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***Neisseria meningitidis* disease-associated clones in Amazonas State, Brazil**

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Abstract

Background: The aim of this study is to describe the molecular epidemiology of *Neisseria meningitidis* invasive disease before the introduction of serogroup C conjugate vaccine in Amazonas State in 2010.

Methods: Meningococcal disease reported cases were investigated in Amazonas State during the period 2000 to 2010. *N. meningitidis* isolates (n = 196) recovered from patients were genotyped by multilocus sequence typing (MLST) and sequencing of *porB*, *porA*, *fetA*, *fHbp*, and *penA*. Antimicrobial susceptibility was determined using E-test.

Results: In the study period, 948 cases were reported; the incidence was 2.8 for the entire state and 4.8 per 100,000 in the capital of Manaus. Most meningococcal disease was caused by *N. meningitidis* belonging to ST-32 (72%; 141/196) or ST-103 (21%; 41/196) clonal complexes. Capsular switching (B→C) was suggested within clonal complex (cc) 32. There were 6 (3%; 6/196) strains with intermediate susceptibility to penicillin and a single strain was resistant to rifampicin. Since 2007, serogroup C strains belonging to the cc103 have predominated and case-fatality has increased.

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Disclosure statement

Dr. Harrison reports serving on a scientific advisory board for GlaxoSmithKline.

Conclusions: We demonstrate a high rate of meningococcal disease in Amazonas State, where, like other parts of Brazil, serogroup C replaced serogroup B during 2000s. These data serve as a baseline to measure impact of serogroup C conjugate vaccine introduction in 2010. This study emphasizes the need for enhanced surveillance to monitor changes in meningococcal disease trends following the introduction of meningococcal vaccines.

Keywords

Neisseria meningitidis, meningococcal disease; Serogroup C conjugate vaccine; MLST clones; Amazonas

Introduction

Sporadic and epidemic meningococcal disease has been reported since the beginning of the last century in Brazil, when reporting of disease became mandatory [1]. In Brazil approximately 30,000 cases of meningococcal disease were reported between 2000 and 2010; 20% of this cases died and survivors with permanent sequelae are unknown [2]. Most notifications (68%) are from the south and south-east regions and metropolitan areas, a fact probably related to a better structure for the diagnosis and reporting of cases, which in some way should mask the importance of the disease in rural or remote areas, where control measures are the biggest challenge.

Serogroup C *Neisseria meningitidis* replaced serogroup B as the predominant serogroup during the 2000s in Brazil, which led to introduction of serogroup C conjugate vaccine into the paediatric immunization schedule in 2010 [3]. This serogroup replacement was associated with outbreaks and high case fatality rates, with outbreak-causing strains belonging genetically to different clonal complexes (cc): cc11, cc32, and cc103 [4].

N. meningitidis infection is a public health problem in Amazonas State, North of Brazil. With a highly-forested and mostly rural land mass of 1,559,149 km² and a population of approximately 3.5 million inhabitants, public health surveillance for meningococcal disease is quite challenging and, because of that, the disease burden is not well defined.

To date, no reports describing the molecular epidemiologic surveillance of meningococcal disease in Amazonas have been published. A single study genotyped a small number of serogroup B *N. meningitidis* isolates (n = 21) recovered from patients living in North of Brazil in 2004, without specifying the state of origin [5].

We report here surveillance data of meningococcal disease and molecular characterization of *N. meningitidis* isolates recovered from patients in Amazonas State, during the period 2000 to 2010.

Patients and Methods

Study design

The study was conducted before introduction of serogroup C conjugate vaccine (MCC-CRM₁₉₇) into the paediatric immunization schedule (< 2 years) in 2010. A retrospective

study was conducted in a series of cases of meningococcal disease reported between 2000 and 2010 to the Foundation of Health Surveillance of Amazonas, with residence in the state of Amazonas. Molecular typing methods were applied to characterize *N. meningitidis* recovered from invasive disease in this period of time.

Cases of meningococcal disease reported

According to the criteria of epidemiological surveillance, patients were defined as confirmed cases by either a *N. meningitidis* positive culture on heated blood agar, a positive meningococcal latex agglutination test or positive direct smear using Gram stain. Suspected cases are those with a clinical picture of fever of abrupt onset and characteristic haemorrhagic skin lesions. Clinical and epidemiological data were analysed using EpiInfo™ (version 3.5.4, Centers for Disease Control and Prevention, Atlanta, GA, USA).

Neisseria meningitidis invasive isolates

N. meningitidis isolates has been stored since 2001, which were obtained from the National Reference Centre for Meningitis (NRCM, Instituto Adolfo Lutz, São Paulo, Brasil), where serogroup was determined by slide agglutination. No changes occurred in the collection and storage protocols or patient population during the study period. There were no significant differences in the number of isolates annually sent by the Central Laboratory of the state of Amazonas, or in the criteria for sending isolates to the NRCM during the period of study.

Sequencing of housekeeping gene fragments and antigen encoding genes

The genetic lineage of *N. meningitidis* was determined by multilocus sequence typing (MLST) and sequencing of outer membrane protein encoding genes: *porB*, *porA*, and *fetA*, *fHbp*, and *penA*; *rpoB* for a single isolate [6,7]. Capsular switching was defined based on MLST and antigen encoding genotypes in clones expressing a serogroup not normally associated with that clone [8]. Sequence types (STs) and alleles at antigenic loci were assigned by the *N. meningitidis* MLST database (www.pubmlst.org/neisseria).

Antimicrobial susceptibility testing

Susceptibility to penicillin, ampicillin, azithromycin, rifampicin, chloramphenicol, and ciprofloxacin was determined using E-test® (bioMérieux, Marcy-l'Étoile, France) and results were interpreted based on the Clinical and Laboratory Standards Institute document M100-S20 [9]. Briefly, a single E-test strip was placed onto Müller-Hinton (Oxoid, Basingstoke, Hampshire, UK) agar plates supplemented with 5% sheep blood, which were incubated at 35°C in a 5% CO₂/95% air atmosphere for 20 to 24 hours. Isolates were stored in freezer vials containing tryptone soya broth with 20% glycerol at -70°C.

Ethical Considerations

This study was approved by the Ethics Committee of the Evandro Chagas Research Institute of the Oswaldo Cruz Foundation (protocol 0029.0.009.000–10).

Results

Surveillance data

Between 2000 and 2010, 948 invasive meningococcal disease cases were reported in the state of Amazonas: 31% were suspected cases and 69% were confirmed cases (culture [40%; 381], direct smear [18%; 174], antigen detection [10%; 94], or necropsy [1%; 7]). The average annual incidence rate was 2.8 cases per 100,000 inhabitants (range, 1.6 to 5.3) in the entire state (Figure 1); in children < 2 years the incidence was 6.7/100,000 (range, 2.8 to 12.5). However, in the capital city of Manaus (1.8 million inhabitants), the average annual incidence was 4.8/100,000 (range, 2.3 to 8.3) (Figure 1); children < 2 years was 8.5/100,000 (range, 5.4 to 22.5). Although cases were reported widely throughout the state, with 58 of 62 cities reporting at least one case, the majority of cases were from Manaus (81%; 769/948) (Figure 2).

The average case-fatality rate was 18% (171/930; 18 with missing data). The case fatality among patients who resided in Manaus, were transferred to Manaus for treatment from the rest of the state, and treated in the rest of the state were 18% (140/757; 12 with missing data), 10% (11/109; 4 with missing data), and 31% (20/64; 2 with missing data), respectively. The case-fatality rate among patients who resided in or were transferred to Manaus was significantly lower than among those who were treated in the rest of the state ($P = 0.01$).

Of 948 total cases, the meningococcal capsular group was determined for 460 (49%) cases: 344 serogroup B (74.8%), 114 serogroup C (24.8%), and 2 serogroup A (0.4%). Serogroup B disease predominated until 2006 (87%; 315/363), after which it was gradually replaced by serogroup C (68%; 66/97) (Figure 3). This serogroup replacement was accompanied by an increase in case-fatality rate, from 16% during 2000–2006 to 25% during 2007–2010 ($p < 0.01$).

Patients with serogroup C infection had a greater risk of dying (12%; 13/109, 5 with missing data), compared with serogroup B disease patients (9%; 29/341, 3 with missing data), although this difference was not statistically significant ($P = 0.1$).

There was also a significant increase in the number of patients over 9 years of age: 52% (2000–2006) versus 64% (2007–2010) ($P < 0.01$).

Molecular typing

All isolates stored in the bacteria collection were selected from each year for this study. The genetic lineage of 196 *N. meningitidis* recovered in culture was determined by sequence of housekeeping and antigen encoding genes (Table 1). Serogroup B isolates belonged to clonal complex (cc) 32 or cc41/44, whereas serogroup C isolates were predominantly assigned to cc103 followed by cc8 and cc32 (Figure 4). ST-5996 and ST-11401 strains were predominant in cc32 (45%; 64/141) and cc103 (61%; 25/41), respectively (Table 1, Figure 4, and Figure S1). The *porA* gene was not detected in 3 cc103 serogroup C strains belonging to ST-3779 and ST-11401, suggesting possible *porA* deletion. The majority of isolates (77%)

had *fHbp* alleles belonging to variant family 1 with remaining isolates belonging to variant families 2 and 3 (14% and 7%, respectively) (Figure S2) [10].

Genotyping results suggested B to C capsular switching in the following strains: 3–1:P1.19,15:F5–1:ST-639 (2 strains from 2004 or 2007), 3–587:P1.19,15:F5–1:ST-639 (2 strains from 2004) and 3–1:P1.19,15:F5–13:ST-5996 (2 strains from 2004) (Table 1, Figure 4).

Antimicrobial susceptibility pattern

All isolates were susceptible to ceftriaxone (MICs, 0.002 – 0.004 µg/ml), chloramphenicol (MICs, 0.125 – 0.75 µg/ml), azithromycin (MICs, 0.094 – 0.75 µg/ml), and ciprofloxacin (MICs, 0.002 – 0.004 µg/ml). Most isolates (97%; 190/196) were susceptible to penicillin (MICs, 0.004 – 0.064 µg/ml) and had the following *penA* alleles: *penA1* (32%), *penA2* (2%), *penA3* (63%), or *penA573–577* (3%). There were six (3%; 6/196) strains with intermediate susceptibility to penicillin (MIC 0.125 – 0.19 µg/ml): five serogroup B (four ST-2503 [*penA54*], one ST-34 [*penA144*]) and one serogroup C (ST-3780 [*penA14*]). A single serogroup C strain (ST-153 [*tpoB14*]) was resistant to rifampicin (MIC > 32 µg/ml).

Discussion

In this study, the overall incidence of meningococcal disease in the state of Amazonas was 2.8/100,000 inhabitants and substantially higher (4.8/100,000) in the capital. Given the difficulties in conducting public health surveillance in a large and complex state such as Amazonas, these estimates likely represent a substantial underestimate of the true meningococcal disease burden. Also, early clinical features of meningococcal disease are indistinguishable from those of many other prevalent viral and parasitic diseases in Amazonas. Therefore, meningococcal disease may not be included as differential diagnosis of acute infectious fever in an early stage, with resulting delayed or mis-diagnosis contributing to underreporting. Despite this limitation, the rates of meningococcal disease observed in this study are in line with studies done in other parts of Brazil [3]. Nearly a third of cases were based on a clinical case definition (suspected cases). However, in a previous study in Brazil using PCR for case ascertainment, we found that the majority of patients meeting this case definition had meningococcal disease [11].

An epidemic of serogroup B meningococcal disease in Amazonas was recorded in 1998 in the capital city of Manaus [21], when serogroup B disease predominated in Brazil and outbreaks of ST-32 complex/ET-5 complex strains were detected [22, 23]. At that time, mass vaccination campaigns were carried out in several states around the country with a vaccine containing outer membrane proteins and group C capsular polysaccharide (Va-Mengoc-BC[®], Instituto Finlay, Habana, Cuba) [24].

We also observed a shift from serogroup B to serogroup C as the predominant serogroup, together with an upward shift in the age distribution. This change was associated with a marked decline in the major serogroup B sub-lineages within cc32 (ST-5996, ST-639 and ST-33) and ongoing persistence of serogroup C strains belonging cc103 (Figure S1A-B). Similarly, serogroup C re-emerged as the major cause of meningococcal disease during the

2000s in other parts of Brazil, where it has been associated with outbreaks and, as in the present study, a high case-fatality rate [4]. The data from this study will serve as a baseline by which to measure the impact in Amazonas of the 2010 introduction of serogroup C conjugate vaccine into the infant immunization program.

Molecular typing demonstrated that most meningococcal disease in Amazonas was caused by *N. meningitidis* belonging to cc32 (69%) or cc103 (21%), which are globally frequent hypervirulent clones. Molecular typing also revealed high genetic and antigenic diversity of the bacterial population, and the peak of ST-11401 (cc103) in 2004, which preceded the serogroup replacement described here. A serogroup B vaccine which includes FHbp protein from variant family 1 as one of its main components (Bexsero™), was licensed in Brazil in 2015 and is available in the private sector. Therefore, inclusion of *fHbp* sequence for fine strain typing is useful for routine meningococcal surveillance in the serogroup B vaccine era [7]. Our data provides an important regional assessment of strain coverage for possible introduction of the serogroup B vaccine in the public sector (Figure S2). Nevertheless, further studies are necessary to monitor the antigenic diversity of strains causing invasive disease after 2010.

A limitation of our study is that the number of isolates (21%) was low compared to the number of meningococcal disease reported during the study period. In addition, the number of culture-proven cases (40%) was also low, which reflects the difficulty of maintaining microbiology laboratories outside the capital city of Manaus. Regardless, we believe that the isolates that were studied are likely representative of the bacterial population of disease-associated strains

Swartley et al. [12], showed that switching from B to C capsule in a cc32 outbreak strain in Oregon, USA was the result of allelic exchange of the polysialyltransferase gene and that capsular switching likely contributed to persistence of the cc32 outbreak strain. Following this report, other authors have described capsular switching events between serogroups B, C, W, and Y, all expressing polysaccharides composed of sialic acid [8, 13–17] and less frequently for serogroups A and X, which express capsular polysaccharides without sialic acid [18–20].

The occurrence of presumed B→C capsular switching was observed among strains recovered from patients in Amazonas, as we reported earlier in Rio de Janeiro State [16] and has been confirmed by whole genome sequence analysis [25]. Capsular switching may have contributed to the long term persistence of cc32 in Brazil by evading population immunity to the original serogroup B strain, emphasizing the need for careful molecular surveillance to monitor trends and inform vaccine policy [25].

Resistance mutations in the *penA* gene that were linked with reduced susceptibility to penicillin was found in a low frequency (3%) among the strains tested in this study. The strains with intermediate susceptibility to penicillin had mosaic *penA* alleles, all of which contained amino acid substitutions in the penicillin binding protein 2 (PBP2) closely associated with reduced susceptibility to penicillin [26].

We found a single strain showing rifampicin resistance, which had the *rpoB14* allele that harbour mutations associated with high rifampicin resistance. The occurrence of resistance to rifampicin is rarely found in *N. meningitidis* isolates recovered from patients. It has been suggested that *rpoB* mutations reduce the ability of these isolates to provoke invasive disease [27].

In summary, we demonstrate a substantial rate of meningococcal disease in Amazonas State and regional changes in strain distribution during the decade preceding the introduction of a serogroup C conjugate vaccine. As in other parts of Brazil, the predominant strains were serogroup C [2]. Although *N. meningitidis* remains susceptible to first-line antibiotics, the management of patients is a challenge in Amazonas, where the mobility of people in the region is made almost exclusively by river [28]. Therefore, the best control measure is vaccination. Additional studies will be required to determine the impact of the introduction of serogroup C conjugate vaccine into the paediatric immunization schedule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Annual incidence rates (cases/100.000 inhabitants) of meningococcal disease in Amazonas State, and the capital city of Manaus, 2000 – 2010.

Figure 2.
Temporal and geographic distribution of meningococcal disease patients, Amazonas State, and the capital city of Manaus, 2000 – 2010.

Figure 3.
Distribution of disease-associated serogroups of *Neisseria meningitidis* in Amazonas State, 2000 – 2010.

Figure 4.

Maximum likelihood tree of MLST data from 196 *Neisseria meningitidis* isolates collected from Amazonas State, 2001–2010. Branches are labeled by ST, serogroup B (▲) serogroup C (△), and FHbp family-1 (●), 2 (●), or 3 (●).

Table 1.

Genotyping results (*porB:porA:fetA:ST*) of 196 *Neisseria meningitidis* recovered from patients in Amazonas State, 2001 – 2010.

Serogroup	Genotype (number of strains)
B	Clonal complex ST-32 (n = 135)
2002	3-1:P1.19,15:F5-1:ST-33 (1)
2001–2005	3-1:P1.19,15:F3-4:ST-33 (15)
2004–2006	3-1:P1.19,15:F2-1:ST-33 (3)
2010	3-593:P1.19,15:F3-4:ST-33 (1)
2006–2009	3-1:P1.19,15:F5-1:ST-34 (6)
2002	3-1:P1.19,15:F3-4:ST-639 (1)
2002–2007	3-1:P1.19,15:F5-1:ST-639 (7)
2001–2008	3-79:P1.7-1,1:F5-1:ST-639 (14)
2010	3-589:P1.19,15:F5-1:ST-639 (1)
2002–2008	3-35:P1.7-2,16:F1-50:ST-2503 (4)
2004	3-1:P1.19,15:F3-4:ST-2763 (1)
2003	3-79:P1.7-1,1:F5-1:ST-3764 (2)
2004	3-1:P1.19,15:F5-13:ST-3765 (1)
2003–2007	3-1:P1.19,15:F5-1:ST-5707 (5)
2002–2006	3-1:P1.19,15:F5-13:ST-5996 (43)
2002–2005	3-1:P1.19,15:F1-37:ST-5996 (2)
2004	3-1:P1.19,15:F5-1:ST-5996 (1)
2004	3-1:P1.19,15:F6-2:ST-5996 (2)
2005	3-1:P1.19,15:F3-15:ST-5996 (1)
2004	3-1:P1.18-1,3:F5-13:ST-5996 (1)
2002	3-79:P1.7-1,1:F5-1:ST-5996 (1)
2002–2004	3-272:P1.19,15:F5-13:ST-5996 (9)
2002	3-591:P1.19,15:F5-13:ST-5996 (1)
2005	3-592:P1.19,15:F5-13:ST-5996 (1)
B	Clonal complex ST-32
2004	3-1:P1.19,15:F5-13:ST-8025 (1)
2002	3-586:P1.19,15:F5-1:ST-11402 (3)
2008	3-79:P1.7-1,1:F5-1:ST-11403 (1)
2002	3-1:P1.19,15:F5-13:ST-11600 (1)
2002	3-1:P1.19,15:F5-13:ST-11608 (1)
2002	3-1:P1.19,15:F5-13:ST-11610 (1)
2003	3-1:P1.19,15:F5-13:ST-11612 (1)
2010	3-1:P1.19,15:F5-1:ST-11913 (1)
2002	3-1:P1.19,15:F5-13:ST-11921 (1)
B	Clonal complex ST-41/44 (n = 7)

Serogroup	Genotype (number of strains)
2004	3-588:P1.18-7,9:F4-38:ST-303 (1)
2003–2004	3-1:P1.5-2,10-11:F5-2:ST-1788 (2)
2003	3-66:P1.18-1,3:F1-76:ST-7042 (1)
2005	3-71:P1.7-1,1:F5-2:ST-11404 (1)
2009	3-1:P1.7-2,13-1:F5-2:ST-11613 (1)
2003	3-590:P1.18-7,9:F1-10:ST-11615 (1)
C	Clonal complex ST-8 (n = 4)
2003	2-30:P1.7-2,13:F5-2:ST-153 (4)
C	Clonal complex ST-32 (n = 6)
2004–2007	3-1:P1.19,15:F5-1:ST-639 (2)
2004	3-587:P1.19,15:F5-1:ST-639 (2)
2004	3-1:P1.19,15:F5-13:ST-5996 (2)
C	Clonal complex ST-103 (n =41)
2002–2009	2-23:P1.18-1,3:F3-9:ST-3779 (6)
2008	2-23:P1.22,14-6:F3-9:ST-3779 (1)
2004–2005	2-23:P1. , :F3-9:ST-3779 (2)
2010	2-23:P1.22,14-6:F3-9:ST-3780 (2)
2004–2010	2-23:P1.22,14-6:F3-9:ST-11401 (23)
2008	2-23:P1.22,14-6:F1-84:ST-11401 (1)
2009	2-23:P1. , :F5-1:ST-11401 (1)
2010	2-23:P1.19,15:F3-9:ST-11405 (1)
2010	2-23:P1.22,14-6:F3-9:ST-11599 (2)
2003	2-23:P1.18-1,3:F3-9:ST-11614 (1)
2002	2-23:P1.18-1,3:F3-9:ST-11616 (1)
B	No clonal complex (n = 2)
2003	3-16:P1.22-1,14:F5-5:ST-11406 (1)
2002	3-79:P1.7-1,1:F5-1:ST11609 (1)
C	No clonal complex (n = 1)
2006	2-23:P1.22,14-6:F3-9:ST-11522 (1)