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Phase Variation Mediates Reductions in Expression of Surface Proteins During Persistent Meningococcal Carriage

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Running title:- Genetic Variation During Meningococcal Carriage

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Abstract

Asymptomatic and persistent colonisation of the upper respiratory tract by *Neisseria meningitidis* occurs despite elicitation of adaptive immune responses against surface antigens. A putative mechanism for facilitating host persistence of this bacterial commensal and pathogen is alterations in expression of surface antigens by simple sequence repeat (SSR)-mediated phase variation. We investigated how often phase variation occurs during persistent carriage by analysing the SSRs of eight loci in multiple isolates from 21 carriers representative of 1-6 months carriage. Alterations in repeat number were detected by a GeneScan analysis and occurred at 0.06 mutations/gene/month of carriage. The expression states were determined by Western blotting and two genes, *fetA* and *nadA*, exhibited trends towards low expression states. A critical finding from our unique examination of combinatorial expression states, 'phasotypes', was for significant reductions in expression of multiple phase-variable surface proteins during persistent carriage of some strains. The immune responses in these carriers were examined by measuring variant-specific PorA IgG antibodies, capsular group Y IgG antibodies and serum bactericidal activity in concomitant serum samples. Persistent carriage was associated with high levels of specific IgG antibodies and serum bactericidal activity whilst recent strain acquisition correlated with a significant induction of antibodies. We conclude that phase variable genes are driven into lower expression states during long-term persistent meningococcal carriage, in part due to continuous exposure to antibody-mediated selection, suggesting localised hypermutation has evolved to facilitate host persistence.

INTRODUCTION

Heightened mutation or recombination rates in specific regions of bacterial genomes are a feature of many bacterial commensals and pathogens (1-4). The mechanisms responsible for this localised hypermutation are diverse (e.g. site-specific recombination and slippage in repetitive DNA tracts), but generate high frequencies of variation in a stochastic manner usually prior to exposure to a selective pressure (5). These hypermutable loci are termed contingency loci and are speculated to have evolved as a mechanism for adaptation to rapid, unpredictable and undetectable fluctuations in selective pressures. Localised hypermutation is therefore likely to contribute to both host adaptation and the disease phenotypes of bacteria but few studies have examined the frequency and types of events occurring during natural infections by these organisms (6-9).

Infections by *Neisseria meningitidis* (Nm) can result in septicaemia and meningitis. Disease is rapid in onset and has high rates of mortality and morbidity with infants exhibiting the highest prevalence of disease. Vaccine-elicited serum bactericidal antibodies (SBA) provide effective protection against Nm infections (10,11). Despite this, Nm normally exists as a commensal on the naso/oropharyngeal surfaces of humans with high frequencies of asymptomatic carriage in teenagers and young adults (12). Meningococci can persist in individual hosts for 6-12 months with carriage eliciting adaptive immune responses against surface determinants (13). The SBA of carriers correlates with protection against disease by homologous strains (14,15) but is also thought to mediate prevention of carriage of homologous strains. Evidence for the latter is based on observations such as sequential carriage and replacement of one strain by another strain with antigenically-mismatched outer membrane proteins (16) and herd protection associated with meningococcal serogroup C (MenC) and serogroup A (MenA) conjugate vaccination (17, 18). This raises a puzzling feature of meningococcal carriage, namely, how does this bacterium persist in the face of an adaptive immune response directed against surface antigens.

Microsatellites or simple sequence repeat (SSR) tracts are a major mechanism of localised hypermutation being subject to high rates of insertions and deletions of repeats during DNA replication (3). The reversible nature of SSR mutations has enabled evolution of phase variation (PV), i.e. high frequency, reversible alterations in phenotypic expression, for a diversity of surface molecules. In Nm, 40-60 genes per genome are subject to SSR-mediated PV with these simple sequence contingency loci (SSCL)

encoding outer membrane proteins, enzymes involved in modulation of the surface exposed-glycans of lipopolysaccharide and pilin, and restriction-modification systems (9,19). The SSRs are present either within the reading frame, resulting in ON/OFF switches in expression, or the promoter, driving modulations of gene expression. The majority of Nm SSRs are polyC or polyG tracts, but there are also tetra-, penta-, hepta- and longer repeat units. The outer membrane proteins encoded by Nm SSCLs include a porin (PorA), iron-acquisition proteins (HmbR, HpuAB, FetA), adhesins and invasins (Opc, Opa, NadA), and autotransporters (MspA/AusI, NalP/AspA, NadA). These proteins have important primary functions – such as iron acquisition (e.g. HpuA, HmbR) and adhesion (e.g. Opc, NadA, MspA) to host tissues - associated with their expression and hence phase-variants in an ON or high expression state are likely to contribute to survival on nasopharyngeal surfaces (20,21). Host persistence may be further facilitated by the secondary functions of some of these proteins, for example, Opc contributes to complement resistance (22,23). Selection for expression of the phenotypes associated with these SSCLs is, therefore, potentially strong during host persistence. Conversely, an opposing 'counter' selection for phase variants in an OFF or reduced expression state is presumed to occur due to adaptive immune responses. The PorA and Opc proteins elicit bactericidal antibodies during carriage (13,24) and *porA* phase variants, exhibiting reduced expression of the PorA protein, can mediate escape of bactericidal antibodies *in vitro* (25). PV of meningococcal SSCLs could therefore confer a major advantage as Nm persists on mucosal surfaces during carriage, however, it is currently unclear whether phase variable switches actually occur during carriage of Nm in native hosts nor whether there are particular patterns of switching.

Characterisation of genetic variation during natural infections provides key indications of how bacterial commensals and pathogens colonise and persist in their hosts. Localised hypermutation is a phenomenon that is presumed to facilitate bacterial adaptation to the fluctuating and opposing selective pressures (e.g. adherence versus immune avoidance) encountered in host environments. In this study we set out to determine how often SSR-mediated PV occurs in meningococci during natural infections and to examine whether changes were driven by adaptive immune responses. Our studies provide the first definitive information on the frequencies of localised hypermutation occurring during long-term persistence of meningococci on the pharyngeal tissues of their native hosts. Critically, our unique combinatorial investigation of multiple phase variable

loci indicates that host persistence is associated with a heightened prevalence of 'phasotypes' with lower expression states for multiple surface proteins. A simultaneous evaluation of the immune responses in these carriers suggests that selection for low expression 'phasotypes' is driven by continuous exposure to immune selective pressures.

MATERIALS AND METHODS

Bacterial isolate growth and characterisation. All meningococcal isolates were obtained from a carriage study performed in Nottingham University between November 2008 and May 2009 as described previously (16). The study was approved by the Nottingham University Medical School Ethics Committee, and written informed consent was obtained from all volunteers. Up to twenty single colonies were re-streaked from initial selective plates onto Columbia chocolate agar plates (Oxoid). After overnight growth at 37^{o}C in 5% CO_2, sweeps of growth were used for preparation of glycerol stocks in BHI broth plus 20% glycerol and genomic DNA by extraction with a DNAeasy Blood and Tissue kit (Qiagen). For expression analyses, strains were initially grown overnight in BHI broth at 37^{o}C and then diluted 10-fold in BHI broth followed by growth for 4-6 hours. Iron-repressed genes were induced by addition of desferal at a working concentration of 30 μM to a mid-log phase culture followed by incubation for an additional 1-2 hours.

Typing of two isolates per time point was described previously (16). Typing of additional isolates was performed by PCR amplification with relevant capsule specific PCR primers and primers specific for the relevant variable regions of the *porA* and *fetA* genes as described previously (see (16) and Table A1).

Enumeration of SSR repeat numbers. The SSRs of each gene (i.e. *fetA*, *porA*, *opc*, *hpuA*, *hmbR*, *nadA*, *mspA*, and *nalP*) for each strain were amplified and sequenced using published or newly-designed primers spanning the repeat tract by previously described methods (16, 25, 26, 27; Table A1). The number of repeats in SSRs of additional isolates for each strain were determined by a GeneScan protocol as described elsewhere (28, 29). Briefly, SSRs were amplified using two oligonucleotides, one of which was labelled on the 5' end with a fluorescent dye (FAM, 6-carboxyfluorescein), and subjected to electrophoresis on an ABI3730 autosequencer in comparison to a GeneScan500LIZ size standard (Applied Biosystems). Product sizes were calculated using PeakScanner v1.0

(Applied Biosystems) and repeat numbers determined by comparison to controls of known size and repeat number from the same strain and hence presumed to have identical flanking sequences. A sub-set of tracts were analysed by sequencing to confirm repeat numbers (see Tables A2 and A3). Mononucleotide repeat tracts of ≥9 units produced two or more labelled products due to slippage during PCR amplification. The ratio of the areas of the primary and secondary peaks was determined and if the ratio was above 1.2 the primary peak was utilised for determining repeat number otherwise the peak with the largest size was selected.

Comparative measurements of gene/protein expression levels. A semi-quantitative measure of protein expression levels for different phase variants of each strain was performed by probing Western blots of meningococcal whole cell lysates with specific antibodies (Table 1A). Meningococcal isolates were grown to mid-log phase either in the presence or absence of an iron chelator in order to induce genes repressed by high iron levels (i.e. *fetA* and *hpuA*). Cells were washed twice before being resuspended in phosphate buffered saline (PBS). Cell numbers were adjusted to a constant OD550 and then mixed with 2X SDS loading buffer at a 1:1 ratio. Cell lysates were electrophoresed on 8% polyacrylamide gels and transferred to PVDF membranes by application of fixed current for 1 hour. Membranes were blocked overnight at 4^{o}C in PBST-Milk (PBS/0.5% Tween20 plus 5% skimmed milk) and subsequently probed with an appropriate primary antibody diluted in PBST-Milk for 2 hours. Membranes were then washed three times with PBST (PBS/0.5% Tween20) and then probed with either a 1:2000 dilution of an anti-mouse or anti-rabbit IgG horseradish peroxidase conjugate for 1 hour. Bound antibodies were detected with an ECL detection kit and X-ray film. Quantification of bands was performed by scanning of blots and quantification using ImageJ.

Surface expression was analysed by flow cytometry as described previously (25). Briefly, meningococcal cells were harvested from mid-log phase cultures, washed and resuspended in assay buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Tween-20) containing primary antibody. Samples were incubated for 1 hr at room temperature, washed thrice and incubated for 1 hour with a 1:100 dilution of an anti-mouse IgG-fluorescin isocyanate conjugate (Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L), Life Technologies^{TM}). After three washes, cells were resuspended in phosphate-buffered saline

containing 0.05% formalin. Samples were then analysed on a fluorescence-activated cell sorter and mean fluorescent intensities were determined for each sample.

Expression of the *porA* gene was assessed by RT-qPCR. Meningococcal strains were grown to mid-log phase in Mueller-Hinton broth before fixing in Bacteria Protect Reagent (Qiagen). Total RNA was then extracted using an RNeasy Mini Kit with an additional RNase-free DNAse step (Qiagen) to remove DNA contamination. Custom primers and probes used for gene expression assays are listed in Table A1. Reverse-transcription PCR assays (RT-PCR) were completed in MicroAmp® Fast Optical 96-Well Reaction Plates using TaqMan® RNA-to-CT™ 1-Step Kit (Applied Biosystems). Reactions were set up in accordance with the Applied Biosystems protocol and analysed on Applied Biosystems 7500. The house-keeping gene, glucose-6-phosphate 1-dehydrogenase (*gdh*) was used as endogenous control gene and H44/76 total RNA as a standard positive control. Fluorescence was recorded at the end of each extension step according to the probes present in the reaction, at wavelengths determined by the Applied Biosystems 7500 Fast System Sequence Detection Software. Relative Quantity (RQ) values were calculated by the using the $2^{-\Delta\Delta CT}$ method. The *gdh* reaction was used as an endogenous control and all samples were calibrated to the positive control sample.

Quantification of anti-PorA and anti-CapY antibodies in serum samples. An immunofluorescence bead-based immunoassay was utilised to quantify variant-specific PorA antibodies in serum samples. Eight PorA variants (P1.5-1,2-2, P1.19,15, P1.19-1,15-11, P1.5,2, P1.5-2,10, P1.7,16, P1.7-2,4, and P1.21,16) were cloned into an expression vector enabling production of His-tagged recombinant PorA proteins. A ninth protein (loopless, P1.-/-) was also produced from which the VR1 and VR2 regions had been deleted. Proteins were produced as inclusion bodies, purified on a His-tag column in the presence of urea, re-folded by droplet dilution and finally dialysed into an appropriate buffer. Polystyrene microspheres labelled with fluorophores (Liquichip Carboxy beads, BioRad) were coated with nickel-NTA (nitrilotriacetic acid). Each recombinant His-tagged PorA variant was bound to a spectrally distinct microsphere.

PorA-coupled fluorescent beads were mixed with four dilutions of each serum sample in Liquichip assay buffer (PBS/0.1% bovine serum albumin/0.05% Triton X-100) in a 96-well filter plate and incubated for 30 minutes at room temperature on a rocking platform. Samples were washed three times and then incubated with a 1:25 dilution of a goat anti-human IgG R-phycoerythrin conjugate (ABD Serotec, Kidlington, UK) in Liquichip

assay buffer. Finally, samples were washed three times and analysed in a LiquiChip200 Workstation. Raw fluorescence data were converted into arbitrary units (AU) by comparison to a standard curve generated using serial dilutions of pooled sera from vaccinees who had been inoculated with either a MenBvac or MenNZB outer membrane vesicle vaccine (derived from two clinical studies MNB1 and MNB2, respectively). Separate standard curves were generated for each PorA variant hence comparisons between AU for each variant are not possible. The quality of each data set was assessed by statistical evaluation of the parallelism of the lines obtained for the standard curves using CombiStats version 5 (European Directorate for the Quality of Medicines and Healthcare, Council of Europe). The AU values for each variant were adjusted for non-specific binding by subtraction of the AU value obtained for the loopless PorA variant, a value of 0.05 was arbitrarily assigned when binding to this control protein exceeded that to a particular PorA variant.

The amount of anti-CapY IgG antibodies in each serum samples were measured by ELISA using purified serogroup Y capsular polysaccharide as a ligand as described in Gheesling *et al*. (30).

Serum bactericidal antibody assays. Serum bactericidal antibody assays were performed by standard techniques (31) in the Vaccine Evaluation Unit using a ST11 meningococcal strain expressing the serogroup Y antigen (M03-241125, CDC S1975, Y:P1.5,2:ST11, serotype 2a, fHBP-Oxford 2.2, NadA 2.49, NHBA 29).

Statistical analyses. Each carrier was examined for significant changes in repeat tract length between a pair of time points for every gene as follows:- repeat numbers were determined for six or more colonies for each time point (apart from four time points where less than six colonies were obtained); repeat numbers were binned into two non-overlapping categories for the two time points; the repeat number of each isolate was assigned to one of the two categories and placed into a contingency table; a difference was then deemed significant if a comparison yielded $P<0.05$ in a Fisher's exact test. Two-tailed Fisher's exact tests were performed using GraphPad (graphpad.com/quickcalcs) or Prism. Chi-squared tests were performed using www.physics.csbsju.edu/stats/. Geometric

mean concentrations were calculated using Prism. Wilcoxon rank signed tests were performed in R using the COINS package.

RESULTS

Frequent changes occur in the SSRs of eight genes encoding outer membrane proteins during persistent meningococcal carriage in the upper respiratory tracts of humans. In order to examine the extent of localised hypermutation occurring during persistent meningococcal carriage, bacterial samples were obtained from the nasopharyngeal tissues of individuals colonised with the same meningococcal strain for up to six months. These samples were from a longitudinal study of meningococcal carriage performed with a cohort of 190 first year students attending six catered halls at the University of Nottingham (16). This study was initiated in November 2008, five weeks after the start of term, and involved four time points (1st, November; 2nd, December; 3rd, February; 4th, May). We have previously described the identification of persistent carriers and characterisation of the colonising strains (16). A sub-set of 21 persistent carriers were selected for further analysis. These carriers were colonised with one of six strains:- Y:P1.21,16:F3-7:ST1466(cc174, 7 carriers); Y:P1.21,16:F3-7:ST8510(cc174, 1 carrier); E:P1.5,2:F1-7:ST1383(cc60, 5 carriers); Y:P1.5-1,10-1:F1-3:ST767(cc167, 3 carriers); Y:P1.5-1,10-1:F4-1:ST1655(cc23, 4 carriers); B:P1.19,15:F5-1:ST5682(cc32, 1 carrier). Ten of these carriers were persistently colonised for 5-6 months with the same strain, whilst six exhibited either clearance or replacement of the initial strain by the 4th time point and the remainder were only examined at early time points. As described previously, oropharyngeal swabs were spread onto selective agar plates and then streaked to single colonies prior to overnight growth. Up to 20 single isolates were obtained by re-streaking single colonies onto non-selective plates for preparation of glycerol stocks and DNA. Thus all isolates were subject to minimal *in vitro* passage to reduce the potential for alterations in PV genotype. Where possible, six or more isolates were analysed as a statistically-representative sample. The similarity of each set of isolates was confirmed by PCR with primers specific for the relevant capsule, PorA and FetA type (data not shown).

A coherent group of eight genes (*porA, fetA, opc, hpuA, hmbR, nadA, mspA* and *nalP*) encoding phase variable outer membrane proteins with known functional attributes were chosen for analysis. Most of the other phase-variable meningococcal genes encode

glycosyltransferases, restriction-modification systems or proteins of unknown function (9, 19). The repeat tract of each gene for each strain was amplified and sequenced. Two genes were not universally present with *hmbR* only in the cc60 and cc32 strains and *nadA* only in the cc174 and cc32 strains (data not shown). The SSR was located in the reading frame for four genes and exhibited a consistent association between the number of repeats and whether the reading frame was 'in-frame' or out-of-frame' and hence predicted to have an ON or OFF expression state, respectively. The predicted ON expression states for each gene were as follows:- 10G/13G, *hpuA*; 9G, *hmbR*; 10C/13C, *nalP*; and 6C/9C, *mspA*. For the other genes, the SSRs were either located upstream of (i.e. the 5'TAAA tract of *nadA*) or within the core promoter (polyC tracts in *fetA* and *opc* and a polyG tract in *porA*; see also Fig. 1A). For these genes, expression state could not be directly predicted from the sequence data. Flanking sequences and repeat tracts were conserved between strains (data not shown) with the exception of the cc60 strains, which contained an interrupted SSR in *fetA* and a 1 nt deletion in the putative -10 of *porA* (Fig. A1). A GeneScan assay was utilised to determine the repeat numbers for each gene in multiple isolates per time point (Tables A2 and A3). Low levels of variation in tract lengths were observed within time points (see Fig. 1 and Fig. A2 for examples). Shifts in tract length between time points were observed for at least one gene in every carrier except V185, with specific trends being evident for some genes. For example, the *fetA* SSR in cc174 strains shifted from 9C to 8C or a mix of 10C and 11C variants in 5/8 carriers whilst the *nadA* SSR remained at or shifted to 9 or 12 5'TAAA in all 8 of the cc174 carriers.

Comparisons between genes indicated very low levels of alterations in the repeat tracts of *hmbR* and *mspA* whilst all the other genes experienced alterations in 30-40% of carriers (Fig. 2A). Both *fetA* and *nalP* exhibited significantly different frequencies of switching between the later as opposed to early time points ($P<0.05$ for comparison of 1^{st}-to-2^{nd} versus 1^{st}-to-4^{th} switching events using a two-tailed Fisher's exact test), indicative of a correlation between length of carriage and propensity for PV. A correlation between mononucleotide repeat tract length and propensity to PV was also observed with no PV in short tracts (6 or 7 G/C repeats), very low levels in 8G/8C tracts, similar levels in tracts of 9 to 11 repeats and a trend towards increasingly higher levels in longer tracts (Fig. 2B).

A switching rate per month of carriage was determined for a combination of the eight phase variable genes with mononucleotide tracts of 9 or more repeats in the $1^{st}/2^{nd}$ time point. A significant switch in repeat tract between the $1^{st}/2^{nd}$ and 4^{th} time points for a particular gene was calculated as described in the Materials and Methods. The number of significant switches was divided by the total number of events analysed (i.e. 21/54) and by the number of months of carriage (i.e. 5 or 6) resulting in an estimate of 0.06 mutations/gene/month of carriage. We conclude that there is a relatively high level of alterations in the mononucleotide repeat tracts of Nm SSCL during carriage and that gene type, repeat number and length of host persistence are important determinants of mutability.

Persistent meningococcal carriage is associated with accumulation of low expression states for some phase variable genes. Alterations in the SSRs provide an indication of mutability but do not test whether there has been a change in phenotype. Detection of any phenotypic variation associated with these SSCL required analysis of whether alterations in SSRs mediated gene expression changes. Translational SSR-mediated switching has a predictable correlation between tract length and expression state. This was confirmed by analysis of SSRs predicted as 'in-frame' (i.e. ON) or 'out-of-frame' (i.e. OFF) for all four genes (see Fig. 3 and Figs. A3-A7 for *hpuA*; Oldfield *et al.* (27) for *mspA* and *nalP*; Tauseef *et al*. (26), for *hmbR*). Transcriptional SSR-mediated switching causes changes in gene expression through modulation of promoter activity and hence is not readily predicted from repeat tract length. A series of Western blots were performed for each strain generating associations between tract length and expression state for *porA*, *fetA*, *nadA* and *opc* (Fig. 3; Fig. A3-A8; Tables A4-A6). All of the genes apart from *porA* exhibited at least three clear expression states ranging from low (>5-fold reduction) to intermediate to high expression (Table A5). For *porA,* high/intermediate levels of expression were detected for multiple tracts whilst the lowest expression state was only ~5-fold below the highest, larger differences but similar trends were detected in a quantitative RT-PCR analysis of a sub-set of strains (Table A7). To correlate surface expression with repeat number, six isolates were investigated by FACs. Variants of a cc32 strain with tracts of 10C or 9C exhibited 9-to-18-fold higher surface expression of FetA than a variant with an 8C tract whilst similar levels of high expression of porA were detected in variants of a cc174 with tracts of 11G, 12G or 13G (Fig. A8).

Expression state data were utilised to classify each SSR/gene combination into an expression code. Translational-SSR were coded into 0 and 2 for OFF and ON respectively and transcriptional-SSR into 0, 1 and 2 representing undetectable/low, intermediate and high expression. Low and intermediate expression were defined as a >5-fold and 1.5-to-5-fold, respectively, reduction relative to high expression as detected by Western blotting for *fetA*, *opc* and *nadA* or a combination of Western blotting and RT-PCR for *porA*. A combined overview of all carriers for all time points detected genic differences in the proportions of carriage isolates with the highest expression states:- PorA (91%); HpuA (66%); NalP (65%); FetA (42%); HmbR (24%); MspA (22%); Opc (18%); and NadA (7%) (Table A6). The largest temporal shift was observed for FetA, which started with 69% of isolates in the high state and ended with only 11% in this state by the 4th time point (i.e. after 5-6 months carriage). A significant reduction in FetA expression (P=0.008; Wilcoxon signed rank test with continuity correction) was observed for a comparison of the mean initial and final expression states observed across all carriers (Fig. A9). Non-significant trends were noted for the other genes with NadA and NalP also exhibiting reductions in expression state (Fig. A9). Expression of NadA was in the lowest state in all of the final time point samples with the absence of a trend towards reductions in expression being due to this gene being in the lowest expression state in many of the initial samples.

Combinatorial reductions in phase variable gene expression occur during persistent meningococcal carriage. A major advantage of our analysis of multiple phase variable genes is an ability to examine the combined expression states within an isolate. A combined code was generated for every isolate using the expression data for the 6 or 7 phase variable genes present and analysed in each clonal group. These codes contain both genotypic and phenotypic information and hence are referred to as 'phasotypes'. Seven genes - three with two states and four with three states - could generate 648 phasotypes. A total of 25 phasotypes was observed for the combined data for the 140 isolates of cc174, indicating a limited exploration of the potential expression states (Table A8).

Each phasotype contains information on the combined expression states of the genes, which is a simple sum of the individual gene states (i.e. 2-2-2-2-2-2-2 has score 14, 2-2-2-2-2-2-0 is 12, etc). The phasotypes were grouped by 'expression score' and the

patterns of change between time points were examined for each carrier (Fig. 4A-C). The mean phasotype score was also calculated for each time point of every carrier (data not shown) and the change in this score plotted against months of carriage (Fig. 4D). The cc167 and cc23 strains were combined as they have identical PorA sub-types (P1.5-1,10-1) and gene combinations (i.e. absence of *nadA* and *hmbR*). Only one carrier exhibited no change in expression score (V185; persistent carrier of a serogroup E, cc60 strain) whilst varying patterns of changes were observed in other carriers. A reduction in phasotype score was observed in 11 of the 21 carriers between the initial and final time point of observed carriage but was variable between strains with a shift towards lower expression phasotypes in 6/8, 4/7, 0/5 and 1/1 of the cc174, cc167/cc23, cc60 and cc32 strains, respectively. A significant change towards a lower mean phasotype score was detected for the cc174 carriers ($P = 0.03$ in a Wilcoxon rank test with continuity correction) but not for the cc167/cc23 or cc60 strains. The absence of a shift towards lower expression scores in the cc60 strains could be due to only one of these carriers exhibiting more than three months observed carriage and three of the genes having short repeat tracts (*fetA*, 6G; *nalP*, 7C; *mspA*, 8C). Overall, persistent carriage is associated with a shift towards lower combined phasotype scores but exhibits a strain bias.

The effect of persistence length on the reduction in phasotype score was examined for all 21 carriers. A trend was observed for reductions in phasotype score as a function of persistent carriage such that 44% (8/18), 50% (8/16) and 70% (7/10) of carriers exhibited reduced scores relative to the initial time point after 1, 2-3 or 5-6 months carriage. Analysis of the phasotype scores for isolates provided further evidence of the effect of prolonged carriage (Table 1). Thus for the cc174 isolates, there was a significant shift from 85% with a score of ≥7 in the initial time point to 100% with ≤6 after 5-6 months carriage ($P>0.001$ in a Chi-squared test). Similarly, there was a reduction in expression score from 65% with ≥7 to 62% with ≤6 for the cc167/cc23 isolates ($P=0.002$). Conversely, the cc60 strains exhibited a significant trend towards rising phasotype scores ($P=0.002$) however, as discussed above, there was a lack of data for more than three months carriage with this clonal complex. Thus, prolonged carriage is associated with reductions in phasotype score and the accumulation of phasotypes with lower expression states.

Variant-specific PorA antibodies can be detected using a multiplex microserology assay in sera from persistent meningococcal carriers. In order to understand why switches in the phase-variable genes are occurring it is important to characterise the selective pressures acting on the products of these genes. Adaptive immune responses exert a strong selective pressure on the surface antigens of meningococci with a significant potential impact on PV expression status. The phase variable PorA protein is a major component of the outer membrane and contains two variable regions, VR1 and VR2, which are the main targets for bactericidal meningococcal antibodies and are also utilised for strain typing with VR specific mAbs (32). We tested for the presence of VR region specific PorA antibodies in serum samples collected concomitantly with nasopharyngeal swabs from the 21 persistent carriers. These sera were complemented with additional sera from the same carriage study (16), this included a set of sera from persistent non-carriers as controls and sera from volunteers exhibiting acquisition of carriage as a test of whether gain-of-carriage was associated with induction of meningococcal specific antibodies.

The levels of PorA-specific IgG antibodies were analysed using a multiplex fluorescent-microsphere protocol and a Liquichip workstation (Qiagen). A single combination of seven PorA variants was utilised for the majority of the assays, which included PorA variants with VRs similar/identical to those present in the carriage isolates and a modified PorA, lacking VR1 and VR2, used as a control for background reactivity (Patel, Chan and Feavers, unpublished data). Antibody levels were measured in arbitrary units (AU) relative to pooled meningococcal vaccinee sera and hence quantitative comparisons of reactivity between variants was not possible.

The specificity and reproducibility of the multiplex PorA assay was previously validated utilising variant-specific PorA monoclonal antibodies but has not been extensively tested with sera from carriers of known PorA variant type (Patel, Chan, Findlow, Borrow, Trotter and Feavers, unpublished data). We, therefore, analysed the results for evidence of specificity and utility in detection of variant specific antibodies (Table A9, Table A10 and data not shown). Importantly, general reactivity to all PorA variants was noted as being very low, and probably due to non-specific background, in persistent non-carriers (Table 2; Fig. 5). High levels of monospecific activity were detected in at least one of the time points for five carriers (V51, V54, V64, V69 and V185) against the PorA variant protein containing one or both VRs matching the carriage isolate. Sera from other carriers exhibited a high level of reactivity mainly to the homologous PorA

variant whilst three carriers (V59, V88 and V124) had a pan-response against all variants and three (V93, V138 and V176) had non-carrier levels of reactivity. Thus some carriers exhibit monospecific responses whilst others elicit varying degrees of cross-reactivity, which could be due to cross-reactive epitopes, recent carriage of other strains or a 'bystander' induction of responses to all previously-encountered PorA variants. The specificity of these responses was confirmed for a selection of samples by using VR specific mAbs to block binding of antibodies to one of the VRs (Table A10). Both VR1 and VR2 specific mAb antibodies reduced binding of serum antibodies but VR2 antibodies were generally more effective at blocking reactivity. Overall, the multiplex assay exhibited utility for detection of variant-specific IgG PorA antibodies in sera from meningococcal carriers.

Persistent meningococcal carriage is associated high levels of adaptive immune responses against surface determinants. The above studies indicated that variant-specific PorA antibodies could be readily detected in the sera of these carriers. However, as variability in the antigenic regions of PorA prevented direct comparisons between strains, antibody responses against the serogroup Y (MenY) capsular antigen were also investigated to facilitate comparisons between volunteers and due to the high prevalence of MenY strains among our persistent carrier samples. Immune responses to the serogroup Y capsular antigen were assessed by a CapY-IgG specific ELISA (i.e. with purified capsular polysaccharide). The sera were also tested for bactericidal antibodies using an ST-11 strain expressing a serogroup Y capsule and a P1.5,2 PorA variant. As the PorA protein was mismatched in either both VRs (cc174) or VR2 (cc167/cc23), this assay will mainly detect bactericidal IgM and IgG anti-CapY antibodies. The expression states and/or sequence variation, as compared to test strains, in the other phase-variable genes were however unknown and may make a minor contribution to bactericidal titre.

Sera from carriers subject to different types of carriage (acquisition, clearance and clonal replacement, and persistent) were analysed to determine the levels and temporal behaviour of antibody responses (Table A9; Fig. 5). As observed for PorA, most of the non-carriers had low levels of CapY IgG antibodies and low SBA titres apart from three who had with either high CapY IgG (V135 and V150) or high SBA (V97) titres, which could be due to carriage of homologous meningococcal strains prior to enrolment in the study or vaccination with a CapY-conjugate vaccine. Acquisition of carriage was associated with a

significant rise in variant-specific PorA antibodies and SBA titre and a non-significant increase in anti-CapY IgG. Antibody levels and SBA titres increased as a function of time post-colonisation. Replacement or clearance of the initial strain was associated with high PorA and CapY IgG antibody concentrations and high SBA titres similar to persistent carriers with no significant overall decrease following loss of carriage, suggesting persistence of antibodies in the absence of on-going stimulation. Significantly higher levels of reactivity were detected in sera from persistent carriers as compared to non-carriers to the homologous PorA type and CapY at all time points (Fig. 5A and 5B). Similarly, SBA titres were higher in persistent carriers than non-carriers although a small but significant drop in SBA titre was found in the fourth time point possibly due to waning of a specific IgM response (Fig. 5C). A major increase between the November (i.e. first) and later time points in both anti-PorA and anti-CapY antibodies was observed in two carriers, V88 and V43, whilst two others exhibited increases in PorA IgG (V64) or CapY IgG (V51). These rises were indicative of recent colonisation and commensurate with the timing of sample collection, which was performed near to the start of term when high levels of spread of meningococcal strains is anticipated among this typical group of university students.

Relative amounts of antibodies were assessed for the 21 carriers subject to PV analysis in groups comparable to those utilised for the phasotype scores (Table 2). The cc174 and cc167/cc23 strains exhibited higher concentrations of CapY IgG and SBA titres than non-carriers at all time points ($P<0.05$ with an unpaired T test) apart from a non-significant difference in CapY IgG at the 1st time point in cc174 carriers probably due to recent colonisation in three of these carriers (V43, V51 and V88) and hence insufficient time for elicitation of antibodies. The levels of variant-specific PorA antibodies were significantly higher in the cc167/23 and cc60 carriers than non-carriers ($P<0.05$). Six of the cc174 carriers had 10-fold higher levels of P1.21,16 specific PorA antibodies levels than non carriers at two or more time points but specific antibodies weren't detectable in two (V59 and V138) carriers.

In nine of the eleven carriers exhibiting a drop in average phasotype score, high variant-specific PorA antibodies, CapY responses and SBA titres were detected at multiple time points during persistence of the meningococcal clone. Of the others, one (V88) exhibited a late rise in PorA levels at the third time point, correlating with a major reduction in phasotype score, whilst in V54 the CapY IgG and SBA response were weak but the PorA specific responses were very high. The exception was for carrier V176 wherein no

PorA antibodies could be detected. Collectively, we detected high levels of surface-antigen specific and bactericidal antibodies in the majority of persistent carriers in which phase variable reductions in OMP expression were observed.

DISCUSSION

Contingency loci are present in many pathogenic and commensal bacteria and are thought to generate high levels of genetic variation enabling adaptation to fluctuations in the stringent selective pressures exerted by the host milieu. A significant gap in our understanding of contingency loci is the extent of their contributions to natural infections whether asymptomatic or disease-associated. This study has determined the frequencies and patterns of SSR-mediated PV occurring during asymptomatic, persistent carriage of Nm, a pathogen/commensal with multiple contingency loci, in the upper respiratory tract of humans - their only hosts - and detected trends towards lower expression states for specific and combinations of phase variable surface proteins (herein termed ‘phasotypes’). Our report highlights the importance of examining bacterial isolates from natural environments and implies a role for PV in facilitating persistent carriage of a bacterial pathogen.

By measuring variation in eight contingency loci, we estimate that the SSR-mediated PV rate for polyG/C tracts of 9 or more repeats is 0.06 mutations/gene/month of carriage for Nm in the upper respiratory tract of humans. In contrast, genetic variation in the VRs of PorA was only detected in one of these 21 carriers and no variation was detected in the FetA VRs (16 and data not shown) indicating an antigenic variation rate of 0.006/gene/month of carriage (1 mutation in 172 months of carriage). Thus changes in the structure of an antigen by point mutation or recombination following lateral gene transfer are infrequent as compared to phase-variable alterations in expression mediated by mutations in SSR tracts. There are 30-40 phase variable genes in each Nm strain most of which modify OMPs or the structures of surface determinants. Thus localised hypermutation is the major source of genetic variability occurring during asymptomatic carriage of a meningococcal clone within an individual person.

Temporal fluctuations in the selective pressures acting on the different expression states of phase-variable loci is likely to be the major force driving any temporal patterns in alterations to the SSR tracts. Thus selection for the ON state may be balanced and exceeded by selection for the OFF state during long-term host persistence of a meningococcal strain resulting in an observation of ON-to-OFF switching. A key finding

from our phenotypic analysis of SSCL was for switches to lower expression states of specific OMPs – namely NadA, FetA and NalP - during persistent carriage. The other phase variable OMPs showed either variability in their expression states or, in the case of PorA, a continuous high expression state. It should be noted that all our results pertain to events on the mucosal surface as sampling of carriers was by swabbing of epithelial surfaces. One implication of the observed accumulation of OFF variants as a function of persistent carriage is that once colonisation has been established within an individual, selection for high/ON expression states of phase variable OMPs is reduced and selection for OFF expression states can drive phase variable loci into minimal expression states. The *nadA* gene was found in a low expression state in all isolates except a few from early time points (Fig. 1A; Table A2). As this gene encodes an adhesin (33,34), our results suggest that NadA is only required for initial colonisation and is rapidly subject to selection for loss of expression as Nm persists in a host. Similar requirements during colonisation may be ascribed to Opc and MspA, which encode known and putative adhesins (35,36) and were also found mainly in low expression states (52% for Opc; 78% for MspA; Table A6). The FetA OMP showed a significant trend towards lower expression states as a function of persistence (Fig. A10) and a high prevalence of ON variants during early time points (Table A6). The prevalence of high-expressing variants of FetA was partially due to clonal expansion of the cc174 strains in one hall of residence (16). However the siderophore-binding iron-uptake attributes of FetA (37) may mean that siderophores are a potent source of iron during initial colonization by Nm but are replaced by other sources (transferrin and haemoglobin) as bacterial numbers increase and perturb the normal mucosal surface. Finally, NalP showed a trend towards an OFF expression state possibly connected with a growing requirement for establishment of a biofilm, which NalP antagonizes by cleavage of other surface meningococcal proteins (38,39).

The above discussion indicates how selection for the functions of phase variable OMPs could result in an elevated prevalence of high/ON expression at specific times during persistent carriage, but, apart from NalP, has not elaborated on how selection for low/OFF expression states is exerted. A novel approach pioneered for *C. jejuni* isolates (40) and utilised in this study was to examine isolates for alterations in the combined expression states of multiple phase-variable loci. The term ‘phasotypes’ has been adopted herein (see also 41) to convey the idea that these types are based on conversion of non-arbitrary genotypic information (i.e. SSR repeat number) into a potential phenotypic state. Phasotype does not indicate an actual *in vivo* phenotypic state as expression of some of

these phase-variable genes may be controlled by external signals (i.e. *fetA* is iron-regulated). Nevertheless changes in the 'phasotype' are indicative of how the bacterial cells are responding to selective forces. We note that the 'phasotype' system is portable and could be utilised by other laboratories for comparisons of the expression states of phase-variable genes.

A novel finding from our analysis of phasotypes is for an overall decrease in surface expression of multiple OMPs during persistent mucosal carriage. This observation implies that selection is acting on the combination of genes present on the Nm surface. One explanation for this finding is that meningococcal cells expressing lower amounts of OMPs have a growth advantage and replace high OMP expressers. This selective pressure is, however, probably very weak and easily counteracted by any selection for expression of a gene. A second explanation is that adaptive immune responses against surface OMPs select for antigenic variation and reduced expression states.

Multiple studies have shown that Nm carriage elicits serum IgG and bactericidal antibodies against a range of OMPs, including PorA, Opc, PilE and NadA, and other surface molecules such as the capsule and lipopolysaccharide (13,42). Mucosal IgA responses to whole Nm cells and to PorA have also been detected (43,44). We demonstrated the presence of high levels of IgG antibodies specific for the homologous PorA variant in the majority of persistent carriers and high levels of anti-CapY specific IgG antibodies (Table 2; Fig. 5). Furthermore we show that these high levels persist through six months continuous carriage, that specific antibodies are rapidly elicited upon acquisition of carriage and that there is also a strong serum bactericidal activity response. Whilst we have yet to confirm whether antibody responses are elicited against all nine OMPs, our results suggest that a robust anti-OMP response is generated as observed in other Nm carriers. As selection of low-expression PorA phase variants has been observed *in vitro* (25), the absence of an effect of the specific antibodies on PorA expression levels is a contraindication for a role of the adaptive immune response. However, adaptive immune responses may exert only a weak effect that could be counteracted by a stronger selection for expression of this OMP. Variability in the strength of selection for the high/ON states of other OMPs may permit weak immune selection to drive these proteins into lower expression states. Variability in the patterns of switching between OMPs may also emanate from another source. Reduction in the combination of surface expressed OMPs implies an effect on the complexity of the bacterial surface rather than the amount of each protein. This could be due to the observed synergistic effects of bactericidal antibodies

against multiple minor OMPs (45) or cross-linking and neutralization of bacterial cells by a polyclonal secretory IgA response (46), resulting in variability in which OMP is down-regulated between isolates and carriers. Overall, our findings are supportive of the hypothesis that prolonged exposure to antibody-mediated selection drives Nm cells towards reduced expression states for phase variable OMPs during persistent carriage in their natural hosts. Thus PV may facilitate host persistence by mediating escape of adaptive immune responses whilst simultaneously rendering the resident Nm strain more sensitive to clearance by innate immune effectors or to competition and replacement by an antigenically-divergent Nm strain.

The reductions in expression of OMPs have potential implications for meningococcal protein vaccines. Two of these proteins, NadA and PorA, are present within the recently-licensed Bexsero® vaccine whilst NalP modifies NHBA another component of this vaccine (47,48). The FetA protein has also been proposed as a potential vaccine candidate (49). The accumulation of low expression variants of NadA and FetA indicates that vaccines including these antigens would not prevent persistent carriage but would be most likely to act during the initial stages of host colonization. Testing for herd immunity by meningococcal vaccines will therefore require a focus on prevention of acquisition of carriage in naïve individuals and a careful design of vaccine trials to monitor the relative times of strain acquisition and vaccine-induced antibody responses.

This study has defined the frequency and patterns of alterations in SSCL encoding eight OMPs during persistent Nm carriage. Our results indicate persistent carriage of Nm populations is associated with reductions in expression of single and combinations of SSCLs with evidence for adaptive immune responses being one of the major selective pressures driving the population into this state. Comparisons of the phasotypes of these carriage isolates with disease isolates of similar strain types can now be performed to determine whether particular phasotypes are required for meningococci to cause disease and if the lack of disease associated with long-term carriage of meningococci is due simply to the specific immune responses or is also prevented by the accumulation of non-invasive phasotypes.

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Figure 1. Changes in repeat tract length of five genes during persistent carriage of cc174 strains. Multiple meningococcal isolates of the same strain were collected from eight volunteers (labelled as V in the figure) persistently colonised with a cc174 serogroup Y strain either ST1466 (V51, V52, V58, V59, V88, V138) or ST8510 (V54; V43 exhibited replacement of ST8510 with ST1466 between the first and second time points). Up to six isolates per time point were analysed for up to four time points (1^{st}-4^{th} which were separated by 1, 2, or 3 months respectively) for the number of simple sequence repeats in five phase variable genes as follows:- panel (A), *fetA* (polyG tract, open circles), *nadA* (tetranucleotide 5'TAAA tract, open triangles), *porA* (polyG tract, filled triangles); panel (B), *hpuA* (poly G tract, filled diamonds) and *opc* (polyG tract, open squares).

Figure 2. Effect of persistent carriage on changes in the repeat tracts of phase variable meningococcal genes. Each gene was examined for significant changes in repeat tract length between a pair of time points for carriers persistently colonised with the same meningococcal strain and plotted as the percentage of carriers with no significant changes. The total number of carrier samples examined for each of the four pairs of time points were:- *fetA* (18, 16, 7, 10); *porA* (18, 16, 7, 10); *opc* (18, 16, 7, 10); *nadA* (9, 8, 4, 5); *hpuA* (18, 16, 7, 10); *hmbR* (5, 3, 1, 2); *nalP* (18, 16, 7, 10); *mspA* (18, 16, 7, 10). Time points were:- 1st to 2nd (1 month), black bars; 2nd to 3rd, (2 months), dark grey bars; 3rd to 4th, (3 months), light grey bars; 1^{st}-4th or 2^{nd}-4th (5 or 6 months respectively), white bars. Panel (A) shows changes per gene. Panel (B) shows changes as a function of the repeat tract length relative to the tract length in the initial time point of each pair.

Figure 3. Comparison of protein expression levels for cc174 phase variants with different tract lengths. Whole cells lysates were prepared from meningococcal cells grown to mid-log phase with (lanes 1-7) or without (lanes 8-14) induction of iron-repressed genes. Western blots were probed with 1:1,000 or 1:2,000 dilutions of primary antibodies/antisera (see Table 1A) followed by an appropiate secondary antibody. Note that the an anti-F1-3 FetA variant mouse polyclonal and an anti-meningococcal serotype P1.16 mouse mAb were used to detect FetA and PorA, respectively, whereas the other antisera recognise a wide range of antigenic types of the relevant protein. Repeat numbers are indicated as either the number of 'G's in a polyG tract or, for *nadA*, the number of 5'TAAA repeats. Lanes 1 and 8, N54.1; lanes 2 and 9, N343.5; lanes 3 and 10, N369.1; lanes 4 and 11, N352.3; lanes 5 and 12, N288.5; lanes 6 and 13, N343.2; lanes 7 and 14, N438.3. These

isolates were from two different cc174 ST-types – ST8510 (N54, N343) and ST1466 (N288, N352, N369, N438).

Figure 4. Longitudinal alterations in the multiplex phase variation expression states during persistent carriage of meningococcal strains. The expression states of phase variable genes were determined from a combination of repeat number and direct assessments of expression state by Western blotting. The expression states of phase variable loci were coded as 0 (OFF/low), 1 (intermediate) and 2 (ON/high) - see text. The combined pattern of expression states (i.e. phasotypes) for six or seven genes (as indicated in each panel) were determined for up to six isolates per time point. A total score was assigned to each phasotype by combining the expression scores of individual genes (i.e. seven genes in their maximum expression state scores 14) and then phasotypes with similar scores were colour-coded and plotted in panels A-C:- red (10-14); orange (8-9), magenta (7); yellow (6); green (4-5); and blue (0-3). A mean score for each time point of each volunteer was calculated from these total phasotype scores. Panel (A), eight cc174 carriers and one cc32 carrier (V176); panel (B), five cc60 carriers; panel (C), three cc167 and four cc23 carriers. Panel (D), graphs for three groups of strains, cc174, cc60 and cc167/cc23, showing the change in mean score relative to the initial time point when carriage was first detected.

Figure 5. Antigen-specific immune responses in serum samples from meningococcal carriers. Volunteers were grouped into four categories according to the type of meningococcal carriage detected by nasopharyngeal swabbing:- Persistent Carriers, same strains detected at all time points; Acquisition, progression from absence to presence of carriage with time points separated into pre-colonisation (Pre) and less than or more than three months after colonisation (i.e. Post(<3) and Post(>3) respectively); Replacement/Clearance, initial strain either replaced by antigenically-different strain or not detected in the 4th time point; Non-carriers, no meningococcal carriage detected at any time point. One to four sera were analysed for each volunteer. Panel A, anti-PorA IgG antibodies detected by a multiplex fluoresecence-bead assay (AU, arbitrary units). Panel B, anti-CapY IgG antibodies detected by ELISA against purified capsular antigen. Panel C, serum bactericidal activity against an ST-11 meningococcal strain expressing a serogroup Y capsular antigen. Values represent the dilution providing 50% killing.

Table 1. Longitudinal changes in expression states of phase variable meningococcal genes

Phasotype Score[1]	Number of Isolates Per Observed Months of Carriage (%)			
	Initial	1 month	2-3 months	5-6 months
cc174 (FetA-Opc-NadA-HpuA-PorA-NalP-MspA)				
10	5 (10%)	2 (5%)	0	0
9	4 (8%)	0	0	0
8	24 (50%)	22 (55%)	14 (38%)	0
7	8 (17%)	1 (3%)	7 (19%)	0
6	7 (15%)	15 (38%)	7 (19%)	15 (63%)
5	0	0	1 (3%)	2 (8%)
0-4	0	0	8 (21%)	7 (29%)
Total	48	40	37	24
cc60 (FetA-Opc-HmbR-HpuA-PorA-NalP-MspA)				
8	6 (20%)	17 (57%)	7 (58%)	6 (100%)
7	5 (17%)	5 (17%)	1 (8%)	0
6	11 (37%)	8 (27%)	4 (33%)	0
5	8 (27%)	0	0	0
Total	30	30	12	6
cc167/cc23 (FetA-Opc-Hpua-PorA-NalP-MspA)				
10-12	8 (21%)	5 (21%)	5 (14%)	0
9	6 (16%)	8 (33%)	11 (31%)	2 (8%)
8	2 (5%)	5 (21%)	8 (22%)	4 (17%)
7	5 (13%)	0	0	3 (13%)
6	5 (13%)	0	1 (3%)	2 (8%)
5	12 (32%)	6 (25%)	8 (22%)	8 (33%)
0-4	0	0	3 (8%)	5 (21%)
Total	38	24	36	24
cc32 (FetA-Opc-NadA-HpuA-PorA-NalP-MspA)				
10-12	6 (100%)	5 (83%)	4(67%)	3 (50%)
9	0	0	2 (33%)	3 (50%)
6	0	1 (17%)	0	0
Total	6	6	6	6

[1]Genotype score was determined from a combination of the expression states for 6-7 genes as outlined in brackets for each clonal complex and Table A8.

902 Table 2. Levels of anti-PorA, anti-CapY and bactericidal antibodies in serum of meningococcal
903 carriers

Antigen/Antibody type or activity[1]	Geometric Mean Antibody Concentration or Titre at Each Time Point (range, number of samples)			
	1st (Nov)	2nd (Dec)	3rd (March)	4th (May)
cc174 (Y:P1.21,16)				
P1.21,16/IgG	1.4 (0.05-40.5; 8)	1.5 (0.05-40.7; 8)	3.4 (0.05-76.1; 8)	1.7 (0.05-25.07; 7)
CapY/IgG	1.8 (0.3-20.5; 5)	ND[2]	3.8 (0.4-17.1; 4)	4.6 (0.6-27.5; 7)
CapY/SBA	1077 (3-8192; 8)	6889 (4096-16384; 4)	2656 (128-8192; 8)	939 (32-4096; 8)
cc60 (E:P1.5,2)				
P1.5,2/IgG	1.5 (0.2-4.1; 3)	2.7 (0.5-7.8; 3)	1.5 (0.4-8.0; 3)	ND
cc167/cc23 (Y:P1.5-1,10-1)				
P1.5-2,10/IgG	1.4 (0.5-6.8; 6)	1.2 (0.3-2.7; 7)	3.2 (0.3-17.2; 6)	3.9 (2.8-8.0; 6)
CapY/IgG	15.2 (8.6-38.5; 4)	9.2 (7.5-11.2; 2)	20.5 (6.4-29.8; 4)	14.9 (4.4-67.3)
CapY/SBA	1465 (3-16384; 5)	2353 (128-16384; 5)	4598 (2048-16384; 6)	1783 (1024-8192; 5)
cc32 (B:P1.19,15)				
P1.19,15	0.1 (NR; 1)	0.1 (NR; 1)	0.2 (NR; 1)	0.3 (NR; 1)
Non-carriers				
PorA/IgG	0.2 (0.1-0.5; 8)	0.1 (0.1-0.6; 6)	0.2 (0.1-0.6; 5)	0.4 (0.2-0.9; 2)
CapY/IgG	0.2 (0.1-0.3; 5)	ND	0.2 (0.1-0.2; 4)	
CapY/SBA	5 (3-128; 8)	7 (3-256; 5)	3 (3-8; 3)	9 (3-512; 8)

904 [1]Antibody concentrations:- PorA (P1) variants, arbitrary units; serogroup Y capsular antigen
905 (CapY), ug/ml; serum bactericidal activity (SBA) for an ST-11/Y strain, highest dilution resulting in
906 ≥50% killing; [2]ND, no data; NR, not relevant.