**APPENDIX**

**METHODS**

**Study design**

Patan Hospital, Kathmandu, Nepal is a major teaching hospital in Nepal and is one of the largest hospitals in Nepal with approximately 320 000 outpatients seen each year. Children and their siblings presenting to the paediatric outpatient department and public vaccination clinic (located adjacent to the outpatient department) were approached for enrolment in the study. Pneumococcal conjugate vaccine coverage among 1-year-olds in Nepal was reported as being, 5% in 2015, 46% in 2016, 80% in 2017, 83% in 2018, 83% in 2019, 80% in 2020, and 84% in 2021.

During the study period three significant events occurred within this population. Firstly, between April and May 2015 there were major earthquakes which resulted in displacement of populations. Secondly, between September 2015 and February 2016, there was a fuel shortage and subsequent use of low-quality fuels resulted in poor air quality. Finally, the SARS-CoV-2 pandemic resulted in the use of public health measures to reduce transmission, as such no swabs were collected in 2020.

**DNA extraction from STGG**

The STGG was mixed in microtubes with 100 μl of pre-lysis solution consisting of; 20 mg/ml of chicken egg lysozyme (Fisher Scientific, Leicester, United Kingdom), 50 Units/ml of mutanolysin (Sigma-Aldrich, Dorset, United Kingdom), and Tris EDTA buffer (Sigma-Aldrich, Dorset, United Kingdom) to make up the remaining volume. Samples were then incubated for one hour at 37°C and then were centrifuged for 30 seconds at 3800 g to remove any condensation from the caps. 20 μl of protein kinase and 50 μl of AL buffer was then added to each sample before mixing and then centrifuging for 30 seconds at 3800 g. Samples were then incubated for one hour at 56 °C and then centrifuged for 30 seconds at 3800g. 150 μl of AL buffer was then added to each sample before incubating at room temperature for 10 minutes. 250 μl of absolute ethanol was then added to each sample before mixing and then transferring each sample to a well on a DNeasy 96 well plate. Plates were then centrifuged for ten minutes at 3800 g. 500 μl of AW1 buffer was then added to each well and the plates centrifuged for five minutes at 3800 g. 500 μl of AW2 buffer was then added to each well and the plates centrifuged for fifteen minutes at 3800 g. 100 μl of AE buffer warmed to 37 °C was then added to each well, plates incubated at room temperature for five minutes and then centrifuged for 2 minutes at 3800 g and the eluted DNA collected. A further 100 μl of AE buffer was again added to each sample well and the plates centrifuged for 2 minutes at 3800 g and the eluted DNA collected. Eluted DNA was stored at -20 °C or below.

**Appendix table 1. Serotypes covered by PCV10, PCV13, PCV15, and PCV20.**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Serotype | 4 | 6B | 9V | 14 | 18C | 19F | 23F | 1 | 5 | 7F | 3 | 6A | 19A | 22F | 33F | 8 | 10A | 11A | 12F | 15B |
| PCV10 | X | X | X | X | X | X | X | X | X | X |  |  |  |  |  |  |  |  |  |  |
| PCV13 | X | X | X | X | X | X | X | X | X | X | X | X | X |  |  |  |  |  |  |  |
| PCV15 | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X |  |  |  |  |  |
| PCV20 | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X |

**Appendix Table 2. Pneumococcal autolysin (*lytA*) primers used to assess pneumococcal density**

|  |  |
| --- | --- |
| Forward primer | 5’-acgcaatctagcagatgaagca-3’ |
| Reverse primer | 5’-tcgtgcgttttaattccagct-3’ |
| Probe | 5’-tgccgaaaacgcttgatacagggag-3’ (5' FAM; 3' MGB) |

**Appendix Table 3. Entire swab collection cohort.**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Collection year | Number of participants | Number of swabs with pneumococcus detected by conventional microbiology (%) | Male % | Age, years median (IQR) | 0 PCV doses | 0 PCV doses carriers (%) | 1 PCV dose | 1 PCV dose carriers (%) | 2 PCV doses | 2 PCV doses carriers (%) | 3 PCV doses | 3 PCV doses carriers (%) |
| 2014 | 1305 | 908 (69.6) | 56.5 | 1.19 (0.76) | 1305 | 908 (69.6) | 0 | 0 | 0 | 0 | 0 | 0 |
| 2015 | 600 | 336 (56) | 55 | 1.15 (0.57) | 600 | 336 (56) | 0 | 0 | 0 | 0 | 0 | 0 |
| 2017 | 1301 | 866 (66.6) | 54.2 | 1.2 (0.52) | 68 | 51 (75) | 53 | 38 (71.7) | 99 | 67 (67.7) | 1081 | 710 (65.7) |
| 2018 | 1303 | 797 (61.2) | 52.6 | 1.08 (0.61) | 45 | 31 (68.9) | 16 | 10 (62.5) | 70 | 44 (62.9) | 1169 | 710 (60.7) |
| 2019 | 1303 | 724 (55.6) | 56.9 | 1.08 (0.6) | 2 | 2 (100) | 8 | 5 (62.5) | 20 | 14 (70) | 1269 | 699 (55.1) |
| 2021 | 548 | 189 (34.5) | 56.8 | 1.03 (0.62) | 1 | 0 (0) | 0 | 0 (0) | 8 | 3 (37.5) | 537 | 186 (34.6) |

**Appendix Table 4. Proportion of non-PCV10 covered serotypes identified on swabs for each collection year.**

|  |  |
| --- | --- |
| Year | **NVT/All serotypes (%)** |
| 2014 | 938/1327 (70.7%) |
| 2015 | 272/392 (69.4%) |
| 2017 | 767/914 (83.9%) |
| 2018 | 707/816 (86.6%) |
| 2019 | 817/891 (91.7%) |
| 2021 | 203/212 (95.8%) |

**Appendix Table 5. Multiple serotype carriage**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 2014, N=908 | 2015, N=336 | 2017, N=743 | 2018, N=710 | 2019, N=720 | 2021, N=189 | Pre, N=1244 | Post, N=2353 | P, Pre vs post |
| No pneumococcus detected | 31 (3.4) | 14 (4.2) | 39 (5.2) | 65 (9.2) | 17 (2.4) | 11 (5.8) | 45 (3.6) | 132 (5.6) | 0.0093 |
| Single serotype | 543 (59.8) | 266 (79.2) | 509 (68.5) | 498 (70.1) | 545 (75.7) | 147 (77.8) | 809 (65) | 1699 (72.2) | <0.0001 |
| Multi-serotype | 334 (36.8) | 56 (16.7) | 186 (25) | 147 (20.7) | 158 (21.9) | 31 (16.4) | 390 (31.4) | 522 (22.2) | <0.0001 |

**Appendix Table 6. Pneumococcal carriage density by collection year and vaccine coverage**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Grouping | 2014, median log10 GE/ml (IQR) | 2015, median log10 GE/ml (IQR) | 2017, median log10 GE/ml (IQR) | 2018, median log10 GE/ml (IQR) | 2019, median log10 GE/ml (IQR) | 2021, median log10 GE/ml (IQR) | Pre-PCV10, median log10 GE/ml (IQR) | Post-PCV10, median log10 GE/ml (IQR) | Pre-PCV10 vs post-PCV10, P |
| PCV10 serotypes |  1.7 (3.2) |  3.3 (2) | 3.5 (2.6) |  2.9 (2.3) | 2.9 (1.4) |  2.5 (0.5) | 2.2 (3.3) | 3 (2.2) | p<0.0001 |
| Non-PCV10 serotypes |  1.4 (3.1) |  3.2 (2.5) |  3 (2.8) |  2.8 (2.6) |  2.3 (2.1) |  2.4 (1.9) | 1.8 (3.3) |  2.6 (2.4) | p<0.0001 |

**Appendix Table 7. Prevalence of antibiotic resistance genes detected on nasopharyngeal swabs processed using a bacterial microarray.**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Gene** | **2014, N=908** | **2015, N=336** | **2017, N=743** | **2018, N=710** | **2019, N=720** | **2021,****N=189** | **Pre, N=1244** | **Post, N=2353** | **P, Pre vs post** |
| **mefA** | 321 (35.4) | 95 (28.3) | 366 (49.3) | 275 (38.7) | 333 (46.3) | 80 (42.3) | 416 (33.4) | 1054 (44.8) | <0.0001 |
| **ermB** | 176 (19.4) | 49 (14.6) | 238 (32) | 226 (31.8) | 254 (35.3) | 74 (39.2) | 225 (18.1) | 792 (33.7) | <0.0001 |
| **ermC** | 59 (6.5) | 24 (7.1) | 54 (7.3) | 57 (8) | 31 (4.3) | 7 (3.7) | 83 (6.7) | 149 (6.3) | 0.7213 |
| **tetM** | 538 (59.3) | 172 (51.2) | 486 (65.4) | 431 (60.7) | 450 (62.5) | 122 (64.6) | 710 (57.1) | 1489 (63.3) | 0.0003 |
| **tetO** | 14 (1.5) | 3 (0.9) | 11 (1.5) | 10 (1.4) | 6 (0.8) | 1 (0.5) | 17 (1.4) | 28 (1.2) | 0.6393 |
| **tetK** | 33 (3.6) | 18 (5.4) | 21 (2.8) | 20 (2.8) | 12 (1.7) | 3 (1.6) | 51 (4.1) | 56 (2.4) | 0.0051 |
| **cat** | 46 (5.1) | 12 (3.6) | 48 (6.5) | 33 (4.6) | 25 (3.5) | 4 (2.1) | 58 (4.7) | 110 (4.7) | 1 |
| **sat4** | 44 (4.8) | 21 (6.3) | 32 (4.3) | 29 (4.1) | 12 (1.7) | 5 (2.6) | 65 (5.2) | 78 (3.3) | 0.0069 |
| **aphA3** | 44 (4.8) | 22 (6.5) | 32 (4.3) | 29 (4.1) | 15 (2.1) | 5 (2.6) | 66 (5.3) | 81 (3.4) | 0.01 |



**Appendix Figure 1. Pneumococcal serotype-specific carriage prevalence among Nepalese children by pre- and post-PCV10 periods and identification as either the major or minor serotype on a swab sample.** Major serotypes were defined as the most abundant serotype detected within a single swab sample. Minor serotypes are defined as all other subsequent serotypes in lesser abundance detected on the swab sample.

**Appendix Figure 2. Pneumococcal carriage densities amongst Nepalese children for PCV10 serotypes for each collection year**. A=2014, B=2015, C=2017, D=2018, E=2019, and F=2021. PCV10 was introduced into the routine Nepalese infant schedule in 2015. Boxplots demonstrate medians and IQR. Dashed vertical line = median overall pneumococcal carriage density for each collection year.