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Title Page

Genomic meningococcal load in the nasopharynx of children with meningococcal disease

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2 HiberGene Diagnostics Ltd. MDS has nothing to disclose.
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11 Meningococcal disease, *Neisseria meningitidis*, bacterial load
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18 Nasopharyngeal bacterial load in meningococcal disease
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22 Running title:

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25 NP meningococcal load
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Abstract

This study demonstrates the feasibility of using quantitative real time PCR to measure genomic bacterial load in the nasopharynx of children with invasive meningococcal disease and shows that these loads are exceptionally high (median 6.6×10^5 (Range 1.2×10^5 to 1.1×10^8) genome copies of *Neisseria meningitidis* per swab).

Introduction

Invasive infection with *Neisseria meningitidis* (NM) causes meningitis and septicemia in children [meningococcal disease]. Most adults have carried meningococcus in their nasopharynx at some stage during their lives¹ and most remain asymptomatic. A very small proportion progress to invasive disease.

The prevalence of meningococcal carriage is often quoted as being ~10%, although it should be emphasised this refers to the average carriage rate across all age groups.² Carriage rates vary dramatically with age, with the lowest rates seen in very young children, who are at highest risk of invasive disease, and the highest rates (often in excess of 20%) seen in late adolescence.^{2,3}

Public Health England (PHE) and National Institute for Health and Care Excellence (NICE) guidelines previously disagreed over the value of nasopharyngeal specimens as a diagnostic test for this infection. PHE recommend that testing of nasopharyngeal specimens may help support a clinical diagnosis alongside other signs and symptoms, and can allow identification of strains in the event of a cluster or outbreak.² Advice not to test nasopharyngeal specimens by any method because of the risk of detecting asymptomatic carriage has recently been removed from UK NICE guidance, to bring it into line with PHE guidance.⁵

Two previous studies from our group demonstrate that molecular testing of nasal and throat swabs has a sensitivity of 81 to 84 % and a specificity of 100% as a diagnostic test for meningococcal disease.^{6,7} With the recent development and licensure of novel meningococcal vaccines, their effect on reducing nasopharyngeal carriage is likely to have a major impact on their effectiveness at inducing herd protection.

Two studies demonstrate a strong association between meningococcal DNA load in blood at presentation and disease severity.^{8,9} To date, no study has attempted to quantify meningococcal DNA load in nasopharyngeal specimens using molecular testing.

Estimation of nasopharyngeal bacterial load is complicated by potential inconsistency in the sampling technique resulting in varying amounts of cellular material being obtained. Specifically, false negative results may arise from poor quality specimens containing little or no cellular material. The RNaseP gene is a proven single copy human gene. Absolute quantification of this gene using real-time TaqMan PCR can be used to accurately estimate the quantity of human DNA (and hence cells) in a clinical sample.¹⁰ This enables quality control of specimens, and can potentially also be used to 'normalise' the bacterial load data.

The aim of this study was to define the meningococcal DNA load in nasopharyngeal specimens in children with meningococcal disease.

Methods

Nasopharyngeal swabs and blood specimens were collected from children with suspected meningococcal disease as part of an ethically approved diagnostic accuracy study between November 1st 2009 and January 31st 2012.⁷ Nasopharyngeal specimens were obtained using rayon tipped throat swabs (Transwab, MWE, Wiltshire, UK) and floc tipped nasal swabs (Copan, Brescia, Italy). Both swabs were then placed into a single container containing 1 ml of eNat nucleic acid transport medium (Copan, Brescia, Italy). For this study paired samples from

children with laboratory confirmed disease [Culture of *NM* and/or detection of *NM* DNA from a normally sterile site; see reference 7] were analysed. Specimens were tested using *ctrA* and *RNAseP* real-time TaqMan PCR and the genomic bacterial load was estimated using external standard curve calibration. Details of the PCR assays, molecular testing protocols and calculation of standard curves is given in Supplementary Digital Content A. Requirement for fluid bolus resuscitation was used as an indicator of more severe clinical presentation.

Statistical methods

Skewed data were log transformed for statistical comparisons. Pearson's correlation coefficient and means were compared using an independent samples two tailed t test. One obvious and statistically significant outlier {linear regression RStudent >2 criteria (outlier) and CooksD >0.5 (undue influence)} was not included in the analyses but the outlier was separately described.

Multiple regression analysis was performed using age (in days), gender, severity score (with/without fluid bolus) and copy number in blood as variables to predict copy number in the nasopharynx.

Results

21 pairs of specimens from children with laboratory confirmed meningococcal disease were analysed.

1 There was a 2:1 female:male ratio (67% female, 33% male). The age range was 6
2 months to 8 years 4 months (mean 2 years 10 months). Using need for fluid bolus
3 resuscitation as a proxy for severity, 10/21 (48%) required at least one bolus.
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10 The nasopharyngeal specimens contained a median of 6.6×10^5 (Range 1.2×10^5 to
11 1.1×10^8) genome copies of meningococcus per ml of transport medium. As the
12 combined nasal and throat swab were inserted into 1 ml of medium this reflects the
13 total amount of bacteria obtained on both swabs. The blood specimens contained a
14 median of 9.9×10^5 (Range 3.7×10^4 to 8.2×10^7) genome copies of meningococcus
15 per ml of original specimen. There was no difference in mean log copy number
16 between nasopharyngeal and blood specimens ($3.49 \log_{10}$ copies/ml versus 3.54
17 $\log_{10}$ copies/ml, $p = 0.9$).
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30 The outlier excluded from the analysis was from a patient who had an exceptionally
31 high nasopharyngeal bacterial load [1.1×10^8] and a moderate blood bacterial load
32 [4.7×10^6] following a short [<6 hours] history. Without this outlier value a moderate
33 correlation ($R = 0.57$ (95% CI: 0.16 to 0.80) [Figure 1]) was found between
34 nasopharyngeal and blood meningococcal copy number.
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42 Analysis of RNaseP in the nasopharyngeal specimens revealed a median of $2.5 \times$
43 10^5 (range 2.1×10^3 to 6.4×10^6) cells per ml of transport medium. Correcting the
44 bacterial load for nasopharyngeal specimen quality by calculating the number of
45 bacteria per host cell weakened the correlation between log nasopharyngeal and log
46 blood meningococcal copy number. A strong correlation between log bacteria per 10
47 cells and the raw log nasopharyngeal copy number was observed (Pearson's
48 correlation coefficient $R = 0.74$, $p = 0.001$) indicating that the uncorrected and
49 corrected nasopharyngeal load are closely related.
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Children who required fluid resuscitation at the time of admission had significantly higher mean blood log copy numbers than those who did not ($3.90 \log_{10}$ copies/ml versus $3.06 \log_{10}$ copies/ml, $p = 0.02$). Nasopharyngeal log copy number also tended to be higher but this did not reach significance. The single patient who died during the study had a nasopharyngeal meningococcal copy number of 5.1×10^6 (seven times higher than the median) and a blood meningococcal copy number of 3.1×10^7 (thirty times higher than the median). This patient had a short history having gone to bed well at 22:00 and awoken in the early hours of the morning with an extensive purpuric rash.

Multiple regression analysis confirmed that copy number in blood was the only statistically significant predictor of copy number in the nasopharynx, after adjustment for age, gender and severity (β -coefficient 0.84; $p=0.024$). None of the other variables were significant, and there was no bias towards more severe clinical presentation.

Discussion

This small study is the first to demonstrate the exceptionally high nasopharyngeal loads of meningococcus in patients presenting with confirmed meningococcal disease. The bacterial load in blood is very similar to previous studies indicating that the methodology used is robust.^{8,9} After removal of one statistical outlier we observed a moderate correlation between nasopharyngeal and blood bacterial load. This potential correlation is based on a small data set and confirmation in a larger study is required. Multiple regression analysis identified blood load as the only statistically significant predictor of nasopharyngeal load.

1 Interpretation of meningococcal load in nasopharyngeal specimens is challenging
2 because of potential inconsistency in sampling. Analysis of RNaseP reveals that the
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4 quantity of human material obtained by a nasopharyngeal swab ranges from a few
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6 thousand to a few million cells. Despite this variation we still demonstrate very high
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8 loads, even in relatively poor quality samples. Furthermore, there is a strong
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10 correlation between the raw meningococcal load data and meningococcal load
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12 corrected for swab quality indicating that correction is probably not necessary.
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17 In this study we only had a single specimen at a single time point and cannot
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19 describe the dynamics of meningococcal copy number or relate nasopharyngeal to
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21 blood copy number as invasive disease progresses. The demonstration of relatively
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23 high nasopharyngeal loads in some patients supports our hypothesis that molecular
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25 testing of nasopharyngeal specimens adds value to diagnostic testing for
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27 meningococcal disease. Testing of both nasopharyngeal and blood specimen by ctrA
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29 real-time TaqMan PCR (as occurs in our unit) has the potential to capture more
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31 cases. Using a molecular test to detect the ctrA gene in nasopharyngeal swabs
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33 ensures that only pathogenic (capsular) strains are detected. In contrast, culture of
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35 nasopharyngeal swabs will also recover carrier (acapsular) strains.
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43 Novel meningococcal B vaccines targeting outer membrane vesicles may have a
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45 much less dramatic effect on carriage¹¹ than conjugate vaccines like Men C which
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47 virtually eliminated carriage. It is clear that any large implementation programme will
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49 require careful monitoring of carriage and we believe that the methodology described
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51 in this study would be ideal for large scale accurate surveillance studies.
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56 The results presented here confirms that quantitative data can be reliably obtained
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58 from nasopharyngeal swab specimens using molecular methods, and that a human
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gene target (RNaseP) can be used as an endogenous control. These data provide further evidence that molecular testing of respiratory specimens is feasible, and may give diagnostically useful information in suspected meningococcal disease. We believe that access to relatively non-invasive rapid molecular testing of nasopharyngeal specimens such as meningococcal LAMP ^{7,12} has potential to increase diagnosis of meningococcal disease in the early stages where nasopharyngeal loads may to be relatively higher as initial replication is taking place.

Conflicts of interest and relevant funding:

TWB received a grant from the Health & Social Care Research and Development Office, Public Health Agency, Northern Ireland. JPM, PVC and DJF hold share options in HiberGene Diagnostics Ltd. JPM, PVC and DJF hold patent US 8465927 B2 related to the meningococcal LAMP assays cited in this study which is licensed to HiberGene Diagnostics Ltd. MDS has nothing to disclose.

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Figure 1. Comparison of *Neisseria meningitidis* genome copy number in nasopharyngeal and blood specimens

Figure

Supplementary digital content A

DNA Extraction

DNA was extracted from EDTA blood (200µl) and eNat medium (200µl, containing combined nasal and throat swabs) using a QuickGene Mini80 system and DB-S DNA kit (Fuji Corporation, Tokyo, Japan) according the manufacturers' instructions.

Standard curves

Standard curves were defined for absolute quantification using real-time TaqMan PCR. *Neisseria meningitidis* serogroup B (NCTC 10026) was cultured, suspended in saline and subjected to DNA extraction using QIAamp DNA Mini Kit. The genomic DNA in the extract was quantified using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, USA). The number of copies of meningococcal DNA per ml of extract was then estimated from the average genomic molecular weight of meningococcus B.¹ For the RNaseP target 200 µl of sterile EDTA blood (male) was extracted using the same extraction method. The genomic DNA in the extract was quantified using a NanoDrop. The number of human genome copies was estimated from the average molecular weight of the haploid human genome (6.294×10^9 base pairs).² Tenfold serial dilutions of the meningococcal and human DNA extracts were then prepared and tested by ctrA and RNaseP real-time TaqMan PCR respectively [see below]. Standard curves were constructed using “trimmed mean” values from 5 replicates (mean of 3 central results from each replicate set). This gives a more robust measure of central tendency than the arithmetic mean.³

PCR assays

PCR assays used Invitrogen Platinum® Quantitative PCR SuperMix-UDG (Life Technologies Ltd., Paisley, UK). Primers and a TaqMan® probe to detect the meningococcal *ctrA* gene were used as described previously.⁴ An additional reverse primer (5'-TTGCCGCGGATTGGCCACCA-3') was used⁵ to ensure that strains with known mutations in the *ctrA* gene could be reliably detected.^{6,7} Primers and probe to detect RNaseP were used as described by the Centre for Disease Control, 2009 [RNaseP F: 5'- AGA TTT GGA CCT GCG AGC G -3'; RNaseP R: 5'- GAG CGG CTG TCT CCA CAA GT -3'; RNaseP P: 5'-FAM - TTC TGA CCT GAA GGC TCT GCG CG -TAMRA 3']

PCR assays were run on a LightCycler® 480II real-time PCR system (Roche Diagnostics Ltd., Burgess Hill, UK) using the following thermal cycling protocol: 50°C (10 minutes); 95°C (2 minutes); 45 cycles of 95°C (15 seconds) and 60°C (30 seconds).

All analysis was conducted on a single plate to avoid inter-assay variation. DNA copy number per µl of extract for each specimen was calculated using the standard curves. Meningococcal bacterial load per ml of blood and meningococcal load and number of human cells per ml of transport medium were then calculated.

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