et al. 35 |
| Streptococcus pneumoniae serogroup 17F | 17F | GTAAAGATTTCATGTCCT | AAAATGTAACCTCTGGTGAGG | This Study |
| Streptococcus pneumoniae serogroup 25A/25F/38 | 25A/25F/38 | GTGGCATTGACCTGATG | GAGGTGCGATCGA TAGGAG | This Study |

0407'04, also registered under Clinical trials number NCT00099658] and all methods were performed in accordance with the relevant guidelines and regulations. The HREC approved further testing of samples (M140907) in this study. At the time of the initial enrolment, written, informed consent had been obtained from the parents/guardians of all the study participants.

Data Availability
The data that support the findings of this study are available from the corresponding author upon request.

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Author Contributions

C.P.O., P.V.A. and S.A.M. conceived and designed the study. C.P.O. was responsible for data curation and analysis. Funding acquisition was undertaken by S.A.M. C.P.O. wrote the first draft of the manuscript and all authors were involved in reviewing and editing the manuscript.

Additional Information

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