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Differences between culture & non-culture confirmed invasive meningococci with a focus on factor H-binding protein distribution

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Summary Objectives: To compare the distribution of capsular groups and factor H-binding protein (fHBP) variants among meningococcal isolates and non-culture clinical specimens and to assess the representativeness of group B isolates amongst group B cases as a whole. Methods: A PCR sequencing assay was used to characterise fHBP from non-culture cases confirmed from January 2011 to December 2013. These were compared to genotypic data derived from whole genome analysis of isolates received during the same period.

Results: Group W and Y strains were more common among isolates than non-culture strains. The distribution of fHBP variants among group B non-culture cases generally reflected that seen in the corresponding isolates. Nonetheless, the non-culture subset contained a greater proportion of fHBP variant 15/B44, associated with the ST-269 cluster sublineage. Conclusions: Differences in capsular group and fHBP distribution among culture and non-culture cases may be indicative of variation in strain viability, diagnostic practice, disease severity and/or clinical presentation. Future analyses combining clinical case information with laboratory data may help to further explore these differences. Group B isolates provide a good representation of group B disease in E&W and, therefore, can reliably be used in fHBP strain coverage predictions of recently-licensed vaccines.

Introduction

Hyper-virulent group B meningococci continue to cause a substantial proportion of meningococcal disease cases, particularly in Europe, North America and Australia.¹ The development and licensure of two novel protein-based vaccines offers the possibility of significantly reducing this disease burden.^{2,3} An antigenic constituent shared by both of these vaccines is factor H-binding protein (fHBP). This outer membrane protein has been shown to elicit immune protection against a diverse array of group B Neisseria meningitidis strains.^{4,5} As a ligand for human complement factor H (fH), the expression of fHBP by meningococci suppresses the alternative complement pathway and promotes bacterial survival in vivo.⁶ Over 900 unique fHBP protein variants have thus far been identified and can be divided into two immunologically distinct and largely non-cross protective subfamilies (A and B)⁷ or divided further into three variant groups.⁸ The antigenic diversity exhibited by fHBP has greatly increased the complexity of vaccine strain coverage predictions as the breadth of protection provided by individual vaccine variants is largely dependent on the presence of cross-reactive epitopes among variants expressed by invasive strains.

Traditionally, the distribution of such antigens among invasive strains has been determined by analysis of cultured isolates, the vast majority of which possess fHBP.9-12 In many disease cases, however, the prompt administration of antibiotics prevents the isolation of a viable culture and laboratory confirmation can only be achieved through the detection of residual meningococcal DNA within 'nonculture' clinical specimens. In England and Wales (E&W), only \sim 50% of cases confirmed by the Meningococcal Reference Unit (MRU) yield a culturable isolate.¹³ All clinical isolates subsequently undergo whole genome sequence analysis, from which the distribution of vaccine antigens such as fHBP can be ascertained. Indexed genomic information from invasive isolates collected over five epidemiological years (July-June inclusive): 2010/11 (n = 513), 2011/ 12 (n = 409), 2012/13 (n = 457), 2013/14 (n = 406) and 2014/15 (n = 521) (total n = 2306) is publicly available at the Meningitis Research Foundation Meningococcus Genome Library (MGL, http://www.meningitis.org/ research/genome).¹⁴ In addition to these genotypic analyses, the expression of fHBP by invasive isolates, a key prerequisite for immune protection, can be confirmed in vitro.^{4,15}

In contrast, for non-culture cases, whole genome sequencing is yet to be established and, in the absence of a viable isolate, fHBP expression cannot be directly quantified. In order to tackle this significant epidemiological knowledge gap, a PCR sequencing assay was developed to sequence *fHBP* from non-culture clinical specimens.¹⁶

Here we present fHBP typing data from non-culture specimens submitted to the MRU over three calendar years

(2011–2013). For the first time we can compare these data with those derived from isolates received during the same period to assess the representativeness of viable isolates among all invasive meningococcal strains in E&W.

Materials and methods

Clinical isolates

To determine the distribution of fHBP among isolates, fHBP peptide data representing E&W culture cases confirmed between January 2011 and December 2013 were downloaded from the MGL (n = 1336).

To assess the association between hyper-virulent clonal complexes and common fHBP variants, Multi-locus Sequence Typing (MLST) data and fHBP peptide data for E&W MGL isolates received from 2010/11 to 2014/15 (n = 2306) were downloaded. These were then combined with corresponding data from isolates received during the 2007/08 epidemiological year (n = 613) generated through previous PCR sequence analysis (total isolates n = 2919).

Clinical specimens

The MRU carries out disease confirmation from submitted culture-negative clinical specimens (e.g. blood and CSF specimens) using an in-house *ctrA*-directed TaqMan real-time PCR assay.¹⁷ Specimens producing PCR cycle threshold (Ct) values of >45 are currently deemed negative. The assay also consists of capsular group-specific primers and probes complementary to sequences within the *siaD* or *mynA* capsular synthesis alleles to allow genogroup determination. DNA extraction and typing of fHBP from non-culture specimens was carried out as previously described.¹⁶

fHBP nomenclature

Differing nomenclatures are currently used for fHBP protein variants. In the system established by Fletcher et al., individual variants are given unique alphanumeric identifiers based upon the subfamily to which each variant belongs.⁷ In an alternative nomenclature system, variants are assigned a unique number preceded by the variant group number, i.e. 1, 2 or 3.⁸ In addition, the PubMLST database (hosted by the University of Oxford, UK) assigns arbitrary, sequential numbers to unique *fHBP* alleles and protein variants as they are submitted.¹⁸ For clarity, fHBP protein variants are here referred to by both their assigned PubMLST peptide ID number and their alphanumeric subfamily ID (e.g. peptide 22/A10).

Statistical analysis

Cochran-Mantel-Haenszel Test¹⁹ and subsequent Fisher's Exact Tests were applied in the analysis of statistical association between the prevalence of variant 15/B44 and source of data (i.e. non-culture diagnoses versus isolates) adjusting for age groups. Results yielding a P-value of less than 0.05 were considered to be statistically significant.

Results

From 1st January 2011 to 31st December 2013, the MRU confirmed 2547 meningococcal disease cases by isolation of a meningococcal strain and/or nucleotide detection from non-culture clinical specimens (Table 1). The proportion of cases from which a viable isolate was obtained ranged from 49.1% in 2011 to 57.3% in 2013. A successive reduction in to-tal MRU-confirmed disease cases (20.7%) was observed between 2011 and 2013.

Capsular group and age profile

Group B was the most common capsular group representing 78.4% of all cases over the three year period. Group Y and W were the second and third most prevalent groups among isolates, respectively. A notable increase in the numbers group W cases was observed, rising from 33 in 2011 to 81 in 2013. Group C and Y prevalence was relatively stable over the three year period, representing $\sim 3\%$ and $\sim 10\%$ of cases, respectively. Fifteen isolates were phenotypically non-groupable.

In infants and children (0-10 years), the vast majority of disease was of group B; only 5.0% of cases were caused by group W or Y strains. In adolescents and adults (>11 years), however, substantial proportions of group W and Y disease were seen (31.5%), with these proportions increasing in each successive age group.

Fig. 1 illustrates the numbers and proportional distribution of culture and non-culture cases of each capsular group/all cases (2011–2013 combined) across different age groups/all ages combined. The age data for three group B, one group C, one group Y and one group W isolate, as well as two group B and a group Y non-culture case were unavailable.

Isolates were more common among those <1 year old (56.2%) and those aged over 25 years (70.0%). In 1–25 year olds, more than half of cases were confirmed using real-

time PCR only (57.5%). Isolates made up the majority of group W and Y strains in all age groups. Overall, only 15.3% of group W and 16.9% of group Y cases were confirmed using PCR only, compared to 45.4% of group B cases (Fig. 1). Indeed, in those aged over 25 years, only 19 group Y cases and six group W cases were confirmed by PCR only over the three year study period.

fHBP typing and subfamily distribution

All but ten isolates (99.3%) possessed alleles coding for full length fHBP peptides. Three 2011 isolates, two 2012 isolates and two 2013 isolates harboured alleles featuring frameshift point mutations resulting in truncated peptides (six ST-11 complex isolates possessing PubMLST nucleotide allele 669 and one ST-32 complex isolate harbouring allele 743). A single isolate received in 2011 and two isolates received in 2012 were fHBP null (i.e. did not possess an *fHBP* allele). None of these three isolates belonged to a defined clonal complex (STs 7395, 9825 and 10277) but they shared MLST alleles with ST-286 at \geq 4 loci and are therefore part of a proposed clonal complex previously associated with fHBP-null isolates.¹²

The numbers of non-culture cases typed for fHBP are listed in Table 1. Successful characterisation of fHBP was achieved for 91.5% of non-culture cases from which sufficient clinical specimen or DNA extract was available (84.9% of all non-culture cases 2011–2013 inclusive). The remaining, unamplified extracts produced relatively high Ct values and, therefore, lacked sufficient DNA concentration for amplification. Four non-culture specimens (two group B and two group C) yielded an *fHBP* allele coding for a truncated peptide (PubMLST allele 669). A total of 52 specimens possessed fHBP nucleotide and/or peptide alleles that were not previously indexed in the PubMLST fHBP curator to be assigned unique numeric IDs.

Subfamily B fHBP variants represented 59.3% of all typed strains. Fig. 2 shows the distribution of the two main subfamilies among culture and non-culture cases of the each capsular group. The vast majority (>95%) of group Y and W cases harboured subfamily A fHBP variants, regardless of culture status. Cases confirmed as groups B, C or non-groupable featured a greater proportion of subfamily B peptides. Subfamily B variants were found among 69.3% and 73.7% of group B isolates and non-culture cases, respectively.

Table 1The number of culture and non-culture confirmed disease cases in England and Wales from 2011 to 2013 and the pro-portion of cases characterised for fHBP.

Calendar year (Jan—Dec)	PCR only (non-culture) cases			Culture cases		Combined (all cases)	
	Total no. of cases	No. of specimens/extracts available	No. typed (% of total)	Total no. of cases	No. typed (% of total)	Total no. of cases	No. typed (% of total)
2011	498	461	411 (82.5)	481	481 (100)	979	892 (91.1)
2012	382	345	319 (83.5)	410	410 (100)	792	729 (92.0)
2013	331	317	298 (90.0)	445	445 (100)	776	743 (95.7)
Combined	1211	1123	1028 (84.9)	1336	1336 (100)	2547	2364 (92.8)



Figure 1 The numbers and proportions of culture and non-culture laboratory confirmations of the major invasive capsular groups and all cases combined stratified by patient age. Each graph illustrates data for a predominant capsular group or all cases as indicated. Within each defined age group, the light and dark grey bars represent the percentage of cases confirmed by culture isolation (Cul) and nucleotide detection (N-Cul), respectively. The corresponding numbers of cases are overlaid each bar.

The distribution of common fHBP peptide variants

The following ten fHBP protein variants (five from each subfamily) were most prevalent and collectively represented 77.9% of all characterised disease cases: 4/B16 (17.1%), 13/B09 (13.1%), 15/B44 (11.1%), 25/A15 (9.3%), 19/A22 (6.7%), 22/A10 (5.6%), 14/B03 (5.2%), 45/A05 (3.5%), 1/B24 (3.4%) and 16/A19 (2.8%). Among the remaining cases, there were 184 distinct fHBP variants, each observed in less than 50 cases. The majority (58.2%) of these miscellaneous variants belonged to subfamily A.

Most group W cases featured 16/A19 or 22/A10 (22.4% and 72.4% of combined typed cases, respectively). Variant 25/A15 dominated the group Y cases (81.3% of combined typed strains). Group C was more diverse with 13/B09, 15/B44, 19/A22 and 22/A10 collectively representing 70.9% of combined typed cases.

Seven of the ten aforementioned fHBP peptides were found in substantial numbers amongst group B isolates and non-culture cases. Variants 1/B24, 4/B16, 13/B09, 14/B03, 15/B44, 19/A22 and 45/A05 collectively represented 73.3% of all characterised group B strains.

Little variation was observed in the distribution of fHBP between group W and Y isolates and the corresponding nonculture cases, with both subsets being dominated by common subfamily A variants. Among group B cases, nonculture typing revealed a very similar fHBP variant profile to that seen in the isolates; however, there was consistent variation in the proportional distribution of three of the predominant variants. Fig. 3 illustrates the changes in the prevalence of these seven variants over the three years studied. When culture and non-culture cases were combined, minimal change (within 3% of the respective datasets) was observed in all seven variants over the three years studied. In each of the three years, variants 4/B16 and 19/A22 were present in greater proportions among the isolates in relation to the non-culture cases (Fig. 3), however there was no statistical significance (Fisher's Exact Test, P > 0.05).

Conversely, variant 15/B44 was significantly more prevalent in non-culture cases than among the isolates overall (age-adjusted Cochran–Mantel–Haenszel Test, P = 0.0094). Using one-tailed Fishers Exact Test, the association between 15/B44 prevalence and confirmation method within each age group was assessed. Variant 15/B44 was significantly more common among non-culture cases within the 11–25 years (P = 0.015) and 26–64 years (P = 0.043) age groups only.



Capsular Group / Culture (Cul) or Non-Culture (N-Cul)

Figure 2 fHBP subfamily distribution among typed culture and non-culture strains of each capsular group. The light and dark grey bars represent the percentage of characterised strains harbouring fHBP variants of each subfamily among culture and non-culture cases of each capsular group and all cases combined. The corresponding numbers of strains are overlaid each bar.

To further characterise 15/B44-harbouring strains, an analysis of the fHBP distribution among isolates of the major hyper-virulent meningococcal lineages received in recent epidemiological years was performed (Fig. 4). The analysis revealed a strong association between 15/B44 and the ST-269 cluster (approximately one half of the ST-269 complex) with 91.3% of 15/B44-harbouring isolates belonging to this lineage.

In the final analysis, the fHBP peptide distribution data were used to assess the representativeness of group B meningococcal isolates among group B disease cases as a whole. The proportional distribution of the seven common fHBP variants among group B isolates were calculated and



Figure 3 Prevalence of the seven most common group B fHBP peptide variants among typed culture and nonculture strains within each calendar year. Each bar represents the percentage of culture (Cul) or non-culture (N-Cul) group B cases received in 2011, 2012, 2013 or all years combined that are represented by a common fHBP variant.



Figure 4 The distribution of fHBP peptide variants among isolates of the major invasive clonal complexes in E&W. The analysis included all isolates belonging to the eight most prevalent clonal complexes (each represented by >50 isolates) received during epidemiological years 2007/08, 2010/11, 2011/2012, 2012/2013, 2013/2014 and 2014/2015 (n = 2340). The ST-269 cluster and ST-275 cluster collectively represent the ST-269 complex.

compared to that of all combined group B disease cases within each age group and for all ages combined (Fig. 5). Whilst minor differences can be seen in the fHBP distributions within several age groups, the isolates exhibited a very similar profile to that seen amongst all disease cases when the age groups were combined.

Discussion

This article describes the first comprehensive characterisation of English and Welsh non-culture meningococcal disease cases beyond the capsular group. Whilst most cases were caused by group B meningococci, increasing numbers of group W and Y disease were observed, partly driven by the recent expansion of a group W ST-11 complex sublineage in E&W.²⁰ In contrast to group B strains, very few group W and Y cases were confirmed through PCR detection alone. This disparity is attributed to the predominance of group W and Y disease in older individuals (>60 years).^{21,22} In these age groups, uncommon or non-specific disease presentations (e.g. pneumonia, septic arthritis) and comorbidities are more frequently observed and, in contrast to cases in younger patients with archetypal meningitic or septic symptoms (e.g. headache/photophobia or petechial rash/fever, respectively), clinicians are less likely to suspect meningococcal disease and submit clinical specimens for laboratory PCR confirmation. These findings suggest that a significant proportion of group Y and W disease is escaping laboratory confirmation and that the disease burden may be under-estimated, especially in older age groups.



💽 4/B16 🚺 13/B09 🔄 15/B44 🔜 19/A22 🔀 14/B03 🛄 45/A05 🔛 1/B24 🔛 Other

Age group (Years) / Culture Only (Cul) or All Cases

Figure 5 Distribution of fHBP among group B isolates compared with all group B disease cases. The bars represent the percentage distribution of the seven most common group B fHBP peptide variants among group B cultured isolates and all typed group B cases (culture and non-culture combined) within each age group and all ages combined.

Among group B cases, less than 3% change was observed in the prevalence of the seven most common fHBP variants over the three years, which is likely to be reflective the relative stability of the clonal complexes harbouring these peptides over this time period. Overall, the distribution of these variants was similar between group B culture and non-culture cases. The prevalence of peptide 15/B44, however, was consistently higher in group B non-culture cases and age analysis of cases featuring 15/B44 revealed a statistically significant difference in the proportions among the two datasets in the 11–25 years and 26–64 years age groups. The age-specific nature of this variation may be indicative of differential disease presentations/severity and/or diagnostic practice, as suggested for group W and Y cases.

An analysis of the fHBP distribution among a large collection of hyper-virulent isolates confirmed an association between 15/B44 and the ST-269 cluster, one of two defined subgroups within the ST-269 complex.²³ These findings may therefore be due to bacteriological factors, inherent to this defined population, influencing the fitness of these strains and thereby reducing the likelihood of obtaining a viable isolate upon sampling. In a recent study, peptide 15/B44 exhibited a ten-fold lower affinity for human fH than variant 1/B24 and a lower growth rate was observed for a mutant expressing 15/B44 in blood and plasma when compared to the same strain expressing 14/ B03 or 1/B24.²⁴ Nevertheless, a direct correlation between fH affinity and survival in blood, plasma or serum is yet to be clearly defined.^{24,25}

The ST-269 cluster differs from other group B hypervirulent lineages in that it lacks NalP, a serine protease autotransporter. Oldfield and colleagues (2013) noted that, among a collection of 641 carriage and invasive strains, deletion of the *nalP* gene had occurred in all isolates of the ST-269 cluster lineage, whilst such deletion was only observed sporadically in other hyperinvasive group B lineages.²⁶ NalP has been shown to increase the survival of meningococci in human serum and aid immune evasion by cleaving bound complement component 3 (C3) from the membrane surface, as well as removing immunogenic surface proteins such as Neisserial Heparin Binding Antigen and Lactoferrin Binding Protein B.^{27–29} The lack of NalP may therefore result in more efficient clearance of an invading ST-269 cluster strain by the host during infection, reducing the likelihood of bacterial isolation through culturing.

Additionally, the transposable insertion sequence IS1301 is frequently observed in the intergenic region of *siaD* and *ctrA* within the capsular synthesis locus of ST-269 cluster isolates, but not among other invasive group B lineages. The up-regulation of capsular expression mediated by IS1301 in cc11 group C isolates is abrogated by associated polymorphisms in ST-269 cluster isolates.^{30,31} Furthermore, limited capsular expression data suggests that one such IS1301 polymorphism may actually reduce capsular expression in these strains, however, a corresponding decrease in serum survival was not observed.³¹

If such a difference in strain fitness exists, it may be revealed in future analyses of the corresponding clinical information, conceivably with relatively milder disease in these cases. Additionally, further laboratory analysis of these strains may reveal other factors influencing bacterial viability *in vivo* (e.g. antimicrobial susceptibility).

It must be noted that immune serum from both of the licensed fHBP-containing vaccines exhibit bactericidal activity against 15/B44-harbouring strains in serum bactericidal antibody assays with human complement (hSBA).^{32,33}

The observed differences are, therefore, unlikely to impact current predictions of strain coverage for these two vaccines.

Finally, the fHBP peptide distribution data presented here suggests that the group B isolates are generally representative of all group B disease in E&W. Genotypic characterisation of isolate collections, as well as *in vitro* assessment of antigenic expression and bactericidal serum susceptibility among clinical isolates are performed to assess vaccine strain coverage.^{4,5,9–11,34} Whilst the current findings support the use of such analyses, this study only focused on one sub-capsular antigen. Characterisation of multiple antigens among non-culture strains and/or the development of protocols to enable whole genome analysis directly from clinical specimens would allow more comprehensive comparisons to be performed.

Conclusions

During the three years studied, the proportions of group W and Y disease were found to be substantially greater among cultured isolates in relation to non-culture cases in all ages, whilst strains harbouring fHBP variant 15/B44 were more commonly observed among the non-culture cases in adolescents and adults. Future analyses of additional laboratory and clinical data may help to explain the observed differences.

The distribution of fHBP peptides was similar among group B culture and non-culture cases. This suggests group B isolates offer a good representation of all group B invasive strains and that isolate-based fHBP coverage predictions can be reliably extrapolated to all cases assuming equivalent antigenic surface expression in non-culture cases.

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