Molecular Targets in Meningococci: Efficient Routine Characterization and Optimal Outbreak Investigation in Conjunction with Routine Surveillance of the Meningococcal Group B Vaccine Candidate, fHBP[∇]†

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In 2007, recommendations were proposed for the molecular typing of meningococci. Multilocus sequence typing (MLST) was recommended to guide national and international disease management and facilitate studies of population biology and evolution. Sequencing of *porA* variable regions (VRs) 1 and 2 and the *fetA* VR was recommended for monitoring antigenic distribution and investigating potential outbreaks. *porB* characterization was recommended if further resolution was required. Several investigational "group B" meningococcal vaccines, including two in the advanced stages of development, incorporate factor H-binding protein (fHBP). The requirement for routine surveillance of *fhbp* places additional pressure on reference laboratories, both financially and in terms of labor. This study investigated the optimal and most efficient molecular typing schemes for (i) routine meningococcal characterization and (ii) the investigation of potential outbreaks, in conjunction with routine surveillance of *fhbp*. All invasive disease isolates received by the Health Protection Agency Meningococcal Reference Unit between July 2007 and June 2008 (*n* = 613) were characterized in terms of capsular group, *porA*, *fetA* VR, *fhbp*, and sequence type (ST). Following capsular grouping and *porA* genosubtyping, several predominant capsular group-*porA* combinations were identified. The levels of additional resolution afforded by *fetA* and *fhbp* were comparable and partially complementary. *fhbp* constitutes an effective substitute for *fetA* as a routine marker of antigenic distribution, thereby reducing costs in conjunction with *fhbp* surveillance. MLST afforded markedly superior resolution overall and is the optimal scheme for investigating outbreaks in which (i) typing data are unavailable for the index case or (ii) the index case possesses a known, predominant capsular group-*porA* repertoire.

Neisseria meningitidis (the meningococcus) causes meningitis and septicemia, with an associated mortality approaching 10% and severe physical and neurological sequelae in approximately 20% of survivors (25). In previously healthy individuals, invasive disease is almost exclusively associated with encapsulated meningococci. It is this capsule (or lack thereof) that forms the basis of the primary classification scheme termed "grouping" (34). The vast majority of invasive meningococcal disease (IMD) is caused by just five (out of 13 known) meningococcal capsular groups, A, B, C, W135, and Y, each displaying distinct geographical tendencies (13). In addition to being the principal epidemiological marker, the existence of glycoconjugate vaccines against groups A, C, W135, and Y (23) bestows upon grouping an important role in directing the management of case contacts. With its relatively low resolution, however, grouping is of limited use when investigating potential outbreaks, especially in countries such as the United Kingdom, in which 90% of IMD is due to group B meningococci

(MenB) (17, 18). Increased resolution has traditionally been obtained by immunological characterization of the major outer membrane proteins (OMPs) PorB (serotyping) and PorA (serosubtyping) (9). Genotypic characterization of these antigens increases resolution further and captures variants for which immunological reagents are unavailable (21). Further resolution may be achieved by genotyping additional antigens, e.g., FetA (previously FrpB), a diverse and immunogenic iron-regulated OMP with a variable region (VR) comparable to those of PorA (30). Another genotypic technique, multilocus sequence typing (MLST), involves genetic analysis of ~500-bp regions of seven housekeeping genes distributed around the genome (6, 20). These genes, unlike those encoding PorA and other surface antigens, are under stabilizing selection, making MLST ideal for tracking long-term epidemiology on national and international scales (2).

In addition to providing enhanced resolution, genotypic methods are rapid, highly portable, and readily adapted for use with noncultured specimens, the proportion of which have increased in recent years (11) probably due to early antibiotic intervention (4). Therefore, where phenotypic data are not specifically required, molecular typing is regarded as the routine typing method of choice (15). To harmonize typing among reference laboratories, recommendations have been published regarding both the choice of targets and the nomenclature employed (15). MLST was recommended for application to (at

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least) a representative proportion of case and carriage isolates to guide national and international disease management and to facilitate studies of population biology and evolution. For investigating potential outbreaks and monitoring antigenic distribution, the *porA* VRs 1 and 2 and the *fetA* VR were recommended. *porB* genotyping was recommended where additional resolution is required, but this was dropped from the recommended nomenclature that takes the following form: sero-/genogroup:*porA* VR1,*porA* VR2:*fetA* VR:MLST sequence type (ST) (clonal complex [cc]), e.g., B:P1.19-1,15-11:F5-1: ST269 (cc269). The *porA* semivariable region VR3 was omitted from the proposed scheme, as it is less diverse and considered to add little information for the additional work its determination entails (15). The recommendation of *porB* genotyping is, perhaps, surprising. Unlike those of PorA, the epitopes of PorB are largely nonlinear and correspond to up to six variable regions (32, 36). The scheme is therefore relatively complex, entails an extended sequencing protocol (15, 31), and currently lacks a standardized nonculture procedure (8). Uptake of *porB* genotyping has been low among European reference laboratories, only two of which returned *porB* genotyping data for the 1st EU-IBIS external quality assessment (EQA) culture distribution. No data were subsequently reported for the 2nd distribution (5). As such, the genotyping of *porB* has not been adopted by the Health Protection Agency Meningococcal Reference Unit (HPA MRU).

Since the MenB capsule has largely been abandoned as a potential vaccine candidate, due to its poor immunogenicity in humans and the potential for eliciting autoimmune antibodies (35), a number of protein alternatives are currently being investigated. Two such investigational vaccines, in the advanced stages of development, are Novartis Vaccines' four-component MenB (4CMenB; previously rMenB-OMV) investigational vaccine (10) and the Pfizer (formerly Wyeth Vaccine Research) investigational MenB vaccine (24), currently undergoing phase III and phase II clinical trials, respectively. A common component to both of these vaccines is factor H-binding protein (fHBP; previously known as genome-derived neisserial antigen 1870 [GNA1870] or lipoprotein 2086 [LP2086]). fHBP is a virulence factor that recruits human factor H to the bacterial surface, thereby downregulating complement-mediated killing (19, 26). It exists in two immunologically distinct subfamilies, subfamily A (comprising phylogenetic variant groups 2 and 3) and subfamily B (comprising phylogenetic variant group 1) (7, 22). Nomenclature schemes for individual subvariants are available on the fHBP allele and peptide database hosted at http://pubmlst.org/neisseria. Another investigational vaccine with an fHBP component (currently undergoing phase I clinical trials) is that of the Walter Reed Army Institute of Research (37).

The Joint Committee on Vaccination and Immunization has, therefore, recommended modification of the current English and Welsh surveillance program (27) to incorporate the routine genotypic characterization of *fhbp*. The expansion of the current system would put additional pressure on reference laboratories both financially and in terms of labor. If *fhbp* (for which an established and extensive database is hosted at http://Neisseria.org) should be found to provide equal or greater resolution compared with *fetA*, then it may be argued that routine characterization of *fetA*, for the purpose of general antigenic monitoring and outbreak investigation, is in excess of the requirement and may be discontinued. This is especially the case if both antigens are found to resolve the same isolates. Conversely, if they were found to complement one another in their resolving power or if a FetA vaccine is introduced, it may be deemed beneficial, or indeed necessary, to retain both in routine typing.

This study aimed to determine the optimal and most efficient molecular typing schemes for (i) routine meningococcal characterization and (ii) the investigation of potential outbreaks, in conjunction with the routine surveillance of *fhbp*. The resolving power of *fetA*, *fhbp*, MLST, and combinations thereof were determined across the full range of invasive disease isolates submitted to the HPA MRU during the epidemiological year from July 2007 to June 2008. The cumulative resolving power of (i) the capsular group, (ii) *porA* VRs 1 and 2, and (iii) *fetA* and/or *fhbp* and/or the ST were also investigated among the predominant lineages identified. The additional resolution afforded by *porA* VR3 was also investigated, and we report on the outcomes of case cluster (two or more spatially related cases of probable or confirmed invasive disease with dates of onset within 4 weeks) investigations carried out by the HPA MRU over the period studied and how these relate to the overall study findings.

MATERIALS AND METHODS

Specimens. Isolates used in the study ($n = 613$) consisted of all English and Welsh invasive disease isolates received by the HPA MRU during the epidemiological year from July 2007 to June 2008, inclusively. Isolates were preserved at $-80°C$ on Microbank cryovials (Prolab Diagnostics, Ontario, Canada). Overnight cultures were performed on Columbia agar plus 5% (vol/vol) horse blood (Oxoid, Basingstoke, United Kingdom) at 37°C in an atmosphere containing 5% CO_2. Additionally, routinely obtained genotypic data (genogroup and genosubtype) pertaining to nonculture cases ($n = 12$) (specimens may include EDTA whole blood, coagulated whole blood, cerebrospinal fluid, serum, or plasma) have been reported for cases in which they formed part of a cluster investigation over the period specified.

Capsular grouping. Sero-/genogroup characterization was performed by the HPA MRU at the point of receipt, as described by Gray et al. (11).

Genomic DNA extraction. Genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Crawley, United Kingdom). Briefly, 1 ml meningococcal suspensions in physiological saline (adjusted to an optical density equal to 0.1 at A_{650}) were heat killed at 60°C for 70 min and then pelleted at $6,000 \times g$ for 10 min. Following aspiration of the supernatant, DNA was extracted using the manufacturer's Gram-negative protocol (Qiagen).

Molecular typing. Primers used for PCRs and sequence analyses are listed in Table S1 in the supplemental material. Thermocycle conditions can be viewed in Table S2 in the supplemental material. Thermocycling was performed on a Veriti 96-well thermal cycler (Applied Biosystems, CA).

PCR. All PCRs were performed using the HotStarTaq DNA polymerase kit (Qiagen). PCR products were electrophoresed on 2% agarose gels. Target-specific methods were as follows: *fhbp* PCRs were performed in 25-μl reactions. Final concentrations were 10× PCR buffer (1×), $MgCl_2$ (3.25 mM), forward primer (0.5 μM), reverse primer (0.5 μM), dNTPs (200 μM per dNTP), and *Taq* polymerase (0.625 units/reaction). Each reaction mixture contained 2 μl of extracted genomic DNA template. *fhbp* PCR products were cleaned using Exo-SAP-IT (exonuclease I and shrimp alkaline phosphatase) (USB Corporation, Cleveland, OH), according to the manufacturer's instructions. Cleaned PCR products were diluted 1 in 3 in nuclease-free water prior to sequencing.

porA, *fetA*, and MLST PCRs were performed in 50-μl reaction mixtures. Final concentrations were 10× PCR buffer (1×), MgCl (1.5 mM), forward primer (0.5 μM), reverse primer (0.5 μM), dNTPs (200 μM per dNTP), and *Taq* polymerase (1 unit/reaction). Each reaction mixture contained 1 μl of extracted genomic DNA template. *porA*, *fetA*, and MLST PCR products were cleaned using the Millipore multiscreen plate (Millipore, MA), according to the manufacturer's protocol.
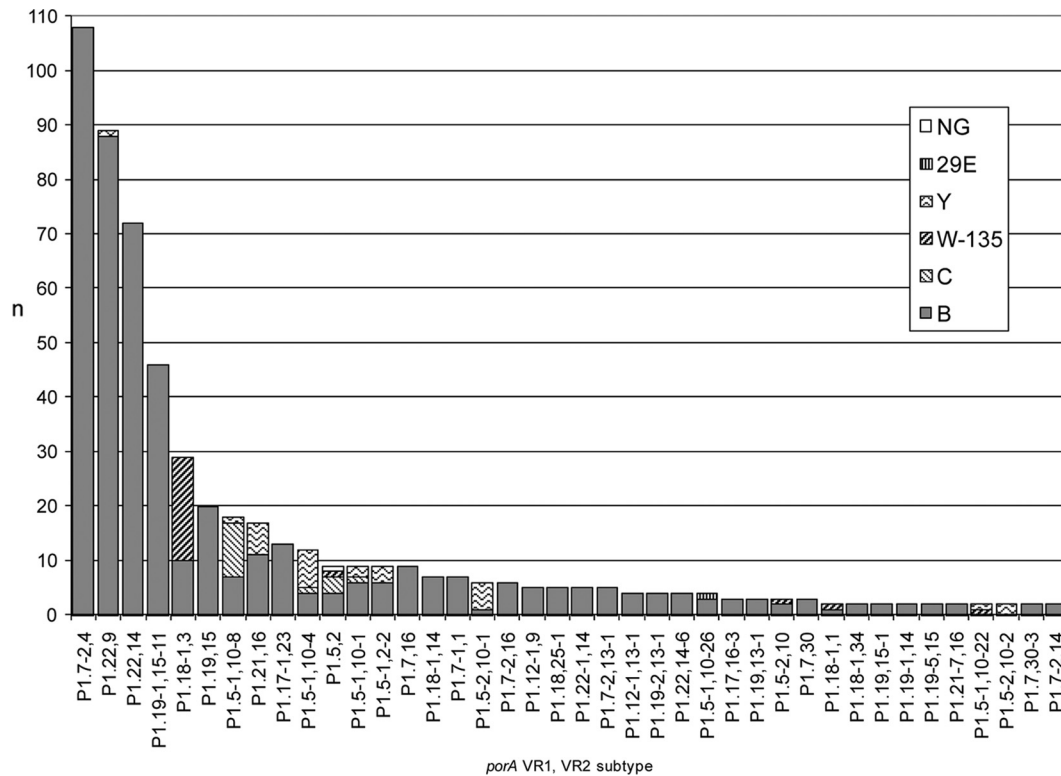
FIG. 1. *porA* subtypes (representing >1 isolate) among English and Welsh meningococcal invasive disease isolates from the epidemiological year 2007-2008, as resolved by capsular group. Collectively, the *porA* subtypes plotted (*n* = 40) represent 554/613 (90.3%) of the isolates under investigation. Each subtype is broken down into its constituent capsular groups. A further 57 *porA* subtypes (not plotted) represented a single isolate each (9.3% of total isolates). Two further isolates (not plotted; 0.3% of total isolates) gave no *porA* PCR product. NG, nongroupable; *n*, number of isolates.

Sequence analyses. *fhbp* and MLST sequence analyses were performed using the BigDye version 3.1 (v3.1) kit (Applied Biosystems) in 1-in-8-strength reactions, according to the manufacturer's instructions. Final primer concentrations were 0.33 μM, and each reaction mixture contained 1 μl of cleaned PCR product as a template.

fetA and *porA* sequence analyses were performed using the BigDye v1.1 kit (Applied Biosystems) in 1-in-16-strength reactions, according to the manufacturer's instructions. Final primer concentrations were 0.33 μM (*fetA*) and 0.66 μM (*porA*), and each reaction mixture contained 1 μl of cleaned PCR product as a template.

All sequencing products were cleaned using ethanol-sodium acetate cleanup, resuspended in 15 μl HiDi formamide (Applied Biosystems), and analyzed on a 3130xl sequence analyzer (Applied Biosystems). Contig assembly and manual adjustment of bases were performed using Sequencher v4.8 (Gene Codes Corporation, MI).

Assessing resolving power among typing schemes. The resolving power of *fetA*, *fhbp*, MLST, and combinations thereof (*fetA-fhbp* and *fetA-fhbp*-ST), were each determined across the full range of isolates using Simpson's index of diversity (*D*) (14, 28), a measure of the probability that two unrelated strains sampled from a test population would be allocated to different types. The resolving power of *fetA*, *fhbp*, MLST, and combinations thereof were also determined within the predominant capsular group-*porA* VR1/VR2 combinations identified. Values for *D* and 95% confidence intervals were obtained according to the methods described by Hunter and Gaston (14) and Grundmann et al. (12), respectively.

RESULTS

Capsular grouping. The capsular groups represented among the isolates were B (87.9%; *n* = 539/613), Y (4.6%; *n* = 28), W135 (3.9%; *n* = 24), C (2.6%; *n* = 16), 29E (0.3%; *n* = 2), A (0.2%; *n* = 1), and Z (0.2%; *n* = 1). Two isolates (0.3%)

[P1.5,2,36-2:ST60 (cc60) and P1.18-23,25-1,38-1:ST198 (cc198), respectively] remained ungrouped, having tested negative in serological assays to detect capsular groups A, B, C, X, Y, Z, 29E, and W135 and in genotypic assays (*siaD*) to detect capsular groups B, C, Y, and W135.

***porA* subtyping.** Two isolates gave no PCR product for *porA*. The 611 confirmed *porA*+ isolates represented 97 different VR1/VR2 combinations, 57 of which accounted for just a single isolate each (9.3% of isolates). Of the 40 remaining subtype combinations (Fig. 1), 30 accounted for between 2 and 10 isolates (21.3%; *n* = 130, collectively). The 10 major subtypes (*n* ≥ 12 isolates) (69.4%; *n* = 424, collectively) were P1.7-2,4 (17.7%; *n* = 108), P1.22,9 (14.6%; *n* = 89), P1.22,14 (11.8%; *n* = 72), P1.19-1,15-11 (7.5%; *n* = 46), P1.18-1,3 (4.7%; *n* = 29), P1.19,15 (3.3%; *n* = 20), P1.5-1,10-8 (2.9%; *n* = 18), P1.21,16 (2.8%; *n* = 17), P1.17-1,23 (2.1%; *n* = 13), and P1.5-1,10-4 (2.0%; *n* = 12).

Capsular group and *porA* VR1 and VR2 combined. *porA* VR1/VR2 combinations representing >1 isolate were initially resolved in terms of capsular group (Fig. 1). Of the 10 major VR1/VR2 combinations identified, P1.7-2,4, P1.22,14, P1.19-1,15-11, P1.19,15, and P1.17-1,23 consisted solely of MenB isolates. P1.22,9 was predominantly MenB, with a single exception (Y:P1.22-9). The remaining major *porA* VR1/VR2 combinations included minor (non-B) capsular groups in greater proportions, with P1.18-1,3 comprising W135 (65.5%;

$n = 19$) and B (34.5%; $n = 10$), P1.5-1,10-8 comprising C (55.6%; $n = 10$), B (38.9%; $n = 7$), and Y (5.6%; $n = 1$), P1.21,16 comprising B (64.7%; $n = 11$) and Y (35.3%; $n = 6$), and P1.5-1,10-4 comprising Y (58.3%; $n = 7$), B (33.3%; $n = 4$), and C (8.3%; $n = 1$) isolates.

Several major ($n > 12$ isolates) capsular group-*porA* VR1/VR2 combinations predominated (representing 366/613 isolates; 59.7%, collectively). These were B:P1.7-2,4 ($n = 108$; 17.6%), B:P1.22,9 ($n = 88$; 14.4%), B:P1.22,14 ($n = 72$; 11.7%), B:P1.19-1,15-11 ($n = 46$; 7.5%), B:P1.19,15 ($n = 20$; 3.3%), W135:P1.18-1,3 ($n = 19$; 3.1%), and B:P1.17-1,23 ($n = 13$; 2.1%). Among these, the four most populous, B:P1.7-2,4, B:P1.22,9, B:P1.22,14, and B:P1.19-1,15-11, accounted for 314/366 isolates (85.8%; 51.2% overall). These constitute the capsular group-*porA* VR1/VR2 combinations requiring the greatest degree of additional resolution from additional typing schemes, e.g., when investigating potential outbreaks.

***fetA*, *fhbp*, and ST.** All isolates were characterized in terms of *fetA*, *fhbp*, and ST. The resolving powers of each scheme and the combinations thereof were compared (i) across all 613 isolates collectively and (ii) within each of the predominant capsular group-*porA* VR1/VR2 combinations identified. Values for the diversity index (D), the number of types resolved (n_t) and the frequency of the predominant type (f_{max}) for each individual scheme/combination are shown in Table 1. Individual frequencies for all identified types within the predominant capsular group-*porA* VR1/VR2 combinations are plotted schematically in Fig. 2. In all cases, where applicable, significance was inferred by the existence of nonoverlapping 95% confidence intervals.

(i) *fetA* versus *fhbp*. Among all 613 isolates collectively, *fhbp* afforded significantly better discrimination than that afforded by *fetA*. *fhbp* also afforded superior D values within four of the seven predominant capsular group-*porA* VR1/VR2 combinations, including the second and third most populous (B:P1.22,9 and B:P1.22,14). In the case of B:P1.22,9, both schemes resolved an equal number of types ($n_t = 13$); however, the isolates were more evenly distributed by *fhbp*, the predominant type of which was therefore less frequent ($f_{max} = 43.2\%$ versus 63.6%). For B:P1.22,14, *fhbp* resolved 61.5% more types ($n_t = 21$ versus 13) and yielded a less-frequent predominant type ($f_{max} = 37.5\%$ versus 54.2%). *fetA* afforded greater D values among two of the remaining three predominant capsular group-*porA* VR1/VR2 combinations, including the fourth most populous (B:P1.19-1,15-11), in which it was marginally superior in terms of D, f_{max}, and n_t. Both schemes performed relatively poorly in this case, however ($D = 20.6\%$ and 4.3%, respectively). Against the most populous capsular group-*porA* VR1/VR2 combination (B:P1.7-2,4), *fetA* and *fhbp* afforded comparable levels of discrimination ($D = 44.7\%$ versus 45.0%, respectively). This was despite *fetA* resolving 45.5% more types overall ($n_t = 16$ versus 11), since the predominant type in each case was similarly (and considerably) frequent ($f_{max} = 74.1\%$ and 73.1%, respectively). Within the predominant capsular group-*porA* VR1/VR2 combinations alone, 95% confidence intervals were unable to confer significance to any differences in D values between *fetA* and *fhbp*.

(ii) *fetA* or *fhbp* versus *fetA-fhbp*. Among all 613 isolates collectively, the combination *fetA-fhbp* afforded significantly better discrimination than either of the antigens individually.

TABLE 1. Resolving power of *fetA*, *fhbp*, *fetA-fhbp*, ST, and *fetA-fhbp-ST* against English and Welsh meningococcal invasive disease isolates from the epidemiological year 2007-2008[a]

Typing scheme/typing scheme combination	All isolates (n = 613)			B:P1.7-2,4 (n = 108)			B:P1.22,9 (n = 88)			B:P1.22,14 (n = 72)			B:P1.19-1,15-11 (n = 46)			B:P1.19,15 (n = 20)			W135:P1.18-1,3 (n = 19)			B:P1.17-1,23 (n = 13)		
	n_t	f_{max}	D (95% CI)	n_t	f_{max}	D (95% CI)	n_t	f_{max}	D (95% CI)	n_t	f_{max}	D (95% CI)	n_t	f_{max}	D (95% CI)	n_t	f_{max}	D (95% CI)	n_t	f_{max}	D (95% CI)	n_t	f_{max}	D (95% CI)
fetA	49	22.8	88.1 (86.7-89.5)	16	74.1	44.7 (33.0-56.4)	13	63.6	57.3 (46.0-68.7)	13	54.2	68.7 (57.6-79.8)	4	89.1	20.6 (5.2-35.9)	5	55.0	68.9 (48.9-89.0)	5	78.9	38.6 (11.6-65.6)	4	46.2	71.8 (57.3-86.3)
fhbp	86	19.6	90.9 (89.7-92.0)	11	73.1	45.0 (33.9-56.1)	13	43.2	69.8 (63.6-76.0)	21	37.5	83.8 (76.5-91.2)	2	97.8	4.3 (0-12.4)	3	65.0	57.9 (34.0-81.8)	3	73.7	43.3 (21.0-65.6)	7	30.8	84.6 (72.4-96.8)
fetA-fhbp	195	16.0	94.9 (93.9-95.9)	26	57.4	66.0 (56.0-76.1)	25	37.5	81.3 (74.9-87.6)	35	31.9	88.9 (82.6-95.2)	9	89.1	20.7 (5.2-36.2)	6	55.0	70.5 (49.2-91.9)	6	57.9	64.3 (43.9-84.7)	8	30.8	89.7 (79.3-100)
ST	267	8.2	98.1 (97.7-98.5)	46	38.0	84.3 (77.8-90.7)	36	22.7	90.3 (86.6-94.0)	29	44.4	79.4 (69.9-88.8)	14	35.0	81.6 (71.2-92.1)	10	21.1	90.6 (84.5-96.8)	7	30.8	84.6 (72.4-96.8)	7	30.8	84.6 (72.4-96.8)
fetA-fhbp-ST	379	7.3	98.9 (98.6-99.2)	56	33.3	88.3 (82.7-93.9)	51	20.5	94.5 (91.3-97.6)	49	22.2	95.0 (91.0-98.9)	23	32.6	87.9 (80.2-95.7)	14	21.1	95.3 (89.7-100)	10	23.1	94.9 (87.9-100)	10	23.1	94.9 (87.9-100)

[a] The resolving power of each typing scheme/combination of typing schemes is given for (i) all of the isolates collectively (all isolates; $n = 613$) and (ii) for all isolates harboring each of the seven predominant capsular groups: *porA* VR1/VR2 combinations identified in the corresponding year (B:P1.7-2,4, B:P1.22,9, B:P1.22,14, B:P1.19-1,15-11, B:P1.19,15, W135:P1.18-1,3, and B:P1.17-1,23). Resolving power is reported in terms of Simpson's index of diversity (D) (%) and is supplemented by the number of individual types resolved (n_t) and the frequency of the predominant type (f_{max}). n_t, number of individual types defined by typing scheme/scheme combination; f_{max}, frequency of predominant type (%).
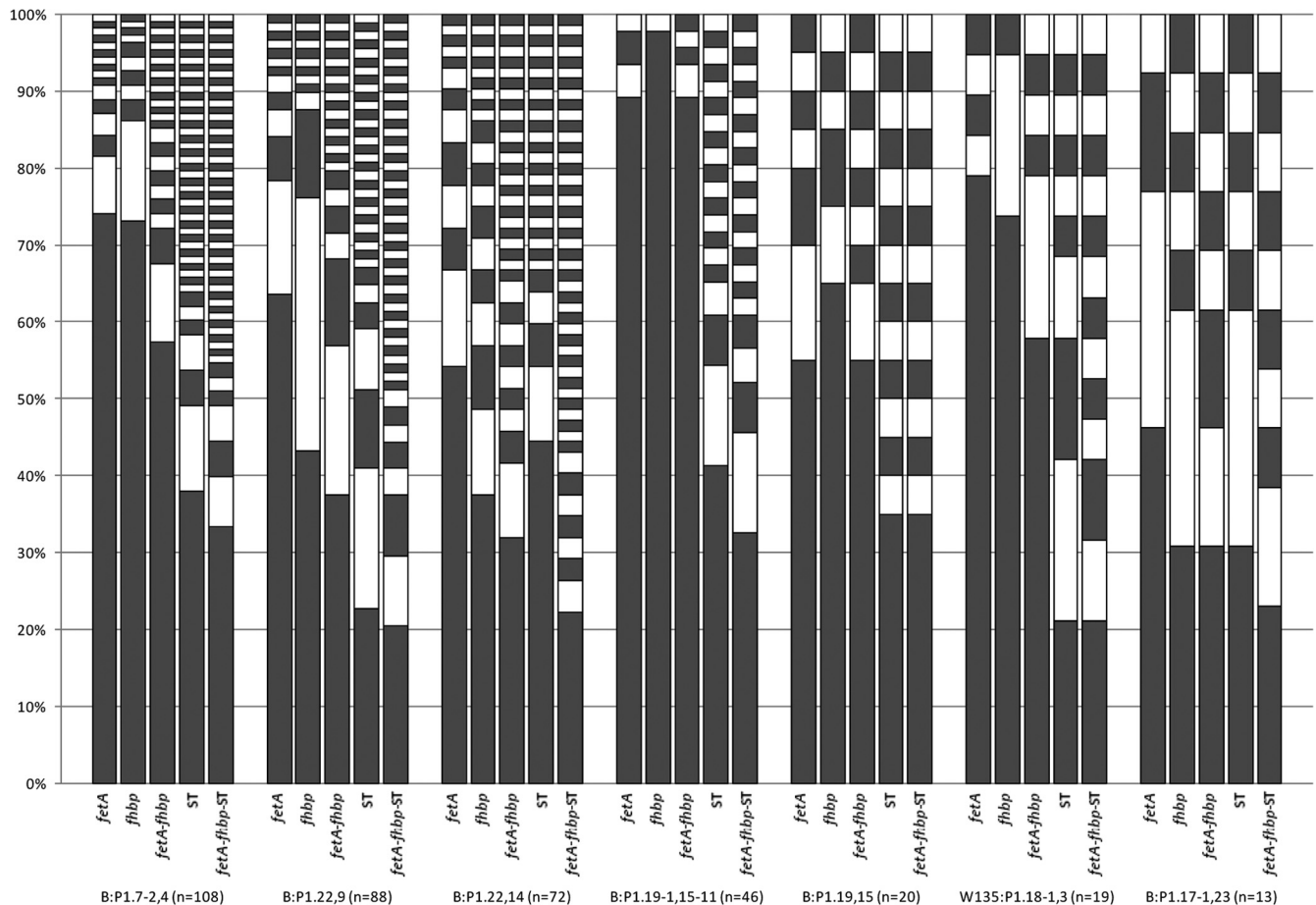
FIG. 2. Resolution of predominant capsular group-*porA* VR1/VR2 combinations by *fetA*, *fhbp*, *fetA-fhbp*, ST, and *fetA-fhbp*-MLST among English and Welsh meningococcal invasive disease isolates from the epidemiological year 2007-2008. For each capsular group-*porA* VR1/VR2 combination, all individual types (each represented by an individual white or gray block) and their respective frequencies (size of block) are plotted for each of the typing schemes/typing scheme combinations under investigation, i.e., *fetA*, *fhbp*, *fetA-fhbp*, ST, and *fetA-fhbp*-MLST. The greater the number of types and the broader the distribution of types, the greater is the resolution afforded by the corresponding typing scheme. White/gray shading is arbitrary and intended to maximize contrast, thereby facilitating comparisons.

fetA-fhbp was also largely superior within the predominant capsular group-*porA* VR1/VR2 combinations. Exceptions were B:P1.19-1,15-11 and B:P1.19,15, in which *fetA-fhbp* performed comparably with *fetA* alone (D = 20.7% versus 20.6%, and 70.5% versus 68.9%, respectively). In all cases, the predominant *fetA-fhbp* type comprised the predominant individual *fetA* and *fhbp* types for the respective capsular group-*porA* VR1/VR2 combination. Nonoverlapping 95% confidence intervals indicated that *fetA-fhbp* afforded significantly better discrimination than *fetA* alone versus the second and third most populous capsular group-*porA* VR1/VR2 combinations, B:P1.22,9 and B:P1.22,14. Significant differences in D values could not be inferred among the remaining predominant capsular group-*porA* VR1/VR2 combinations, with respect to *fetA-fhbp* versus *fetA* or *fhbp* alone.

(iii) *fetA* or *fhbp* versus ST. Among all 613 isolates collectively, the ST afforded significantly better resolution than that of either *fetA* or *fhbp*. Against the predominant capsular group-*porA* combinations, the D values afforded by MLST exceeded those of *fetA* and *fhbp* for seven and five of the predominant capsular group-*porA* VR1/VR2 combinations, respectively.

The difference was shown to be significant (against both individual antigenic typing schemes) for three of the four most populous capsular group-*porA* VR1/VR2 combinations (B:P1.7-2,4, B:P1.22,9, and B:P1.19-1,15-11) and the sixth most populous (W135:P1.18-1,3). Against the third most populous capsular group-*porA* VR1/VR2 combination (B:P1.22,14), *fhbp* afforded superior D and f_{max} values versus those of MLST, despite MLST defining 38.0% more types (n_t = 29 [MLST] versus 21 [*fhbp*]). In this case, however, the difference was not shown to be significant. MLST and *fhbp* performed equally in terms of D, f_{max}, and n_t against the least populous capsular group-*porA* VR1/VR2 combination, B:P1.17-1,23.

(iv) *fetA-fhbp* versus ST. Among all 613 isolates collectively, ST afforded significantly better resolution than that of combined *fetA-fhbp*. ST also afforded greater D values within five of the seven predominant capsular group-*porA* VR1/VR2 combinations. Nonoverlapping 95% confidence intervals conferred significance in the case of the most populous combination (B:P1.7-2,4) and the fourth most populous (B:P1.19-1,15-11), for which the difference was considerable (D = 81.6% versus 20.7%). *fetA-fhbp* afforded greater D values for the remaining

TABLE 2. Resolution of *porA* VR3 outliers within predominant capsular group-*porA* (VR1 andVR2) populations among English and Welsh meningococcal invasive disease isolates from the epidemiological year 2007-2008[a]

Population (*n*)	Total no. of *porA* VR3 outliers	No. of *porA* VR3 outliers further discriminated by VR3 after initial typing with:				
		fetA	*fhbp*	*fetA-fhbp*	MLST	*fetA-fhbp-MLST*
B:P1.7-2,4 (108)	6	6	6	4	2	2
B:P1.22,9 (88)	5	3	5	3	4	2
B:P1.22,14 (72)	2	2	2	1	1	0
B:P1.19-1,15-11 (46)	1	1	1	1	1	1
W135:P1.18-1,3 (19)	1	0	1	1	1	0

[a] Where applicable, the total number of *porA* VR3 outliers, i.e., those not harboring the respective predominant *porA* VR3 subtype, is reported for each of the predominant capsular group-*porA* populations. Also reported are the number of these *porA* VR3 outliers which, following initial resolution of their respective capsular group-*porA* population by a given typing scheme, can still be more finely discriminated by virtue of their outlying *porA* VR3. For example, in the case of B:P1.7-2,4 there are a total of six *porA* VR3 outliers among 108 isolates. Following initial resolution of B:P1.7-2,4 by MLST, however, only two of the original *porA* VR3 outliers can be more finely discriminated by virtue of their *porA* VR3.

two combinations, i.e., those for which *fhbp* alone was superior. In neither case, however, was the difference shown to be significant.

(v) *fetA-fhbp*-ST versus all. Among all 613 isolates collectively, a combination of all three schemes (*fetA-fhbp*-ST) afforded significantly better resolution than any of the respective schemes individually or the combination *fetA-fhbp*. *fetA-fhbp*-ST also afforded superior D, f_{max}, and n_t values within all of the predominant capsular group-*porA* VR1/VR2 combina-

tions except B:P1.19,15, for which ST alone performed equally in all respects, and W135:P1.18-1,3, for which ST afforded an equal value for f_{max}. With respect to the predominant capsular group-*porA* VR1/VR2 combinations, nonoverlapping 95% confidence intervals were able to confer significance to the superiority of *fetA-fhbp*-MLST over ST alone only in the case of B:P1.22,14.

porA **VR3.** Among the seven predominant capsular group-*porA* VR1/VR2 combinations described, B:P1.19,15 and B:P1.17-1,23 were each homogeneous with respect to *porA* VR3, which therefore afforded no further resolution. The remaining five combinations were also relatively conserved in terms of VR3; however, a number of VR3 "outliers" existed for each, comprising 6/108 (5.6%) B:P1.7-2,4 isolates (across four additional VR3s), 5/88 (5.7%) B:P1.22,9 isolates (across five additional VR3s), 2/72 (2.8%) B:P1.22,14 isolates (across two additional VR3s), 1/46 (2.2%) B:P1.19-1,15-11 isolates, and 1/19 (5.3%) W135:P1.18-1,3 isolates. With the exception of *fetA* in W135:P1.18-1,3 and of *fetA-fhbp*-ST in B:P1.22,14 and W135:P1.18-1,3, the resolution afforded by VR3 was, at least partially, complementary to that of the additional typing schemes compared herein (Table 2).

Cluster investigation. Over the period studied, the HPA MRU investigated 20 clusters in terms of capsular group and *porA* VR1, VR2, and VR3 (11). Seven clusters were incompletely characterized due to negative or indeterminate PCR results. Details of the 13 characterized clusters can be viewed in Table 3. The constituent cases of four of the clusters could be differentiated on the basis of capsular group-*porA* VR1/VR2 and so were deemed to be unrelated. With the exception of several more-diffuse regional cases, the constituent cases of

TABLE 3. Meningococcal case clusters characterized by the HPA MRU over the epidemiological year 2007-2008

Cluster no.	No. of cases	Nature of specimens	Typing data	Spatiotemporal data (interval [days])
1	2	Culture	B:P1.22,14,36	Patients attended same school (12)
		Culture	B:P1.22,9,35-1	
2	2	Culture	29E:P1.18-7,9,35-1	Patient/career contact (16)
		Culture	W135:P1.18-1,3,38	
3	2	Nonculture	B:P1.5-1,10,36-2	Patients lived locally, close-knit community (3)
		Culture	B:P1.7-2,4,37	
4	2	Culture	Y:P1.5,10	Patients attended same college (2)
		Culture	B:P1.4	
5	2	Nonculture	B:P1.7-2,4,37	Extended-family members (<1)
		Nonculture	B:P1.7-2,4,37	
6	2	Nonculture	B:P1.19,13-1,35-1	Patients attended same school (2)
		Nonculture	B:P1.19,13-1,35-1	
7	2	Nonculture	B:P1.22,14,36	Extended family with mutual friends (29) Previous, indistinguishable case occurred on same road (156)
		Nonculture	B:P1.22,14,36	
8	2	Nonculture	B:P1.17-1,23,37	Patient/health professional contact (5)
		Culture	B:P1.17-1,23,37	
9	2	Culture	B:P1.22,9,35-1	Patients attended same nursery (9)
		Nonculture	B:P1.22,9,35-1	
10	2	Nonculture	B:P1.7-2,4,37	Patients attended same work party (7)
		Nonculture	B:P1.7-2,4,37	
11	2	Nonculture	B:P1.7-2,4,37	Work colleagues, same department (1)
		Culture	B:P1.7-2,4,37	
12	2	Culture	B:P1.7-2,4,37:F1-5:ST41 (cc41/44); *fhbp*, 1.4	Extended-family members, attended same school (1)
		Culture	B:P1.7-2,4,37:F1-5:ST41 (cc41/44); *fhbp*, 1.4	
13	2	Culture	B:P1.19-5,15,36:F5-1:ST479 (cc269); *fhbp*, 1.2	Patients attended same school, different classes (8)
		Culture	B:P1.19-5,15,36:F5-1:ST479 (cc269); *fhbp*, 1.2	

the nine remaining clusters were not differentiated on this basis. Two of these provided cultures in all constituent cases and so (in the course of this study) were further characterized in terms of *fetA*, *fhbp*, and ST. In neither case did the additional characterization serve to differentiate the constituent isolates. The remaining seven undifferentiated clusters provided only noncultured or a mixture of cultured and noncultured specimens and so were not characterized in terms of *fetA*, *fhbp*, and ST.

DISCUSSION

Several investigational MenB vaccines, including two in the advanced stages of development, incorporate fHBP. The antigen has therefore been recommended for routine genotypic surveillance in England and Wales. This study aimed to evaluate the discriminatory value of *fhbp* and several existing (sero-/genogroup, *porA* subtype, and MLST) and recommended (*fetA*) typing schemes to investigate the optimal and most efficient typing repertoire for (i) routine antigenic monitoring and (ii) the investigation of potential outbreaks. All invasive disease isolates received by the HPA MRU in 2007-2008 were characterized in terms of capsular group, *porA* VRs 1, 2, and 3, *fetA* VR, *fhbp*, and ST.

When attempting to differentiate between unrelated isolates, the ideal typing scheme would afford a diversity index of 100% (complete diversity); however, a value of >90% is widely regarded as desirable (14, 33). In this respect, *fhbp* performed significantly better than *fetA* against all invasive disease isolates received by the HPA MRU in the epidemiological year 2007-2008. Both loci are comparable in terms of their proximity to the *porA* typing locus (~506 kb versus ~626 kb) (29), and both are covered by extensive databases at http://pubmlst.org. They each also encode candidate MenB vaccine antigens; however, FetA-based vaccines are in the relatively early stages of development, whereas fHBP-containing vaccines are likely to be licensed in the near future. The ensuing requirement for the routine surveillance of *fhbp* and its comparability with *fetA* in terms of diversity and genomic proximity to the core antigenic typing locus (*porA*) suggest that *fhbp* would be an effective substitute for *fetA* as a means of routine antigenic monitoring, thereby saving on costs and labor. Therefore, the HPA MRU shall not be adopting *fetA* as a routine target for the purpose of monitoring antigenic distribution. A nonculture protocol is currently being devised for *fhbp* to account for the high proportion of nonculture, laboratory case confirmations in England and Wales (approximately 60%).

The ability to distinguish between unrelated isolates is crucial when investigating potential outbreaks, so as to avoid unnecessary intervention beyond close contacts. Against all invasive disease isolates received by the HPA MRU in the epidemiological year 2007-2008, ST afforded significantly better discrimination than either *fetA* or *fhbp*. The combination *fetA-fhbp* performed significantly better than either *fetA* or *fhbp* individually; however, the individual schemes often differentiated the same groups of isolates, such that their respective predominant types tended to coincide. As such, ST also performed significantly better than the combination *fetA-fhbp*. The superiority of ST was magnified among the predominant capsular group-*porA* combinations in which *fetA* and *fhbp* af-

forded inconsistent and often poor levels of discrimination. The range of *D* values afforded by ST against these populations was 79.4% to 90.6% (versus 20.7% to 89.7% for *fetA-fhbp*). Even against the predominant capsular group-*porA* combination B:P1.7-2,4 (representing approximately 1 in 6 isolates across the entire year), *fetA-fhbp* afforded a *D* value of only 66.0% compared with 84.3% for ST. Thus, when investigating a potential outbreak (or cluster) in which (i) typing data are not yet available for the index case or (ii) the index case possesses a known predominant capsular group-*porA* VR1/VR2 repertoire, it seems prudent to immediately apply MLST (alongside routine characterization, including *fhbp*) to all potentially related cases. Conversely, where the index case is non-MenB or possesses a minor capsular group-*porA* subtype (approximately 40% of isolates in 2007-2008), MLST may not be required since routine antigenic typing would likely differentiate any unrelated cases. The finding that MLST affords greater resolution than either *fetA* or *fhbp* is not, perhaps, surprising, given that there are seven loci characterized by MLST (versus just one or two loci for *fetA* or *fhbp* or *fetA-fhbp*). While this bias undoubtedly contributed to the superiority of MLST in resolving the isolates in this study, it is possible that combinations of housekeeping alleles are inherently better suited to this purpose. A study by Buckee et al. (3) showed strong temporal associations between the *fetA* VR and those of *porA*, with minimal overlap between combinations. Conversely, the MLST alleles (which displayed a strong lineage structure and temporally fluctuating antigenic associations) demonstrated considerable overlap among the various combinations, thus allowing more combinations among isolates with a common *porA* subtype.

From a practical point of view, MLST is already adapted for nonculture work (1) and so is available as a tool for outbreak investigation both in the presence and absence of laboratory culture. The relative resource and labor intensity of MLST are unlikely to preclude its use in outbreak investigations since the indication for its immediate use is likely to be a relatively rare occurrence (over the epidemiological year studied, the HPA MRU investigated 20 clusters as potential outbreaks), thus minimizing additional costs. The extended turnaround time associated with MLST applies to genotypic schemes in general. The HPA MRU, for example, quotes a minimum 3-working-day turnaround for *porA* genotyping (currently recommended for outbreak investigations). Therefore, any genotypic data pertaining to outbreak investigations may often be examined retrospectively after the initiation of management action based on, e.g., spatiotemporal evidence and more rapidly attainable serogrouping and serosubtyping data (routinely performed on cultures within the HPA MRU). A potential drawback pertaining to nonculture MLST in particular is its relatively high consumption of DNA template. Therefore, when DNA content is low, or in cases involving infants (from whom relatively low blood/cerebrospinal fluid [CSF] volumes are obtained), there may be insufficient sample remaining following preliminary molecular testing. Therefore, while MLST constitutes the optimal technique for investigating potential outbreaks, the scope of genotypic investigations may ultimately be limited by the volume/quality of the specimen received. A possible solution may be to employ a targeted approach in which the most discriminatory MLST alleles are prioritized, based on previous

data (local epidemiological knowledge), as has been alluded to previously (20).

Inclusion of *porA* VR3 further resolved up to 5.7% of the isolates among five of the predominant capsular group-*porA* VR1/VR2 combinations. Up to 100% of this additional resolution complemented that of *fetA*, *fhbp*, and ST, either alone or in combination. Therefore, while the additional resolution afforded by VR3 is limited, it seems wasteful not to process the data when it already exists (having been generated alongside that of VR2), especially when an outbreak is suspected. Consistent with the recommendations on molecular targets for meningococci (15), however, the limited potential for resolving clusters does not warrant the extra work of specifically pursuing VR3.

The finer aspects of typing applied in this study (*fetA*, *fhbp*, and MLST) were undertaken largely retrospectively. During the period under investigation, clusters were typically investigated by the HPA MRU using only sero-/genogrouping and *porA* genosubtyping (VR1, VR2, and VR3). The number of clusters investigated (*n* = 20) was too small to enable any statistical analyses; however, they serve to illustrate several important points. First, approximately a third of the clusters were incompletely characterized due to negative/indeterminate PCR results, reflecting, in some cases, poor specimen quality. This represents an area for future development in (i) increasing the sensitivity of nonculture laboratory methods and (ii) educating clinicians to ensure that optimal specimens are submitted. Second, the constituent cases of four of the clusters were differentiated on the basis of capsular group and *porA* alone, illustrating that even apparently strong spatiotemporal links can often be coincidental. Third, nine of the clusters were unresolved, which would serve to bolster decisions (initially based on strong spatiotemporal associations) regarding intervention among case contacts and possibly the wider community. Seven of these comprised cases with predominant capsular group-*porA* repertoires, thus reducing any certainty the cases were indeed linked and highlighting the need to apply MLST in order to maximize discriminatory potential. Even when MLST fails to discriminate, however, it should be remembered that, while an outbreak may be discounted with 100% certainty, the same is not true of outbreak confirmation, which therefore relies heavily on spatiotemporal and circumstantial evidence.

In summary, *fhbp* is to be included within the routine meningococcal typing scheme in England and Wales to monitor the potential coverage of fHBP-containing vaccines. The present study has shown *fhbp* and *fetA* to be comparable in terms of diversity. *fhbp* shall therefore provide a practical and effective substitute for *fetA* as a routine marker of antigenic variation in England and Wales. MLST outperformed *fetA*, *fhbp*, and *fetA-fhbp* when resolving the predominant capsular group-*porA* VR1/VR2 combinations. Therefore, in cases in which (i) typing data are not yet available for the index case or (ii) the index case possesses a predominant capsular group-*porA* VR1/VR2 repertoire, MLST should be the method of choice for investigating possible outbreaks. Genetic diversity tends to be higher among invasive capsular group B isolates (13); therefore, the situation described may be different in countries experiencing a greater balance among, e.g., capsular

groups, or indeed if additional targets are required for routine surveillance.

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