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Pharyngeal carriage of *Neisseria* species in the African meningitis belt



Kanny Diallo [a,i,*], Caroline Trotter [b], Youssouf Timbine [a], Boubou Tamboura [a], Samba O. Sow [a], Bassira Issaka [c], Ibrahim D. Dano [c], Jean-Marc Collard [c], Marietou Dieng [d], Aldiouma Diallo [d], Adane Mihret [e], Oumer A. Ali [e], Abraham Aseffa [e], Stephen L. Quaye [f], Akalifa Bugri [f], Isaac Osei [f], Kadidja Gamougam [g], Lodoum Mbainadji [g], Doumagoum M. Daugla [g], Galadima Gadzama [h], Zailani B. Sambo [h], Babatunji A. Omotara [h], Julia S. Bennett [i], Lisa S. Rebbetts [i], Eleanor R. Watkins [i], Maria Nascimento [j], Arouna Woukeu [j], Olivier Manigart [j], Ray Borrow [k], James M. Stuart [j], Brian M. Greenwood [j], Martin C.J. Maiden [i]

[a] *Centre pour les Vaccins en Développement, Bamako, Mali*
[b] *Department of Veterinary Medicine, University of Cambridge, Cambridge, UK*
[c] *Centre de Recherche Médicale et Sanitaire, Niamey, Niger*
[d] *Institut de Recherche pour le Développement, Dakar, Senegal*
[e] *Armauer Hansen Research Institute, Addis Ababa, Ethiopia*
[f] *Navrongo Health Research Centre, Navrongo, Ghana*
[g] *Centre de Support en Santé International, N'Djamena, Chad*
[h] *University of Maiduguri, Nigeria*
[i] *Department of Zoology, University of Oxford, Oxford, UK*
[j] *London School of Hygiene & Tropical Medicine, London, UK*
[k] *Vaccine Evaluation Unit, Public Health England, Manchester, UK*

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Non-meningococcal *Neisseria*;

Summary *Objectives: Neisseria meningitidis*, together with the non-pathogenic *Neisseria* species (NPNs), are members of the complex microbiota of the human pharynx. This paper investigates the influence of NPNs on the epidemiology of meningococcal infection.

* Corresponding author. Zoology Department, University of Oxford, The Tinbergen Building, S Parks Rd, Oxford, OX1 3PS, UK. Tel.: +44 1 865271205.
E-mail address: kanny.diallo@zoo.ox.ac.uk (K. Diallo).

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Pharyngeal carriage;
African meningitis belt

Methods: *Neisseria* isolates were collected during 18 surveys conducted in six countries in the African meningitis belt between 2010 and 2012 and characterized at the *rplF* locus to determine species and at the variable region of the *fetA* antigen gene. Prevalence and risk factors for carriage were analyzed.
Results: A total of 4694 isolates of *Neisseria* were obtained from 46,034 pharyngeal swabs, a carriage prevalence of 10.2% (95% CI, 9.8—10.5). Five *Neisseria* species were identified, the most prevalent NPN being *Neisseria lactamica*. Six hundred and thirty-six combinations of *rplF*/*fetA_VR* alleles were identified, each defined as a *Neisseria* strain type. There was an inverse relationship between carriage of *N. meningitidis* and of NPNs by age group, gender and season, whereas carriage of both *N. meningitidis* and NPNs was negatively associated with a recent history of meningococcal vaccination.
Conclusion: Variations in the prevalence of NPNs by time, place and genetic type may contribute to the particular epidemiology of meningococcal disease in the African meningitis belt.
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Introduction

The human pharynx hosts a complex microbiota, including bacteria belonging to the genus *Neisseria*. Most members of this genus are non-pathogenic commensals (non-pathogenic *Neisseria*, NPNs), which very rarely cause invasive disease, but *Neisseria meningitidis* (*Nm*), the meningococcus, is an exception.[1] Despite advances in vaccine development, invasive meningococcal disease remains a public health challenge globally and especially in the African meningitis belt, where very large epidemics continue to occur,[2,3] despite the recent widespread deployment of a serogroup A meningococcal conjugate vaccine (PsA-TT, MenAfriVac®).[4—8]

Most pharyngeal carriage studies in the African meningitis belt have focused on the meningococcus,[9—12] with little attention paid to other *Neisseria* species apart from *Neisseria lactamica* (*Nl*). In northern Nigeria, pharyngeal carriage of *Nl* was common, especially in young children[13] and genetic exchange among *Nm*, *Nl* and unspecified *Neisseria* species has been demonstrated in The Gambia.[14] Molecular epidemiological studies of *Nm* and *Nl* conducted in Burkina Faso from 2009 to 2012 reported a high overall prevalence of *Nl* carriage (18.2%), a higher prevalence of *Nl* in males than in females, except in those aged 18—29 years, no change between dry and rainy season and no significant changes following vaccination with PsA-TT.[10,15—17] It has long been considered likely that carriage of NPNs influences host susceptibility to infection and invasion by *Nm*, a view supported by the fact that healthy subjects inoculated with *Nl* exhibit some protection against colonization with *Nm*.[18] Until recently, *Nl* was considered to be the NPN genetically most similar to *Nm*. However, recent whole genome sequence (WGS) studies have shown that *Neisseria polysaccharea* (*Np*) and *Neisseria bergeri* (*Nb*) are more closely related to *Nm* than *Nl*.[19] Consequently, these bacteria may also influence the epidemiology of *Nm* colonization and invasion. For this reason we have studied the prevalence of carriage with various *Neisseria* species in six countries of the African meningitis belt and investigated risk factors for their carriage.

Materials and methods

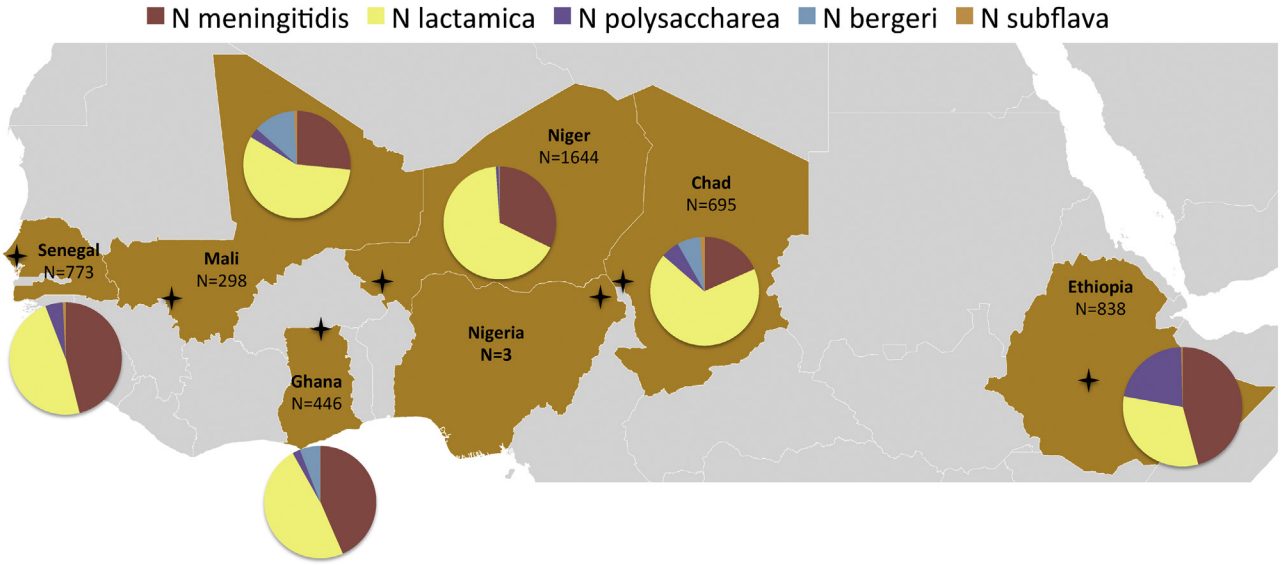
The methods employed in the MenAfriCar surveys, during which the isolates described in this paper were collected, have been described in detail previously[11] and are summarized briefly here. Ethical approval for the surveys was obtained from the ethics committee of the London School of Hygiene & Tropical Medicine and from ethical committees in each partner country. The study was registered with ClinicalTrials.gov (NCT01119482).

Carriage surveys

Bacteria were isolated during 18 cross-sectional surveys conducted in Chad, Ethiopia, Ghana, Mali, Niger and Senegal during 2010—2012 (Fig. 1). Pre-vaccination surveys in Mali, Niger and Chad had a target of 5000 participants and post-vaccination surveys a target of 2000 participants in Mali and Niger and 6000 in Chad. The remaining surveys aimed to recruit 2000 participants. A representative sample of households was obtained either from an updated demographic surveillance system (DSS) or from a census conducted for the study. Within selected households, individuals in four different age groups (0—4 years, 5—14 years, 15—29 years and 30 years or more) were chosen randomly until the required sample size was reached, with a maximum of 5 individuals recruited per household. Once written consent had been obtained, standardized household and individual questionnaires inquiring about risk factors for meningococcal infection were administered. Pharyngeal swabs were obtained using a standardized technique that involved swabbing both the posterior-pharyngeal wall and the tonsils.[20]

Bacteriology

NPNs were isolated using the same conventional microbiology techniques that were employed for the detection of *Nm* described previously.[11] Briefly, pharyngeal swabs were plated onto modified Thayer Martin agar plates and incubated 24—48 h at 37 °C in 5% CO_2; oxidase and gram stain testing identified oxidase positive, Gram-negative



A map of the African meningitis belt with pie charts depicting the prevalence of *Neisseria* species identified in each country.
N correspond to the total number of *Neisseria* isolates identified and is the denominator.

✦ Correspond to the sampling area

Figure 1 Frequency distribution of *Neisseria* species in each country of the study.

diplococci. Further biochemical tests (ortho-nitrophenyl-β-galactoside, γ-glutamyl transpeptidase and tributyrin) were used in each site to differentiate between putative *Nl* and *Nm* isolates and members of the genus *Moraxella*.

Molecular methods

A boiled cells suspension of each oxidase positive, Gram negative diplococci (OPGND) isolate was sent to the Department of Zoology at the University of Oxford for molecular typing, where Sanger sequencing was used to characterize gene targets as described in the Supplement. Sequences were assembled using SeqSphere (http://www.ridom.de/seqsphere/) and imported into the isolate's record previously created in a BIGSdb database.[21]

Neisseria speciation

Amplification and sequencing of a 413 bp fragment of the *rplF* gene was used to differentiate among *Neisseria* species as described previously.[22] A phylogeny based on the *f_rplF* alleles from Bennett et al. (2014) and the unique alleles found in this study was reconstructed using the Neighbor-Joining algorithm[23] and the Kimura 2-parameter substitution model[24] in MEGA version 6.0.[25] For isolates that did not yield results for the *rplF* assay, sequencing of the *rrna* gene, encoding 16S rRNA,[26] was used to confirm the presence or absence of a bacterium and to determine its genus. Only *rplF* confirmed NPNs were included in this study with the exception of four *Nm* speciated on the basis of the 16S rRNA and the *porA* sequences. New alleles were investigated by a BLAST of obtained sequences against the 16S rRNA sequence data of the EzTaxon server (http://www.ezbiocloud.net/eztaxon;[27]).

Genetic diversity

Sequencing of the variable region of the *fetA* gene (*feta_VR*) was used to assess the genetic diversity of the *Neisseria* identified as described in the Supplementary methods.[28] The sequences were assembled as for the other targets using SeqSphere (http://www.ridom.de/seqsphere/). The trace files of each new allele were manually curated in MEGA version 6.0,[25] the quality of the sequences were assessed and the modified bases checked in both the forward and reverse trace file. The corresponding protein sequences were also aligned and compared with known sequences stored in the *Neisseria* sequence definition database of pubMLST[21] before being entered into the allele database and the appropriate isolate record.

For isolates for which *fetA_VR* could not be amplified, an assay identifying the absence of the *fetA* gene (*fetA* null, *fnl*) was employed, using primers placed on genes on each side of the *fetA* gene: *thdF* and *fetB* as described in the Supplementary methods.[29]

Statistical methods

Analyzes were performed using Stata v12.0 (StataCorp, Texas). Survey design and potential household clustering were taken into account using the survey commands in Stata. Carriage prevalence of each of the different *Neisseria* species, together with 95% confidence intervals, was calculated for each country and each survey. Risk factors

for carriage of *Nm* and NPNs were assessed simultaneously using multinomial logistic regression. Each risk factor was considered in turn using univariable, multinomial logistic regression. A multivariable model was then constructed including country, age group and sex *a priori* and any variable with a p-value <0.1 in the univariable analyses; only the variables with a p value < 0.05 in the multivariable analysis were kept in the final model. As a final check, dropped variables were re-entered into the model one at a time and the p-values re-examined; the variable was retained as significant if the p value was <0.05.

Results

Prevalence of carriage with *Neisseria* species

A total of 4694 of the 46034 pharyngeal swabs collected yielded a *Neisseria* species, giving a carriage prevalence of 10.2% [95% CI, 9.8—10.5%]: 696 *Neisseria* were identified out of the 946 OPGND samples received from Chad; 838 out of the 994 received from Ethiopia; 446 out of the 544 received from Ghana; 298 out of the 504 from Mali; 1644 out of 2321 from Niger and 773 out of the 971 received from Senegal. The most frequently isolated species was *Nl* with a 5.6% point prevalence [95% CI, 5.3—5.8%], followed by *Nm* at 3.6% [95% CI, 3.4—3.8%]), *Np* at 0.6% [95% CI, 0.5—0.7%], *Nb* at 0.2% [95% CI, 0.2—0.3%] and *Neisseria subflava* (*Ns*) at 0.05% [95% CI, 0.03—0.1%] (Supplementary Table 1). Twenty isolates from Chad were identified as belonging to the *Neisseria* genus but did not cluster with any known species on the Neighbor-Joining Tree (NJT; data not shown); they were designated *Neisseria* sp. and will be investigated further by whole genome sequencing methods.

Factors influencing the prevalence of carried *Neisseria* species

Country, season, age, gender, and a history of recent vaccination against *Nm* were associated with carriage of NPNs. Additionally, area of residence, household crowding

Table 1 Factors associated with carriage of *Neisseria meningitidis* and non-pathogenic *Neisseria*; results from a multinomial multivariable logistic regression.

Factor		Number (carriers)	Adjusted RRR *Neisseria meningitidis* (95% CI)	Adjusted RRR Non-pathogenic *Neisseria* (95% CI)
Age	<1 year	2074 (207)	0.40 (0.29, 0.55)	3.11 (2.60, 3.73)
	1—4 years	8291 (1355)	0.70 (0.59, 0.82)	5.90 (5.23, 6.65)
	5—14 years	12,563 (895)	1.49 (1.31, 1.69)	2.59 (2.29, 2.94)
	15—29 years	11,863 (4372)	1.0	1.0
	30 + years	11,243 (206)	0.59 (0.50, 0.68)	0.51 (0.43, 0.61)
Sex[a]	Female	26,619 (1701)	1.0	1.0
	Male	19,296 (1313)	1.34 (1.21, 1.48)	0.87 (0.80, 0.94)
Season	Rainy (XS1 and XS2)	30,522 (220)	1.0	1.0
	Dry (XS3)	15,512 (815)	1.53 (1.35, 1.74)	0.78 (0.70, 0.86)
Country	Chad	13,396 (584)	1.0	1.0
	Ethiopia	5970 (450)	7.11 (5.43, 9.29)	1.70 (1.44, 2.01)
	Ghana	5209 (253)	4.81 (3.64, 6.35)	1.25 (1.03, 1.51)
	Mali	8837 (219)	1.45 (1.05, 1.99)	0.48 (0.40, 0.58)
	Niger	8213 (1112)	11.39 (9.00, 14.43)	3.58 (3.13, 4.01)
	Senegal	4409 (427)	10.85 (8.28, 14.23)	2.39 (1.97, 2.89)
Area	Urban	19,462 (1398)	1.0	1.0
	Rural	26,572 (1637)	1.44 (1.09, 1.60)	0.97 (0.88, 1.06)
Crowding[b]	<2 people per room	16,299 (889)	1.0	1.0
	≥2 people per room	29,679 (2127)	1.27 (1.12, 1.45)	1.08 (0.98, 1.19)
Kitchen location	Open air	17,805 (1274)	1.0	1.0
	Inside house	12,741 (1027)	1.32 (1.09, 1.60)	0.90 (0.79, 1.02)
	Separate hut	14927 (677)	0.94 (0.76, 1.17)	0.96 (0.84, 1.09)
	Missing information	504 (38)	1.06 (0.57, 1.98)	0.77 (0.51, 1.17)
Vaccinated recently with meningitis vaccine	No	31,338 (2060)	1.0	1.0
	Yes, <1 year ago	9048 (545)	0.71 (0.59, 0.84)	0.82 (0.73, 0.92)
	Yes, 1—3 years ago	4543 (356)	0.51 (0.42, 0.63)	0.94 (0.81, 1.10)
	Don't know/missing	1020 (55)	0.68 (0.47, 1.00)	0.62 (0.46, 0.84)

The final multivariable logistic regression model included age group, sex, country, season area, crowding, kitchen location and recent vaccination.

Other risk factors used in the univariable model but not significant in the adjusted model: smoking, living in a house with smokers, cooking fuel, respiratory symptoms and attendance of social gatherings.

[a] Sex not reported for 119 individuals.

[b] Data not reported for 57 individuals.

and kitchen location were significant risk factors for carriage of *Nm* (Table 1).

Country: The prevalence of carriage of *Neisseria* species overall varied significantly among countries with Niger having the highest point prevalence (19.9% [95% CI, 18.9–20.9]), followed by Senegal (17.5% [95% CI, 16.1–18.8]), Ethiopia (13.8% [95% CI, 12.8–14.8]), Ghana (8.5%[95% CI, 7.6–9.4]), Chad (5.3%[95% CI, 4.9–5.7]) and Mali (3.4% [95% CI, 2.9–3.8]) (Supplementary Table 1). The distribution of the different species of *Neisseria* by country is shown in Fig. 1. The prevalence of *Nm* carriage varied significantly among countries, being highest in Senegal (8.0% [95% CI, 7.1–9.0]) and lowest in Mali (0.9% [95% CI, 0.7–1.1]). There were also major differences in the prevalence of carriage of NPNs by country and some NPNs were not identified in some countries, for example no *Nb* were isolated in Ethiopia and Senegal and no *Ns* in Ghana. Ethiopia was the only country where the prevalence of *Nm* carriage was higher than that of *Nl*.

Year and season: The prevalence of carriage of *Neisseria* species overall varied little over the three years of the study: 10.0% [95% CI, 9.4–10.5%] in the first survey, 11.1% [95% CI, 10.6–11.7%] in the second survey and 9.4% [95% CI, 8.9–9.9%] in the third survey (Table 2). The prevalence of carriage with both *Nm* and NPNs was also similar over time, with *Nl* being the most carried species, regardless of survey or season. There was, however, variation in the prevalence of carriage of NPNs over time at the country level: for example, there was an increase in the prevalence of *Nl* between surveys 1 and 2 in both Chad (from 1.1% to 5.9%) and Ghana (from 0.8% to 6.3%) and an increase in *Nm* prevalence between survey 2 and 3 in Senegal (from 3.1% to 19.8%[4]) (Supplementary Fig. 1). The relative risk of carriage of NPNs was significantly lower during the dry compared to the rainy season with an adjusted Relative Risk Ratio (aRRR) of 0.78, [95% CI, 0.70–0.86], whereas the opposite was true for *Nm* with an aRRR of 1.53, [95% CI, 1.35–1.74] in the dry season.

Age and gender: The relative risk of carrying NPNs overall was higher in children aged less than 15 years compared to the young adult age group (age 15–29 years) with an aRRR of 3.11 [95% CI, 2.60–3.73] for those <1 year, 5.90 [95% CI, 5.23–6.65] for the 1–4 year olds and 2.59 (95% CI, 2.29–2.94) for the 5–14 year olds. The oldest age group >30 years had a lower aRRR of 0.51 (95% CI, 0.43–0.61). The prevalence of carried *Nl* was highest in the 1–4 year old age group, reaching a peak of 14.1% [95% CI, 13.3–14.8%] in contrast to carriage of *Nm,* which reached a peak of 5.2% [95% CI, 4.8–5.6%] in the 5–14 year age group. Similarly to *Nl*, *Np* carriage also reached a peak prevalence of 1.7% [95% CI, 1.4–1.9%] in the 1–4 year old age group (Fig. 2). Carriage prevalence of *Nb* and *Ns* was too low to identify an overall trend in age group distribution. Males had a lower risk of carrying NPNs than females (aRRR of 0.87 [95% CI, 0.80–0.94]) but a higher risk of carrying *Nm* (aRRR of 1.34 [95% CI, 1.21–1.48].

Vaccination history: A history of vaccination within the past 12 months with any meningococcal vaccine was associated with a decrease in *Nm* carriage, as reported previously,[8] but additionally with a decreased risk of carrying NPNs (aRRR of 0.82 [95% CI, 0.73–0.92]. In the three countries where MenAfriVac® was introduced during the course

Table 2 Number of isolates identified as a *Neisseria* species per survey.

	N. meningitidis	Prevalence [CI]	*N. lactamica*	Prevalence [CI]	*N. polysaccharea*	Prevalence [CI]	*N. bergeri*	Prevalence [CI]	*N. subflava*	Prevalence [CI]	Total	Prevalence [CI]
Survey 1	577	3.6 [3.3–3.9]	896	5.7 [5.3–6.1]	79	0.5 [0.4–0.6]	17	0.1 [0.1–0.2]	4	0.03 [0–0.05]	1573	10.0 [9.4–10.5]
Survey 2	454	3.0 [2.7–3.3]	1010	6.7 [6.3–7.2]	117	0.8 [0.62–0.9]	62	0.4 [0.3–0.5]	17	0.1 [0.1–0.2]	1660	11.1 [10.6–11.7]
Survey 3	648	4.2 [3.8–4.5]	682	4.4 [4.0–4.7]	94	0.6 [0.5–0.7]	34	0.2 [0.1–0.3]	3	0.02 [−0.002 to 0.04]	1461	9.41 [8.9–9.9]
Total	**1679**	**3.6 [3.4–3.8]**	**2588**	**5.6 [5.3–5.8]**	**290**	**0.63 [0.5–0.7]**	**113**	**0.2 [0.2–0.3]**	**24**	**0.05 [0.03–0.1]**	**4694**	**10.2 [9.8–10.5]**

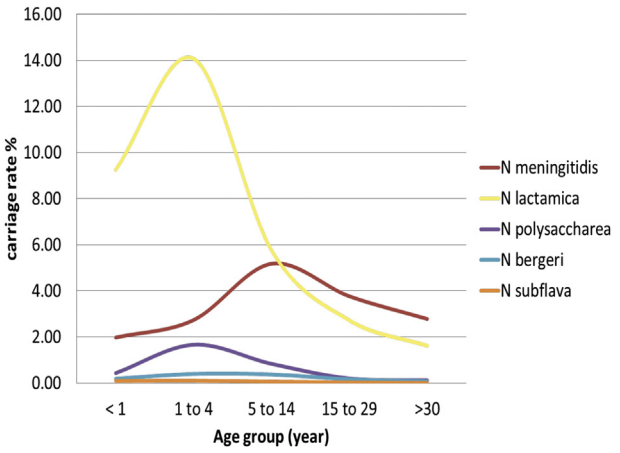


Figure 2 Prevalence of *Neisseria* species carried by age group.

of the study (Chad, Mali, and Niger) a significant decrease in the prevalence of carriage of *Nl* from 6.4% to 4.9% was observed. An overall decrease in carriage of *Nm* was also observed in the three countries but this reduction was not consistent in all countries with Mali experiencing an increase from 0.6% to 1.2% (Supplementary Fig. 2).

Other risk factors: Area of residence (rural vs urban), crowding, cooking with cow dung or straw, kitchen location, and attendance at social gatherings in the past week all had a significant impact on the odds of carrying NPNs in the univariable regression model, but their effect was not significant in the multivariable model. Some of these risk factors were retained in the final model as, although they had no effect on NPN carriage, they were significant for *Nm* carriage.

Genetic diversity of identified *Neisseria* species

Forty-two different alleles were identified for the *rplf* fragment (*f_rplf*). *Nl* was the most diverse species with 17 *f_rplf* alleles, the most frequent of which was *f_rplf 6* (1453 isolates, 56.1%). Eleven alleles were found for *Nm* the most frequent being *f_rplf 2* (749 isolates, 44.6%) and *f_rplf 1* (707 isolates, 42.1%); four *f_rplf* alleles were identified in *Np,* the most frequent of which was *f_rplf 9* (207 isolates, 71.4%). Four alleles were also found among the *Nb* alleles with *f_rplf 62* (57) and f_rplf 69 (46) representing 91.2% of these isolates. Finally, seven alleles were observed for *Ns*, with predominance of *f_rplf 43* (15 isolates, 62.5%) (Supplementary Table 2). A Neighbor Joining tree was used to represent the phylogeny of the *rplF* fragment present in the *Neisseria* isolates of this study in relation to the original isolates used to create the *f_rplF* assay[22] (Supplementary Fig. 3).

The diversity of *fetA* alleles varied by country (Fig. 3A; Supplementary Table 3); Niger had the highest number of *fetA_vr* alleles (111) and Ghana had the least (55). Some allele variability was seen between surveys, for example the proportion of the F1-1 allele increased from 61 (3.63%) in survey 2 to 342 (23.38%) in survey 3 and similar changes were observed for others alleles in survey 3 (Fig. 3B; Supplementary Table 4). A total of 234 different alleles were identified across all species: 75 of these were found in only one isolate; 184 in fewer than 20 isolates. A total of 194 alleles were observed for *Nl*, 80 for *Nm*; 35 for *Np*; 21 for *Nb*; and 16 for *Ns*. Most alleles were found predominantly in only one species, for example 99.61% of *fnl* alleles were found in *Nm*,[29,30] all F5-84 variants were only detected in *Nl*, all F5-1 were exclusive to *Nm*, all F11-4 were found only in *Np* and all F1-169 and all F1-193 variants were only observed in *Ns*. Some variants, however, were shared among different species, e.g. F1-72, F1-21, F2-24, F6-3 (Fig. 3C; Supplementary Table 5). Neither the *fetA_VR* nor the *fnl* fragment was successfully amplified in 643 isolates, which were designated Not Determined (ND). As defined by *f_rplf* and *feta_VR* alleles, there were 636 different *Neisseria* strain types identified, with almost half of these (297, 46.70%) observed only once. There was appreciable variation in the frequency of the strain types observed more than 20 times (Table 3).

Discussion

Although the introduction of PsA-TT into the African meningitis belt has had a major impact on serogroup A epidemics,[8,31] the region will remain at risk of meningococcal disease until comprehensive vaccines targeting all serogroups are available.[3] The reasons for the unique epidemiology of the African meningitis belt remain poorly understood,[32] making it difficult to predict when and where epidemics caused by non-serogroup A meningococci might occur. Variations in the prevalence of NPN species, which potentially contribute cross immunity through subcapsular antigens, could play a role in determining susceptibility to a potentially epidemic strain. A number of studies have indicated the movements of genes encoding various protein antigens from NPN to *Nm*[14,33] and variants of the FetA antigen have been previously shown to be shared amongst *Neisseria* species.[14,34] This antigen is known to generate protective responses in humans.[35] Colonization with NPNs also affects colonization of humans in experimental studies.[18]

The novel sequence based techniques employed in this study enabled *Nm* and NPN to be identified and characterized rapidly and cost effectively from the very large numbers of samples obtained in the African centers. The *rplF* assay[22] achieved reliable speciation, which would not have been possible with conventional methods such as 16S rRNA gene sequencing. An indication of diversity within species was achieved by sequencing the variable region of the gene encoding FetA (*fetA_VR*), an outer membrane protein (OMP) found in most *Neisseria*, and which is involved in iron metabolism and been shown to elicit protective immune responses.[36] Further characterization of the meningococcal isolates has been previously published.[12]

Of the five known *Neisseria* species identified, with a possible novel species present in Chad, the most common were *Nl* and *Nm*. These are the species that have been observed mostly frequently in previous investigations in the African meningitis belt and they are known to have antagonistic interactions in colonization.[18,37] It is possible that the isolation of some of the other species was affected by the selective media used in this and other studies. Although growth of *Np*,[38] *Nl*[17,39–41] and *Ns*[42] on colistin containing agar (such as modified Thayer–Martin or New York

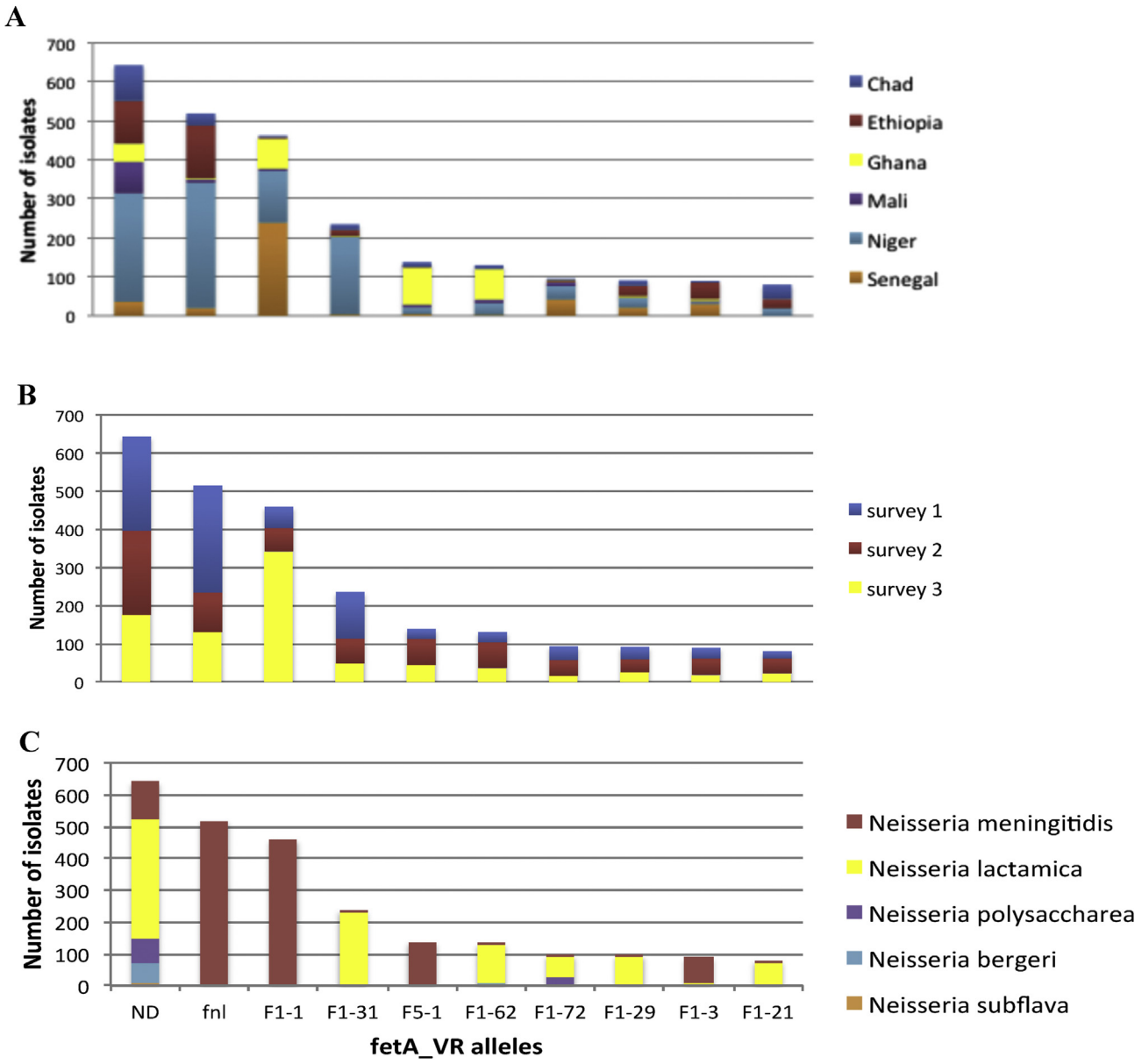


Figure 3 Frequency distribution of *fetA_VR* alleles. Frequency distribution of different alleles of the variable region of *fetA*. Only the 10 most common alleles are displayed; distribution by country (A), by survey (B) and by species (C).

city medium) has been reported, other species such as *Neisseria perflava*, *Neisseria sicca*, *Neisseria mucosa*, *Neisseria cinerea* may not have been identified using the culture technique employed in this study.[43] The impact of these media on *Nb* is unknown. In future, the identification of NPNs could be enhanced by the use of molecular approaches, although these will have to have species-level resolution, such as the *rplF* assay.[22] The *Neisseria* identified were highly diverse at both the strain and the species level and varied markedly over time and place. This is consistent with other carriage studies in the meningitis belt, but is different from the relatively stable carriage observed in countries that do not experience large-scale epidemic disease.[44]

The most common NPNs recovered, *Np* and *Nl*, were isolated predominantly from young children as opposed to *Nm*, which was isolated most frequently from older children. *Nl* is known to colonize infants and young children preferentially in many settings[45] and this is consistent with carriage of NPNs having a role in the rapid acquisition of antibody against *Nm* among children in the meningitis belt, although given the age distribution of meningococcal disease these antibodies may not be protective.[46]

The risk factors for Nm and NPNs carriage were not the same and in some cases (eg. age, sex and season) were inversely related. Individuals were more likely to carry NPNs during the rainy season if they were a female and under 5 years old whereas they were more likely to carry *Nm* during the dry season, if they were male and between 5 and 29 years old.[12] These results suggest that the physical presence of an NPN in the pharynx may prevent the colonization by *Nm* although this may not apply to the hyper-invasive meningococci since the incidence of meningococcal disease in the African meningitis belt is highest in

under five year olds. Recent vaccination with a meningitis vaccine (primarily MenAfriVac®, which was used in Mali, Niger and Chad during the course of our study) appeared to be protective both against carriage of Nm and NPNs overall in the regression analysis. This effect could possibly be explained by disturbance of the microbiome following vaccination but could also be due to residual confounding or a reflection of the naturally dynamic patterns of carriage. In Mali, an increase in Nm was observed post-vaccination, due to an expansion of serogroup W.[12] This seems unlikely to be an effect of MenAfriVac® but it is not known how the removal of one serogroup from the nasopharyngeal environment could affect the other serogroups. The influence of host factors on carriage of *Nm*

Table 3 Number of isolates for the most common commensal strain types per species.

Commensal strain_type "*f_ rplf:fetA*"	*Neisseria bergeri*	*Neisseria lactamica*	*Neisseria meningitidis*	*Neisseria polysaccharea*	Total
1:fnl	–	–	470	–	470
2:F1-1	–	–	452	–	452
6:F1-31	–	205	–	–	205
2:F5-1	–	–	135	–	135
6:ND	–	122	–	–	122
32:ND	–	88	–	–	88
52:ND	–	82	0	–	82
2:F1-3	–	–	74	–	74
1:ND	–	–	68	–	68
9:ND	–	–	–	66	66
6:F1-29	–	62	–	–	62
34:F1-62	–	56	–	–	56
6:F1-62	–	47	–	–	47
52:F3-60	–	46	–	–	46
6:F1-100	–	46	–	–	46
6:F1-72	–	46	–	–	46
1:F3-1	–	–	45	–	45
6:F5-133	–	45	–	–	45
6:F4-5	–	43	–	–	43
6:F4-17	–	42	–	–	42
9:F7-3	–	–	–	37	37
6:F5-84	–	36	–	–	36
33:F2-17	–	35	–	–	35
52:F1-21	–	35	–	–	35
62:ND	35	–	–	–	35
33:ND	–	34	–	–	34
88:fnl	–	–	32	–	32
9:F2-24	–	–	–	32	32
1:F5-5	–	–	31	–	31
34:ND	–	31	–	–	31
69:ND	31	–	–	–	31
6:F4-6	–	29	–	–	29
6:F5-12	–	29	–	–	29
2:ND	–	–	28	–	28
6:F5-18	–	26	–	–	26
2:F6-3	–	–	25	–	25
52:F1-29	–	25	–	–	25
6:F1-21	–	25	–	–	25
9:F11-4	–	–	–	25	25
4:F1-7	–	–	23	–	23
63:F1-72	–	–	–	23	23
6:F6-2	–	23	–	–	23
1:F4-23	–	–	22	–	22
33:F4-6	–	22	–	–	22
33:F5-34	–	22	–	–	22
6:F2-23	–	22	–	–	22
6:F1-120	–	21	–	–	21
6:F1-101	–	20	–	–	20

and NPNs has not been investigated in this study but the IgA secretory status of the individuals sampled could also play a role as previously shown.[47]

Few published studies have characterized pharyngeal NPNs in the African meningitis belt. One recent study in Burkina Faso, which did not survey adults over the age of 30 years or identify species other than *Nm* and *Nl*,[17] reported a number of differences compared to the MenAfriCar surveys described here: there was a higher overall prevalence of *Nl* carriage (18.2%); a higher prevalence in males, although it was significantly higher in women (9.1% vs 3.9%) for the 18—29 year age group and no significant changes between seasons or post vaccination. The reasons for these inconsistencies are unclear, although both studies indicated fluctuations in prevalence among surveys and these differences may reflect the highly dynamic nature of *Neisseria* carriage.

Some of the *fetA_VR* alleles found were shared among the different species, as reported previously.[14,34] Since the variable region of *fetA* plays an important role in its immunogenicity,[28] the shared protein variants could create a cross-reactive immunity at the subscapular level. The inclusion of *fetA_VR* in the meningococcal typing system also increased discrimination between strains (supplemental Table 6). Although there was correlation between *porA* and *fetA_VR* alleles for some *Nm* strains (e.g. *cnl*:P1.18-11,42-1:*fnl*), in others the *fetA_VR* (e.g. W:P1.5,2:F1-1 and W:P1.5,2:F6-3) or both OMPs (e.g. W:P15-1,2-36:F5-1) sequences varied, suggesting that the serogroup W *Nm* strains are antigenically diverse. This has potential implications for using proteins such as *porA* or *fetA* in vaccine formulations.[48] The Neighbor Joining phylogeny (Supplemental Fig. 3) shows the clustering of all of the isolates with the appropriate species except for one, which has the *f_rplF* allele 58 defined previously as *Ns*[22] but clusters more closely, in this study, with the *Nb* species which was not discovered at the time of the previous study. This particular isolate may also be a *Ns* with a *f_rplF* allele similar to *Nb*. More sequence data from this isolate will clarify this issue.

This study has demonstrated the dynamic nature and high diversity of the genus *Neisseria* in pharyngeal carriage in countries of the African meningitis belt. The results are consistent with the idea that the carriage of NPNs may influence invasive meningococcal disease epidemiology in the African meningitis belt. Although more research is needed to elucidate such effects, understanding these organisms could potentially contribute to meningococcal disease control. This is of particular importance given the absence of comprehensive vaccines against all meningococcal serogroups and the continuing interest in the development of protein based vaccines.

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Conflict of interest

Caroline Trotter reports that she received a Consulting payment in 2013 for a critical review of health economic model of meningococcal ACWY vaccine by GlaxoSmithKline (GSK); Ray Borrow reports that he performed contract researches on behalf of Public Health England for Novartis Vaccines and Diagnostics, Baxter Biosciences, Sanofi Pasteur, Serum Institute of India and GSK. All other authors report no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jinf.2016.03.010.

References

1. Stephens DS, Greenwood B, Brandtzaeg P. Epidemic meningitis, meningococcaemia, and *Neisseria meningitidis*. *Lancet* 2007 Jun 30;**369**(9580):2196—210. PubMed PMID: 17604802.
2. Maurice J. Vaccine shortage threatens spread of meningitis in Niger. *Lancet* 2015 Jun 6;**385**(9984):2241. PubMed PMID: 26088485.
3. WHO. *Meningitis outbreak response in sub-Saharan Africa: WHO guidelines*. 2015 [accessed 11.06.15].
4. Lee CH, Kuo WC, Beri S, Kapre S, Joshi JS, Bouveret N, et al. Preparation and characterization of an immunogenic meningococcal group A conjugate vaccine for use in Africa. *Vaccine* 2009 Jan 29;**27**(5):726—32. PubMed PMID: 19063929.
5. Frasch CE, Preziosi MP, LaForce FM. Development of a group A meningococcal conjugate vaccine, MenAfriVac(TM). *Hum Vaccin Immunother* 2012 Jun;**8**(6):715—24. PubMed PMID: 22495119.
6. Djingarey MH, Barry R, Bonkoungou M, Tiendrebeogo S, Sebgo R, Kandolo D, et al. Effectively introducing a new meningococcal A conjugate vaccine in Africa: the Burkina Faso experience. *Vaccine* 2012 May 30;**30**(Suppl. 2):B40—5. PubMed PMID: 22607898.
7. Caini S, Beck NS, Yacouba H, Maiga I, Chaibou I, Hinsa I, et al. From Agadez to Zinder: estimating coverage of the MenAfriVac conjugate vaccine against meningococcal serogroup A in Niger, September 2010—January 2012. *Vaccine* 2013 Mar 15;**31**(12):1597—603. PubMed PMID: 23337027.
8. Daugla DM, Gami JP, Gamougam K, Naibei N, Mbainadji L, Narbe M, et al. Effect of a serogroup A meningococcal conjugate vaccine (PsA-TT) on serogroup A meningococcal meningitis and carriage in Chad: a community study [corrected]. *Lancet* 2014 Jan 4;**383**(9911):40—7. PubMed PMID: 24035220. PMCID: 3898950. Epub 2013/09/17. eng.
9. Trotter CL, Greenwood BM. Meningococcal carriage in the African meningitis belt. *Lancet Infect Dis* 2007 Dec;**7**(12):797—803. PubMed PMID: 18045562.
10. Kristiansen PA, Diomande F, Wei SC, Ouedraogo R, Sangare L, Sanou I, et al. Baseline meningococcal carriage in Burkina

Faso before the introduction of a meningococcal serogroup A conjugate vaccine. *Clin Vaccin Immunol* 2011 Mar;**18**(3): 435—43. PubMed PMID: 21228139. PMCID: 3067389. Epub 2011/01/14. eng.
11. MenAfriCar. Meningococcal carriage in the African meningitis belt. *Trop Med Int Health* 2013;**18**(8):968—78. PubMed PMID: 23682910. Eng.
12. MenAfriCar c. The diversity of meningococcal carriage across the African meningitis belt and the impact of vaccination with a group A meningococcal conjugate vaccine. *J Infect Dis* 2015;**212**(8):1298—307. PubMed PMID: 25858956.
13. Blakebrough IS, Greenwood BM, Whittle HC, Bradley AK, Gilles HM. The epidemiology of infections due to *Neisseria meningitidis* and *Neisseria lactamica* in a northern Nigerian community. *J Infect Dis* 1982 Nov;**146**(5):626—37. PubMed PMID: 7130749.
14. Linz B, Schenker M, Zhu P, Achtman M. Frequent interspecific genetic exchange between commensal *Neisseriae* and *Neisseria meningitidis*. *Mol Microbiol* 2000 Jun;**36**(5):1049—58. PubMed PMID: 10844690.
15. Kristiansen PA, Ba A, Ouedraogo AS, Sanou I, Ouedraogo R, Sangare L, et al. Persistent low carriage of serogroup A *Neisseria meningitidis* two years after mass vaccination with the meningococcal conjugate vaccine, MenAfriVac. *BMC Infect Dis* 2014 Dec 4;**14**(1):663. PubMed PMID: 25472422. PMCID: 4267149.
16. Kristiansen PA, Diomande F, Ba AK, Sanou I, Ouedraogo AS, Ouedraogo R, et al. Impact of the serogroup A meningococcal conjugate vaccine, MenAfriVac, on carriage and herd immunity. *Clin Infect Dis Off Publ Infect Dis Soc Am* 2013 Feb; **56**(3):354—63. PubMed PMID: 23087396.
17. Kristiansen PA, Diomande F, Ouedraogo R, Sanou I, Sangare L, Ouedraogo AS, et al. Carriage of *Neisseria lactamica* in 1- to 29-year-old people in Burkina Faso: epidemiology and molecular characterization. *J Clin Microbiol* 2012 Dec;**50**(12):4020—7. PubMed PMID: 23035186. PMCID: 3503018.
18. Deasy AM, Guccione E, Dale AP, Andrews N, Evans CM, Bennett JS, et al. Nasal inoculation of the commensal *Neisseria lactamica* inhibits carriage of *Neisseria meningitidis* by young adults: a controlled human infection study. *Clin Infect Dis Off Publ Infect Dis Soc Am* 2015 May 15;**60**(10):1512—20. PubMed PMID: 25814628.
19. Bennett JS, Jolley KA, Earle SG, Corton C, Bentley SD, Parkhill J, et al. A genomic approach to bacterial taxonomy: an examination and proposed reclassification of species within the genus *Neisseria*. *Microbiology* 2012 Jun;**158**(Pt 6):1570—80. PubMed PMID: 22422752. PMCID: 3541776.
20. Basta NE, Stuart JM, Nascimento MC, Manigart O, Trotter C, Hassan-King M, et al. Methods for identifying *Neisseria meningitidis* carriers: a multi-center study in the African meningitis belt. *PloS one* 2013;**8**(10):e78336. PubMed PMID: 24194921. PMCID: 3806823.
21. Jolley KA, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 2010;**11**:595. PubMed PMID: 21143983. PMCID: 3004885. Epub 2010/12/15. eng.
22. Bennett JS, Watkins ER, Jolley KA, Harrison OB, Maiden MC. Identifying *Neisseria* species by use of the 50S ribosomal protein L6 (rplF) gene. *J Clin Microbiol* 2014 May;**52**(5): 1375—81. PubMed PMID: 24523465. PMCID: 3993661. Epub 2014/02/14. eng.
23. Saitou N, Nei M. The neighbor-joining method — a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987 Jul; **4**(4):406—25. PubMed PMID: ISI: A1987J406700007. English.
24. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980 Dec;**16**(2):111—20. PubMed PMID: 7463489. eng.
25. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013 Dec;**30**(12):2725—9. PubMed PMID: 24132122. PMCID: 3840312.
26. Harmsen D, Singer C, Rothganger J, Tonjum T, de Hoog GS, Shah H, et al. Diagnostics of neisseriaceae and moraxellaceae by ribosomal DNA sequencing: ribosomal differentiation of medical microorganisms. *J Clin Microbiol* 2001 Mar;**39**(3): 936—42. PubMed PMID: 11230407. PMCID: 87853.
27. Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, et al. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* 2012 Mar;**62**(Pt 3):716—21. PubMed PMID: 22140171.
28. Thompson EAL. Antigenic diversity of meningococcal enterobactin receptor FetA, a vaccine component. *Microbiology* 2003;**149**(7):1849—58.
29. Claus H, Elias J, Meinhardt C, Frosch M, Vogel U. Deletion of the meningococcal fetA gene used for antigen sequence typing of invasive and commensal isolates from Germany: frequencies and mechanisms. *J Clin Microbiol* 2007 Sep;**45**(9):2960—4. PubMed PMID: 17626167. PMCID: 2045310. Epub 2007/07/13. eng.
30. Findlow H, Vogel U, Mueller JE, Curry A, Njanpop-Lafourcade BM, Claus H, et al. Three cases of invasive meningococcal disease caused by a capsule null locus strain circulating among healthy carriers in Burkina Faso. *J Infect Dis* 2007 Apr 1;**195**(7):1071—7. PubMed PMID: 17330799.
31. Gamougam K, Daugla DM, Toralta J, Ngadoua C, Fermon F, Page AL, et al. Continuing effectiveness of serogroup A meningococcal conjugate vaccine, Chad, 2013. *Emerg Infect Dis* 2015 Jan;**21**(1):115—8. PubMed PMID: 25536336. PMCID: 4285275.
32. Greenwood B. Manson Lecture. Meningococcal meningitis in Africa. *Trans R Soc Trop Med Hyg* 1999 Jul—Aug;**93**(4):341—53. PubMed PMID: 10674069.
33. Zhu P, van der Ende A, Falush D, Brieske N, Morelli G, Linz B, et al. Fit genotypes and escape variants of subgroup III *Neisseria meningitidis* during three pandemics of epidemic meningitis. *Proc Natl Acad Sci U. S. A* 2001 Apr 24;**98**(9):5234—9. PubMed PMID: 11287631. PMCID: 33193.
34. Bennett JS, Thompson EA, Kriz P, Jolley KA, Maiden MC. A common gene pool for the *Neisseria* FetA antigen. *Int J Med Microbiol* 2009 Feb;**299**(2):133—9. PubMed PMID: 18718812. PMCID: 3968273. Epub 2008/08/23. eng.
35. Marsay L, Dold C, Green CA, Rollier CS, Norheim G, Sadarangani M, et al. A novel meningococcal outer membrane vesicle vaccine with constitutive expression of FetA: a phase I clinical trial. *J Infect* 2015 Sep;**71**(3):326—37. PubMed PMID: 25982025. PMCID: 4535279.
36. Thompson EA, Feavers IM, Maiden MC. Antigenic diversity of meningococcal enterobactin receptor FetA, a vaccine component. *Microbiology* 2003 Jul;**149**(Pt 7):1849—58. PubMed PMID: 12855736. Epub 2003/07/12. eng.
37. Gagneux SP, Hodgson A, Smith TA, Wirth T, Ehrhard I, Morelli G, et al. Prospective study of a serogroup X *Neisseria meningitidis* outbreak in northern Ghana. *J Infect Dis* 2002 Mar 1;**185**(5): 618—26. PubMed PMID: 11865418.
38. Boquete MT, Marcos C, Saez-Nieto JA. Characterization of *Neisseria polysaccharea*e sp. nov. (Riou, 1983) in previously identified noncapsular strains of *Neisseria meningitidis*. *J Clin Microbiol* 1986 May;**23**(5):973—5. PubMed PMID: 3086373. PMCID: 268766.
39. Saez-Nieto JA, Dominguez JR, Monton JL, Cristobal P, Fenoll A, Vazquez J, et al. Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in a school population during an epidemic period in Spain. *J Hyg (Lond)* 1985 Jun;**94**(3):279—88. PubMed PMID: 3924995. PMCID: 2129489.
40. Cartwright KA, Stuart JM, Jones DM, Noah ND. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria*

lactamica. *Epidemiol Infect* 1987 Dec;**99**(3):591—601. PubMed PMID: 3123263. PMCID: 2249239.

41. Kremastinou J, Tzanakaki G, Levidiotou S, Markou F, Themeli E, Voyiatzi A, et al. Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in northern Greece. *FEMS Immunol Med Microbiol* 2003 Oct 24;**39**(1):23—9. PubMed PMID: 14556992.
42. Sheikhi R, Amin M, Rostami S, Shoja S, Ebrahimi N. Oropharyngeal colonization with *Neisseria lactamica*, other nonpathogenic Neisseria species and Moraxella catarrhalis among young healthy children in Ahvaz, Iran. *Jundishapur J Microbiol* 2015 Mar;**8**(3): e14813. PubMed PMID: 25964847. PMCID: 4418171.
43. Saez Nieto JA, Marcos C, Vindel A. Multicolonization of human nasopharynx due to Neisseria spp. *Int Microbiol* 1998 Mar;**1**(1): 59—63. PubMed PMID: 10943342.
44. Maiden MC, Ibarz-Pavon AB, Urwin R, Gray SJ, Andrews NJ, Clarke SC, et al. Impact of meningococcal serogroup C conjugate vaccines on carriage and herd immunity. *J Infect Dis* 2008 Mar 1;**197**(5):737—43. PubMed PMID: 18271745.
45. Bennett JS, Griffiths DT, McCarthy ND, Sleeman KL, Jolley KA, Crook DW, et al. Genetic diversity and carriage dynamics of *Neisseria lactamica* in infants. *Infect Immun* 2005 Apr;**73**(4): 2424—32. PubMed PMID: 15784588. PMCID: 1087434.
46. Trotter CL, Yaro S, Njanpop-Lafourcade B-M, Drabo A, Kroman SS, Idohou RS, et al. Seroprevalence of bactericidal, specific IgG antibodies and incidence of meningitis due to group A *Neisseria meningitidis* by age in Burkina Faso 2008. *PloS one* 2013;**8**(2):e55486.
47. Blackwell CC, Weir DM, James VS, Todd WT, Banatvala N, Chaudhuri AK, et al. Secretor status, smoking and carriage of *Neisseria meningitidis*. *Epidemiol Infect* 1990 Apr;**104**(2): 203—9. PubMed PMID: 2323355. PMCID: PMC2271764. eng.
48. Koeberling O, Ispasanie E, Hauser J, Rossi O, Pluschke G, Caugant DA, et al. A broadly-protective vaccine against meningococcal disease in sub-Saharan Africa based on generalized modules for membrane antigens (GMMA). *Vaccine* 2014 May 13;**32**(23):2688—95. PubMed PMID: 24704334.