Bacterial meningitis in Salvador, Brazil

Five Years of Active Hospital-Based Surveillance

by

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I. PREFACE

Bacterial meningitis is one of the top infectious causes of death worldwide, and imposes a high burden of morbidity and mortality on infants and young children of both the developed and developing worlds. Historically, *Haemophilus influenzae* type b was the most common pathogen responsible for meningitis in children of industrialized nations. Introduction of the conjugate Hib vaccine in the late 1980s has dramatically reduced the incidence of meningitis due to *H. influenzae* type b, and increased the proportion of meningitis cases in infants and children due to other pathogens, such as *Neisseria meningitidis. N. meningitidis* is also an important cause of bacterial meningitis in young adults, and is responsible for both outbreaks and sustained epidemics globally.

Mass vaccination with the conjugate Hib vaccine was begun in Brazil in the late 1990s and has significantly reduced incidence of *H. influenzae* meningitis. However, the full impact of the Brazilian vaccination campaign remains to be described. In addition, mass Hib vaccination campaigns could potentially increase the incidence of invasive disease due to other serotypes of *H. influenzae,* as a result of decreased inter-species competition, although this phenomena has not been documented to date.

Vaccines have not proven effective in the prevention of disease due to *N. meningitidis* in Brazil. Disease caused by serogroup B *Neisseria meningitidis* is endemic and has caused large outbreaks in Brazil since the 1980s. In contrast to other serogroups of *N. meningitidis,* no highly effective vaccine against serogroup B organisms is currently available. The epidemiologic features of serogroup B meningococcal disease in Brazil remain poorly

characterized, and little population-based information is available for serogroup B disease in the developing world. Such information is crucial to guiding the development of new serogroup B vaccines and to decisions regarding the use of currently available vaccines.

Active hospital-based surveillance for *N. meningitidis* and *H. influenzae* meningitis was carried out in Salvador, Brazil starting in 1996. Salvador is the capital of the northeastern state of Bahia and has over three million inhabitants. We report findings from five years of surveillance for meningitis due to *N. meningitidis* and 4.5 years of surveillance for meningitis due to *H. influenzae*, a period spanning introduction of the Hib vaccine in 1999. All cases of meningitis were prospectively identified and were confirmed by laboratory culture. Antisera and monoclonal antibodies were used to determine serogroup and serotype of *N. meningitides* isolates. *H. influenzae* isolates were analyzed using serological and polymerase chain reaction-based methods to determine serotype and pulsed field gel electrophoresis to evaluate clonality.

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I. Meningococcal meningitis in Salvador, Brazil: *Five years of active hospital-based surveillance*

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II. *Haemophilus influenzae* type a meningitis: *Population-based evidence for serotype replacement following introduction of Hib vaccination*

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II. MENINGOCOCCAL MENINGITIS IN SALVADOR, BRAZIL: FIVE YEARS OF ACTIVE HOSPITAL-BASED SURVEILLANCE

INTRODUCTION

Neisseria meningitidis is a leading cause of meningitis worldwide, responsible for significant morbidity and mortality in infants and young children [1]. Without appropriate antimicrobial treatment, most cases of meningococcal meningitis are fatal [2], and even with prompt intervention case fatality ratios approach 10% [3]. Among those who survive, permanent sequelae, including deafness, cognitive impairment, and paralysis, are common [4-6]. Given the rapid progression of the disease, the high cost of treatment, and the significant risk of an adverse outcome, development of effective meningococcal vaccines has been a high priority for many years.

A quadrivalent meningococcal vaccine including the capsular polysaccharides of serogroups A, C, Y and W-135 has been available since the 1980s, and polysaccharide-protein conjugate vaccines for groups A and C have been developed [7-10]. In contrast, serogroup B capsular vaccines have not proven effective, due to the polysaccharide's poor immunogenicity and mimicry of a human epitope [11-14]. Alternative strategies to develop serogroup B vaccines have focused instead on outer membrane proteins (OMP) contained in outer membrane vesicles (OMV) [15]. Antibody against the PorA OMP is the primary determinant of bactericidal activity following vaccination with OMV-based vaccines [16-18]. To date, however, efforts to develop effective OMV-based vaccines have been limited by the heterogeneity of the PorA protein, changes in the distribution of serogroup B strains over time, and incomplete understanding of the factors affecting the level of protection induced by these vaccines [18-20].

The epidemiologic features of endemic disease caused by serogroup B *N. meningitidis* in the developed world have been extensively described; although temporal and geographic variation in serogroup and serosubtype distribution and the dynamics of group B epidemics remain incompletely understood. Very few population-based data about the epidemiologic features and serosubtype diversity of serogroup B meningococcal disease are available for the developing world. Serogroup B meningococcal disease is endemic in Brazil, and since the 1980s serogroup B has caused the majority of meningococcal disease. For reasons that remain incompletely understood, the massive epidemics of serogroup A and C disease in Brazil during the 1970s [21] were followed by a decline in the incidence of disease caused by these serogroups and an increase in the incidence of cases caused by serogroup B. Introduction of the ET-5 complex of serogroup B, an invasive clonal group responsible for sustained epidemics globally [22-25], played a major role in this serologic shift. Specific serotype/ serosubtype combinations associated with the ET-5 complex, including B:4,7:P1.19,15, have been present in Brazil since at least the early 1980s, and have caused epidemics [17, 26]. Despite these important changes and the continued high morbidity and mortality caused by *N. meningitidis*, no active surveillance studies and very few data examining trends in the epidemiologic features of meningococcal disease in Brazil have been reported since the 1970s [17, 27]. We report the results of active surveillance for meningococcal meningitis conducted in the Metropolitan Region of Salvador, Brazil from February 1996 through February 2001.

METHODS

Surveillance

Active hospital-based surveillance for meningococcal meningitis was performed in Salvador, Brazil, a city of more than 2 million inhabitants located in the northeastern state of Bahia. It is mandatory under state health department protocol that all suspected cases of meningitis in the metropolitan region of Salvador be referred to a single state-run infectious disease referral hospital. This hospital reports more than 95% of the meningitis cases in the region (unpublished case notification records of the Secretary of Health for the State of Bahia).

Between February 1, 1996 and January 31, 2001, project personnel reviewed daily clinical laboratory records at the infectious disease referral hospital to identify all cases of meningococcal meningitis. A case was defined as culture of *N. meningitidis* from the cerebrospinal fluid (CSF) of a patient with the clinical signs and symptoms of meningitis. Following entry into the study in accordance with protocol approved by the Institutional Review Boards of the Brazilian Ministry of Health and Cornell University, a standardized data-collection form was completed for each case. Demographic and clinical history data were collected through interview and medical chart review.

Laboratory Methods

N. meningitidis were isolated from cultures of cerebrospinal fluid incubated at 37 degrees Celsius on sheep red blood cell agar. Slide agglutination with serogroup-specific sera was

used to identify the serogroup of isolates. Monoclonal antibodies were used to identify serotype and serosubtype of isolates, using the dot-blot method [28].

Analysis

Cases were included in incidence calculations only if they reported residing in the city of Salvador or in one of the 29 municipalities located within 75 km of Salvador, a region with an aggregate population > 3 million, and without any other major metropolitan centers or infectious disease hospitals. All incidence calculations were based on the 1996 Brazilian population estimates without adjusting for population growth, provided by the Brazilian Institute for Geography and Statistics (IBGE; http://www.ibe.government.br).

Data were entered and managed using Epi-Info Version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA), and analyzed using SAS for Windows Version 8.02 (SAS Institute, Cary, NC). Rate ratios and confidence intervals were calculated using Poisson regression. Statistical significance was assessed using $\chi 2$ or Fisher's exact test. Median ages were compared using Kruskal-Wallace. All p-values were based on two-sided tests.

RESULTS

During the five-year study period, 408 cases of meningococcal meningitis were detected. Three hundred and four (74.5%) of these cases occurred in individuals who lived within 75 km of Salvador and were included in the incidence calculations within 75 km of Salvador and were included in incidence calculations, yielding a mean annual incidence of 1.89 per 100,000 inhabitants. The incidence was 2.2 in year 1, 1.7 in year 2, 2.5 in year 3, 1.7 in year 4, and 1.5 in year 5 ($\chi 2$ test for trend, P=0.062) (Figure 1).

The monthly number of cases of meingococcal meningitis varied by season (Figure 2), with the highest proportion of cases observed in the month of May (10.9%), and the lowest proportion of cases occurring in January and February (5.6% each). Incidence during the rainy months of April to September (2.17/100,000; N=235; 58%) was higher than incidence during the drier season from October to March (1.62/100,000; N=173; 42%; RR, 1.32; 95% confidence interval [CI], 1.05-1.66). Outcome information was available for 390 (95.6%) of the 408 cases. Of these, 34 cases died (case fatality ratio = 8.7%) and neurologic sequelae occurred in 13 (3.7%) of the survivors.

Children ages 5-14 years accounted for the largest proportion of cases (35%), while 21% of cases were in children ≤ 2 two years of age, 17% were in children ages 2 -4 years, 18% were in those 15-24 years of age, and 9% were in adults ≥ 25 years of age. The age - specific incidence rate was highest in children less than one year of age (16.9/100,000), peaking at age 4-5 months (29.4/100,000) and declining with increasing age thereafter. Fifty eight percent of cases were in males, and the incidence per 100,000 was

significantly higher in males (2.2 vs. 1.6; RR, 1.38; 95% CI, 1.10-1.73, P=.0059). Male cases were older than female cases (median age 9 vs. 5 years; Kruskal-Wallis [KW] $\chi 2$, P=.049), attributable to a higher incidence in males (.531/100,000) vs. females (.299/100,000) in the population 5-25 years of age (RR, 1.93; 95% CI, 1.39-2.67, P<.0001) (See Figure 3).

Of the 408 cases detected during the study period, serogroup was determined for the isolates from 383 (94%). Of these cases, 314 (82.0%) were caused by serogroup B, 60 (15.7%) were caused by serogroup C, 8 (2.1%) were caused by serogroup W135; and 1 (0.26%) was caused by serogroup Y. During the study period, declines in the incidence of both serogroup B disease ($\chi 2$ test for trend, P=.078) and serogroup C disease ($\chi 2$ test for trend, P=.011) were observed. (See figure 5). Patients with serogroup C disease were less likely than cases caused by other serogroups to be younger than one year of age (3.3% vs. 17.0%; P=.005), and more likely to live farther than 75 km from the city of Salvador (39.0% vs. 19.6%; P=.0011). The median ages of patients with serogroup B, C, and W135 disease were 7.0 years, 10.0 years, and 7.0 years, respectively. Patients with Serogroup B disease were more likely than patients with disease caused by other serogroups to live within 75 km of Salvador (80.8% vs. 61.7%; P=.0007). Serogroup was not associated with sex, season, having a positive blood culture, case fatality ratio, development of sequelae, presence of fever, seizures, nausea/vomiting, neurologic findings or skin lesions on presentation, or development of seizures or focal neurologic findings during the hospital course.

Serotype and serosubtype were determined for 381 (93%) of the 408 study cases. Of these cases, 256 (67%) were serosubtype P1.19,15, and 244 (64%) were serotype 4,7 and serosubtype P1.19,15. The five most common serosubtypes, P1.19,15; P1.7,1; P1.3; P1.2; and P1.5,2, accounted for 81.9% of all cases (Table 1) A greater proportion of cases with P1.19,15 isolates occurred during the rainy season of April-September (62.5% vs. 51.2%; P=.035). Serosubtype P1.19,15 was not associated with a difference in age, sex, case fatality ratio, having a positive blood culture, development of sequelae, presence of fever, seizures, nausea/vomiting, neurologic findings or skin lesions on presentation, or development of seizures or focal neurologic findings during the hospital course.

The case fatality ratio was significantly higher in cases less than one year of age (16.4% vs. 7.5%, P= .039); in cases presenting with petechiae (11.5% vs. 3%, P=.003); and in cases with a hospital course including seizures (14.6% vs. 6.0%, P=.05). In only 27% of cases (N=113) were blood cultures performed; of those, 36 cases (32%) had blood cultures that were positive. Case fatality ratio was marginally higher in patients with a positive blood culture (17.4% vs. 5.19%, P=.068). The case fatality ratios and clinical course of study subjects are summarized in Table 2.

The incidence of meningococcal meningitis during the six-month period between August 1998 and January 1999 (2.81/100,000) was higher than the average annual incidence during the study period as a whole (1.89/100,000, RR, 1.48; 95% CI, 1.07-2.00; P=0.014). The largest and only statistically significant increase in incidence during this period occurred in the population ages greater than 4 years, in whom the incidence

increased from 1.20/100,000 to 2.20/100,000 (RR, 1.83; 95%CI: 1.25-2.68; P=0.003). Changes in age distribution over time are shown in figure 4. This six month span was not distinguishable from the rest of the study period by the sex distribution, serogroup or serotype/serosubtype distribution, proportion of cases with P1.19,15 serosubtype, or case fatality ratio of the cases.

DISCUSSION

Active surveillance in Salvador detected a mean annual incidence of 1.89 cases of meningococcal meningitis per 100,000. In a recent report from the United States, invasive meningococcal disease occurred at a mean annual incidence of 1.1 cases per 100,000; however, *N. meningitidis* was isolated from the CSF of only 35% of these cases, indicating an incidence of CSF culture-positive meningococcal meningitis cases of .39 per 100,000 [29]. Despite the high incidence and fewer health care resources available per capita, the case fatality ratio of 8.7% in Salvador is comparable to that seen in the United States and Europe [29, 30]; however, this estimate may underestimate the true case fatality ratio due to the exclusion of cases of pure meningococcemia, which is associated with increased fatality. An increased risk of death was associated with younger age, as has been described elsewhere [29, 30].

The seasonal fluctuation in cases was similar to the pattern observed in other endemic settings, with an increase in cases during the winter and spring months (April-September) [21, 29]. As has been described previously, the incidence of meningococcal meningitis was highest in young children [21], peaking in infants 4-5 months of age [29]. Males over age 4 years had higher rates of meningococcal meningitis than did females, a pattern not reported in a recent study in the United States [29]. Further investigation of risk factors that may be more prevalent in males of this age group is needed to explain this difference in sex-specific rates.

The incidence of meningococcal meningitis was not higher in teenagers and young adults; an elevated rate of meningococcal disease has been reported in this age group in the United States and Europe [29, 31]. It has been suggested that the increased incidence of meningococcal disease in young adults in these regions is due, in part, to changes in risk factors, such as crowding and exposure to diverse strains of *N. meningitidis,* associated with college attendance [30, 31]. In Salvador it may be that this age group is not at increased risk of meningococcal meningitis because only a small percentage attend college and there is less movement to new cities.

Over 80% of meningococcal meningitis cases in Salvador were caused by Serogroup B strains of one of five dominant serosubtypes, and 67% were caused by a single serosubtype, P1.19,15. These figures support findings from passive surveillance from throughout Brazil, in which 60% of cases were caused by serogroup B, and 66% were caused by serosubtype P1.19,15 [17]. The heavy predominance of a single serogroup and serosubtype and the sustained moderately high incidence throughout the study period are characteristic of extended outbreaks, as described in other parts of the world following introduction and establishment of strains from the ET-5 complex [3, 22-25]. Unlike recent trends in the United States [29, 32], we found no evidence for increasing rates of disease caused by serogoup Y or W135.

The overwhelming dominance of serogroup B disease shows that there is no indication for administration of a capsular vaccine in Salvador at this time. In contrast, an effective OMV-based vaccine against the dominant serosubtypes could prevent significant

morbidity and mortality, considering the relative homogeneity of the PorA protein in the strains causing disease in the region. A hexavalent OMV-based vaccine developed in the Netherlands against six common serosubtypes (P1.19,15; P1.7,16; P1.7,4; P1.5,2; P1.5,10; P1.12,13) appears to be safe and immunogenic in adults and older children, [33-35]. This vaccine, which includes strains with serosubtypes that account for 70.6% of cases of meningococcal meningitis in Salvador, could be a candidate for use in Salvador if it proves to be efficacious.

The potential limitations of OMV-based meningococcal vaccines developed to date include lower efficacy in young children [36-42], inability to induce immunologic memory [36, 43, 44], and failure to provide cross-protection against non-vaccine strains [16, 33, 45]. In Salvador, although the incidence of meningococcal meningitis was highest in infants, 63% of serogroup B cases occurred in individuals over the age of 4 years, a group in which the immunogenicity of OMV-based vaccines is more promising.

Of greater concern is failure of OMV-based vaccines to induce cross-protection against non-vaccine strains. OMV-based vaccines are generally considered to be protective against *N. meningitidis* strains of the same porA phenotype, and generation of porA-specific antibodies has been considered a marker for immunity against strains of the same serosubtype [17, 18]. However, several lines of evidence have called the assumption of serosubtype-specific immunity into question. Non-vaccine strains of the same serosubtype as vaccine-strains are less immunogenic in vaccinated individuals [40], and sera from a single individual may vary significantly in bactericidal activity against

different strains with identical serosubtype [46]. PorA sequence variability not captured by the serosubtype classification system may be partially responsible for the variable efficacy of OMV-based vaccines [35, 47, 48]. In addition, immunity in both vaccinated and non-vaccinated individuals may depend on membrane components other than the PorA protein [17, 18, 43, 46]. PorA sequencing and multi-locus sequence typing (MLST) of isolates from this study would provide a better indication of the heterogeneity of meningococcal strains and the degree of PorA antigenic variation in Salvador, with implications for the potential success of an OMV vaccination campaign.

The incidence of meningococcal meningitis increased significantly in Salvador from September 1998 to February 1999, particularly in the older age groups. An increased incidence, a shift to an older age distribution, and increased predominance of a single bacterial strain are characteristic of a transition from an endemic to an epidemic pattern of meningococcal disease [22, 49]. The changes observed in Salvador in late 1998 are consistent with the first two criteria; however, the majority of endemic disease prior to this period was already due to *N. meningitidis* with a single serologic profile (B:4,7;P1.19,15), and the increase in incidence in 1998 was not accompanied by a shift in the serosubtype distribution. More discriminatory methods of distinguishing between strains, such as multi-locus sequence typing (MLST) and PorA sequencing, are needed to determine whether the excess cases were caused by a single bacterial strain.

The surveillance method and case definition we employed most likely led us to underestimate the incidence of invasive meningococcal disease in Salvador. Surveillance

was restricted to cases of suspected meningitis, which generally represents about 50-70% of invasive meningococcal disease [29]. While reliance on a positive CSF culture for diagnosis ensured inclusion only of cases caused by *N. meningitidis*, the price for this high specificity was undoubtedly exclusion of some cases of culture-negative meningococcal meningitis, reducing the sensitivity of our case detection. In addition, reliance on a single reference hospital for surveillance of a relatively large region raises the possibility of incomplete case ascertainment, although the reference hospital is the only infectious disease hospital in the region, and reports over 95% of meningitis cases in the region.

To the best of our knowledge, this is the first active surveillance study of meningococcal meningitis in Brazil since the 1970s. The epidemiologic features of meningococcal meningitis have changed dramatically over the past three decades in Brazil, with the disappearance of large-scale epidemics caused by serogroups A and C and the introduction and establishment of serogroup B strains of the ET-5 complex. This study demonstrates that potentially important differences exist between the epidemiologic features of meningococcal disease in Brazil and in the developed world. Improved understanding of these differences and of the changing trends in the epidemiologic features of the disease in the region are essential to planning and implementation of improved control and prevention strategies to reduce the high morbidity and mortality caused by *N. meningitidis*.

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Table 1. Serosubtype classification of study cases.

Serosubtype	No. Cases (%) (N=381)
P1.19,15	256 (67.2)
1.7,1	20 (5.2)
1.3	16 (4.2)
1.2	10 (2.6)
1.5,2	10 (2.6)
1.5	6 (1.6)
1.9	6 (1.6)
1.12	5 (1.3)
1.22-1,14	5 (1.3)
1.14	3 (.79)
1.10	3 (.79)
1.4	3 (.79)
1.5	3 (.79)
1.16	2 (.52)
1.19	2 (.52)
1.7,16	2 (.52)
1.1	2 (.52)
1.7,13	1 (.26)
1.5,10	1 (.26)
Nontypable	25 (6.6)

Table 2: Clinical presentation, hospital course and case fatality ratios.

Presenting Symptoms (N=326)	No. of Cases (%)	Case Fatality Ratio	Case Fatality Ratio (comparison group)	P
Fever	320 (98.2%)	6.1	16.6	.33
Seizures	51 (15.6%)	7.8	.38	.20
Nausea/ vomiting	57 (17.5%)	5.7	8.9	.35
Presenting signs				
Skin lesions (N=345)	142 (41.2%)	11.5	3.0	.0028
Focal neurological signs (N=283)	13 (4.6%)	16.7	5.7	.16
Hospital course				
Seizures (N= 376)	41 (10.9%)	14.6	6.0	.051
Neurologic signs (N=341)	23 (6.7%)	13.0	5.7	.16
Positive blood culture (N=113)	36 (32%)	17.4	5.2	.068
Demographics/Season (N=408)				
Male sex	230 (56.5%)	9.6	7.6	.49
Season (April-September)	235 (57.6%)	8.4	9.2	.77
Residence w/in 75 km of Salvador	304 (74.5%)	8.4	7.1	.71
Age less than one year	61 (15.0%)	16.4	7.5	.039
Serogroup/Serosubtype				
Serogroup B (N=383)	314 (82.0%)	9.7	6.1	.48
Serogroup C (N=383)	60 (15.7%)	3.5	10.0	.13
Serosubtype P1.19,15 (N=381)	256 (67.2%)	9.5	7.4	.49

Figure 1. Incidence of Meningococcal Meningitis by 6 Month Period, February 1996- January 2001, Salvador, Brazil

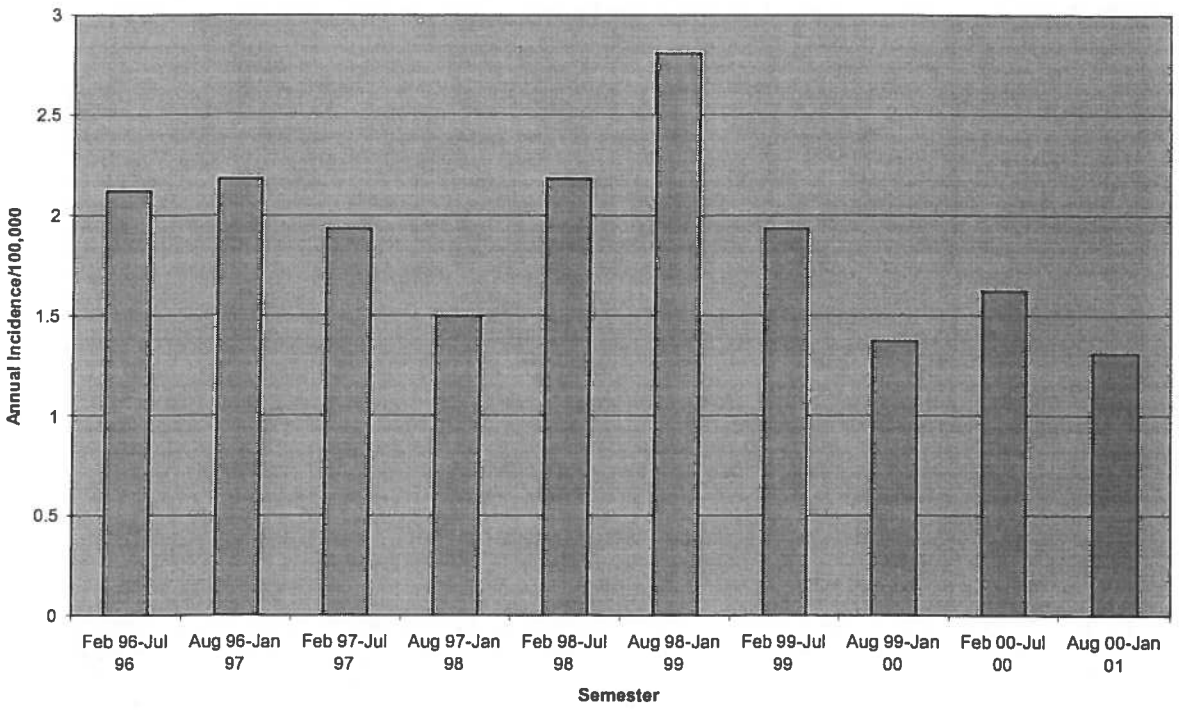


Figure 2. Division of Meningococcal Meningitis Cases by Month, February 1996- January 2001, Salvador, Brazil

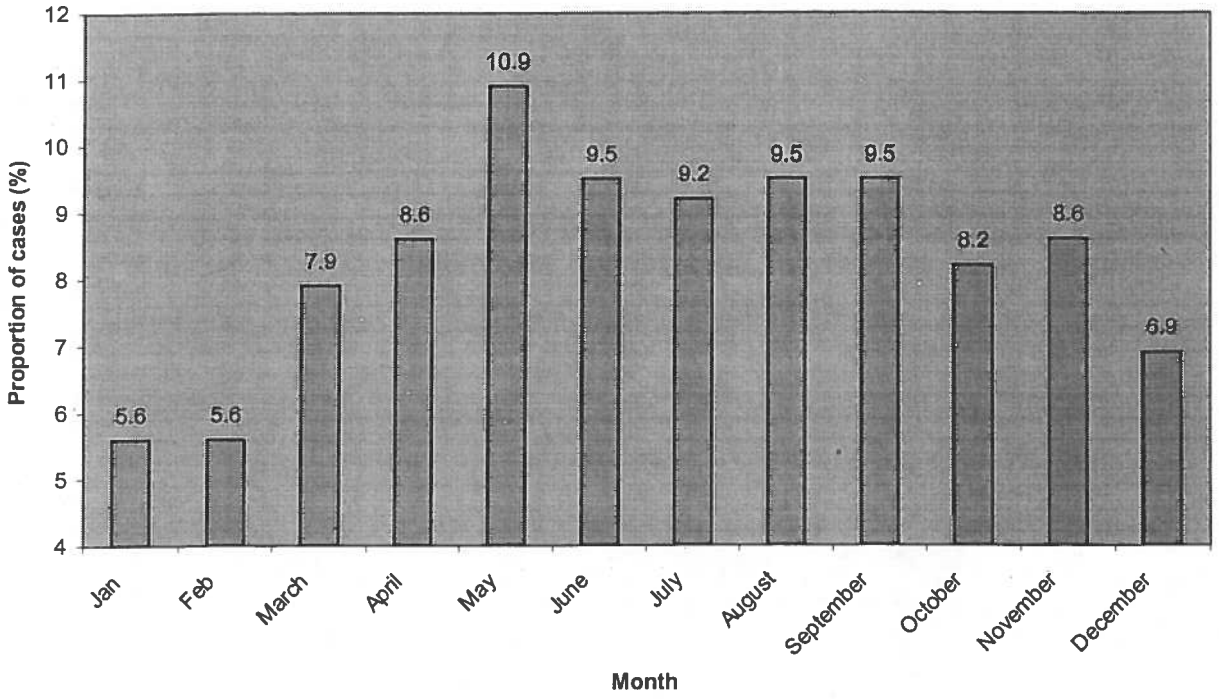
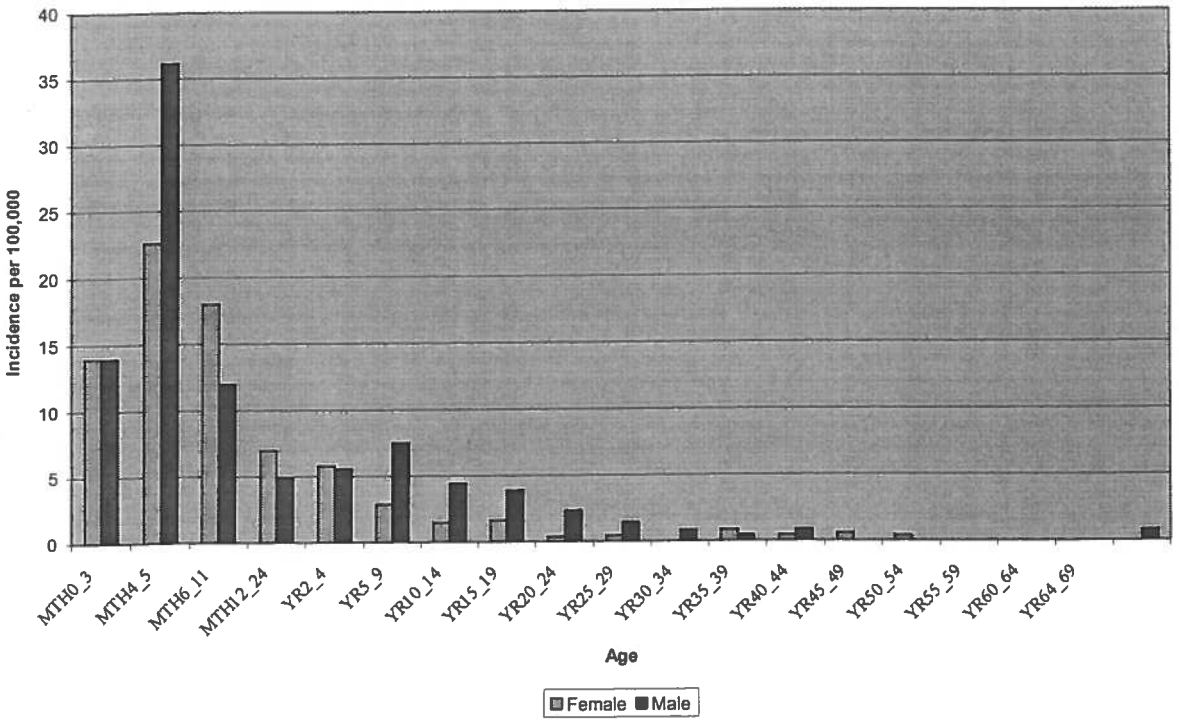


Figure 3. Sex and Age Specific Cumulative Incidence Rates of Meningococcal Meningitis, February 1996-January 2001, Salvador, Brazil



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The above findings from five years of active hospital-based surveillance for meningococcal meningitis supplement currently sparse data about the epidemiological characteristics of disease caused by *N. meningitidis* in Brazil. Surveillance data also have implications for the design of future vaccines targeted against serogroup B *N. meningitidis,* and will help to guide public health decisions in Brazil about the implementation of vaccination campaigns using current PorA vaccines. Unfortunately, a highly effective vaccine against serogroup B *N. meningitidis* is not yet available.

In contrast, the conjugate Hib vaccine is safe and highly effective at preventing invasive disease due to serotype b *H. influenzae*. Mass Hib vaccination in Brazil has resulted in a dramatic decline in the incidence of *H. influenzae* meningitis, and has substantially reduced overall morbidity and mortality due to meningitis. Although clearly effective, the impact of Hib vaccination on the epidemiological characteristics of disease caused by *H. influenzae* in Brazil, and specifically on the incidence of disease due to other serotypes of *H. influenzae,* remains to be fully described.

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III. *HAEMOPHILUS INFLUENZAE* TYPE A MENINGITIS: *POPULATION-BASED EVIDENCE FOR SEROTYPE REPLACEMENT FOLLOWING INTRODUCTION OF Hib IMMUNIZATION*

INTRODUCTION

A major public health advance has been the development and implementation of *Haemophilus influenzae* type b (Hib) polysaccharide conjugate vaccines. Hib is an important cause of meningitis, pneumonia and acute otitis media in the pediatric population and responsible, each year, for more than 2 million cases of invasive disease and 400,000-700,000 deaths worldwide among children less than 5 years of age. [1] Reduction in Hib infection rates have been dramatic in countries that have implemented conjugate vaccines as part of routine immunization [1] and have changed the epidemiology of diseases such as bacterial meningitis [2]: within 10 year period following licensure of conjugate vaccines in the late 1980s, cases of Hib meningitis among children less than 5 years of age decreased from more than 20,000 to less than 200 per year in the US. [3, 4]

In addition to preventing Hib invasive disease, conjugate vaccines are effective in reducing nasopharyngeal colonization [5-7] and therefore, confer protection to populations not targeted for immunization through herd immunity. [8] On the other hand, reduction of Hib carriage may open ecological niches for non-type b serotypes, therefore potentially increasing the risk of colonization and invasive disease with these strains. [9, 10] Non-type b *H. influenzae* are generally believed to be less pathogenic than Hib and an infrequent cause of invasive disease. However, reports have shown that these strains can cause of outbreaks of meningitis and bacteremia. [11, 12] Furthermore, in the conjugate vaccine era, the incidence of non-type b invasive disease has increased in certain geographical locations. [13-15] In Salvador, Brazil, through active surveillance for meningitis, we had an opportunity to examine the incidence of non-type b disease before and after

introduction of routine Hib immunization. In this report, we provide evidence for serotype replacement with *Haemophilus influenzae* type a associated with the use of the Hib conjugate vaccine.

METHODS

Study site

Salvador is a city with more than 2 million inhabitants located in Northeast Brazil. State health secretary protocol require that suspected cases of meningitis from the metropolitan region be referred to the state infectious disease hospital for diagnosis evaluation and assessment of the need for isolation procedures. Notification of a case of meningitis to state health officials is mandatory and this hospital reports more than 98 percent of the cases that reside within the metropolitan region (case notification records, Secretary of Health for the State of Bahia).

On August 9, 1999, Hib conjugate vaccine was introduced as part of the routine immunization program in Brazil. Between August and December 1999, 71,213 vaccine doses (HbOC, Wyeth-Lederle) were administered to a target population of 58,412 children with less than one year of age (estimated coverage of the three dose schedule given at 2 month intervals, 5 percent) and 22,488 doses to a target population of 60,051 children with age between 12 and 23 months (estimated coverage of the single dose schedule, 31 percent) (immunization records, Secretary of Health for the State of Bahia). In 2000, 162,303 doses (PRP-T, Pasteur-Merieux) were administered to a target population of 59,261 children with age less than one year (estimated coverage, 72 percent) and 14,461 doses to a target population of 60,486 children with age between 12 and 23 months (estimated coverage, 24 percent).

Surveillance and Definitions

Active surveillance for *H. influenzae* meningitis was performed at the state infectious disease hospital in Salvador between March 9, 1996 and September 8, 2000. A case was defined as the isolation of *H. influenzae* from the blood or cerebrospinal fluid of a patient with clinical signs and symptoms of meningitis. Clinical laboratory records were reviewed daily to identify culture-positive cases. Case was selected into the study according to guidelines of the Institutional Review Boards of the Oswaldo Cruz Foundation, Brazilian Ministry of Health and Weill Medical College of Cornell University. A standardized data entry form was used to extract information on demographics characteristics and clinical presentation and outcome during medical chart review. After introduction of Hib immunization, family members or guardians were interviewed for the patient's vaccination status. Timing and number of administered doses of Hib conjugate vaccine were confirmed through review of immunization cards. The pre and post-vaccine period was defined as the 3.5 year interval before and 1 year after September 9, 1999, respectively. Cases were included in incidence calculations if they resided within the metropolitan region that included the city of Salvador and 29 municipalities located within a 75 km radius (total population, 1996; 3,208,893 inhabitants [16]).

Laboratory Investigation

H. influenzae was identified according to gram stain morphology and growth requirement for factors V and X. Isolates from primary cultures were stored at –70° C for subsequent

analyses in order to avoid multiple passaging. Biotyping was performed with the indole spot (Difco Laboratories), ornithine decarboxylase and urease (BBL Microbiology Systems) tests. Commercial antisera (Difco Laboratories) were used to determine capsular serotype at the Gonçalo Moniz Research Center and the National Meningitis Reference Laboratory, Adolfo Lutz Institute. Each isolate was tested for slide agglutination with the complete panel of type a to f-specific antisera and a saline control. Isolates identified as non-type b and those with discordant results were reanalyzed at the Center for Disease Control and Prevention, USA. A semi-nested polymerase chain reaction (PCR) method was used to amplify serotype-specific and nonspecific DNA sequences from the *H. influenzae* capsular loci. [17] Isolates were defined as non-capsulated if agglutination was not observed with the six type-specific antisera and if PCR amplification of capsular loci sequences conserved amongst serotypes were not detectable. [17]

Pulsed-field gel electrophoresis (PFGE) was performed with *SmaI* digested DNA [18] from isolates identified during surveillance in Salvador and with type a strains identified from national reference laboratory-based surveillance in Brazil and the US. PFGE fingerprint patterns were defined according to the criteria of Tenover et al. [19] Closely-related (1 to 3 band difference) and identical patterns were assigned a unique letter and number code, respectively.

Statistical Analysis

Data entry and statistical analyses was performed with the Epi-Info version 6.04 software (Centers for Disease Control and Prevention, USA). The chi-square or Fisher's exact test was used to compare proportions and the Kruskal-Wallis test to compare continuous data. Incidence densities were calculated based on information obtained from the national census bureau population count in 1996. [16] and used to obtain risk ratios and their 95 percent confidence intervals. Rates from the pre-vaccine period were used as the expected value to calculate the probability, according to the Poisson distribution, of observing post-vaccine period rates. RIDIT (Relative to an Identified Distribution) analysis was used to compare the distributions of administered conjugate vaccine doses between type a and Hib meningitis cases. [20]

RESULTS

Active surveillance identified 522 cases of *H. influenzae* meningitis during the 4.5-year period between March 1996 and September 2000 (Figure 1). Among the 483 (93 percent of 522) cases that had serotyped isolates, 467 (96.7 percent) had type b, 13 (2.7 percent) type a, 2 (0.04 percent) non-capsulated and one (0.02 percent) type f strains. PCR-based detection of capsular loci sequences confirmed the serotype status of the 13 type a, 1 type f and 2 non-capsulated isolates, as determined by conventional slide agglutination (Figure 2). Isolates were serotyped from 431 of 467 (92 percent) and 52 of 55 (95 percent) of the cases identified during pre and post-vaccine periods, respectively. Whereas the proportion of cases with Hib isolates decreased from 98.1 (423 of 431) to 84.6 (44 of 52) percent following introduction of routine Hib immunization, the proportion of cases with type a isolates significantly increased from 1.2 (5 of 431) to 15.4 percent (8 of 52) ($P<0.001$). A significant difference was not observed in the proportion of cases with type f and non-capsulated isolates.

Based on the 357 cases (70 percent of 522) that resided within the metropolitan region of Salvador, the overall incidence of *H. influenzae* meningitis decreased 64 percent after the introduction of Hib immunization (pre and post-vaccine rates; 2.88 and 1.03 cases per 100,000 population, respectively; $P<0.001$) (Table 1). This decline was due to the significant reduction in incidence of Hib meningitis among children less than one year (77 percent) and between 12 and 23 months of age (49 percent). However, the incidence of type a meningitis increased eight-fold following introduction of routine Hib immunization (pre and post-vaccine rates; 0.02 and 0.16 cases per 100,000 population,

respectively; P=0.008). There were significant differences between rates of type a meningitis between pre and post-vaccine periods in the target population for Hib immunization, children with age less than 24 months (0 and 1.77 cases per 100,000 population, respectively; P=0.049), but not in other age groups (Table 1).

Type a meningitis cases did not spatially cluster with respect to the neighborhood of residence during pre- or post-vaccine periods. There were no significant differences with respect to age, gender, prior hospitalization, attendance at day care centers and underlying chronic diseases between type a and non-type a meningitis cases (Table 2).

Type a isolates from meningitis cases belonged to two distinct groups of closely-related PFGE fingerprint patterns (A [4 isolates] and B [9 isolates] in Figure 3 and Table 3). Pattern A isolates were biotype I whereas those with pattern B were biotypes II or III (Table 3). Both type a patterns were unrelated to the four fingerprint patterns found in PFGE analyses of 15 (of a total of 44) Hib isolates obtained during the post-vaccine period (results not shown). One of 4 pattern A and three of 9 pattern B type a strains were isolated from cases identified before the introduction of Hib immunization.

Furthermore, 7 of the 8 type a clinical isolates identified between 1990 and 1998 during national laboratory-based surveillance in Brazil had PFGE patterns (A and B, 3 and 4 isolates, respectively) identical to those of type a isolates obtained during surveillance in Salvador (Figure 3). The 7 strains were isolated from cases that resided in Brazilian

cities other than Salvador. PFGE patterns A and B found in Brazilian type a strains were unrelated to those of type a clinical isolates or reference strains from the US.

Acquisition of type a meningitis was significantly associated with increasing number of Hib conjugate vaccine doses administered prior to hospitalization (P=0.003). Information on Hib immunization status was obtained from 14 (52 percent) of 27 cases that were identified in the post-vaccine period and belonged to the vaccine target population. For four interviewed cases with type a meningitis, two had completed their three dose Hib immunization schedule and two received 2 vaccine doses. In contrast, among the 10 interviewed cases with Hib meningitis, 4 did not receive the conjugate vaccine and 6 received one dose before acquiring their illness.

The clinical manifestations of type a meningitis cases were similar in severity to those of non-type a cases (Table 2). Among 13 type a cases, 3 died during hospitalization (case fatality rate, 23 percent) of 13) and 2 of the 10 (20 percent) survivors had neurological sequelae, such as hydrocephalus and auditory deficits, on hospital discharge (Tables 2 and 3). Type a cases did not have outcomes different from those of non-type a cases with respect to admission to the intensive care unit (ICU) or duration of hospitalization, although those who were admitted to the ICU did have a longer duration of stay than did non-type a cases (7 vs. 2 days, P=0.02).

DISCUSSION

Although there have been increasing reports demonstrating *H. influenzae* non-type b as the cause of invasive disease, [11-15, 21, 22] serotype replacement has not been identified since Hib conjugate vaccines were introduced in the late 1980s. [10] In this study, the evidence that serotype replacement occurred after introduction of routine Hib immunization in Salvador, Brazil is as follows: 1) a significant increase in both the proportion and incidence of type a meningitis cases was observed following the start of Hib conjugate vaccine campaign; 2) the increased was not associated with any recognized outbreaks or with the introduction of a new hypervirulent strain into the community; 3) type a strains belonged to two clonal groups present in Salvador before the introduction the Hib conjugate vaccine; and 4) the risk group for acquiring type a meningitis was the population targeted for the Hib immunization campaign and type a meningitis was documented in subjects who had previously been immunized with conjugate vaccine.

Improved surveillance is an alternative explanation for the detected serotype shift that followed introduction of the vaccine. However, rate increases would be expected for disease due to several non-b types, rather than the type a-specific phenomenon that was observed. Although outbreaks of type a disease have been reported, [11, 12] type a cases in this study were not clustered in space or time and did not belong to specific risk groups other than the pediatric target population for the immunization campaign. In addition, the similarity of host characteristics between type a and non-type a cases indicates that increased serotype a disease was not associated with a change in the population at risk for *H. influenzae* meningitis. Introduction of a new hyper-virulent strain was not observed

since type a isolates from the post-vaccine period had isolates with identical or closely related PFGE patterns to those from the pre-vaccine period. Finally, the demonstration of a positive association between the number of administered conjugate vaccine doses and acquisition of type a meningitis supports a causal role for Hib immunization.

Potential study limitations include the reliance on a single surveillance site, raising the possibility of incomplete case ascertainment and introduction of bias. The surveillance hospital is the only site in the region that provides isolation procedures for cases of meningococcal disease; therefore state health regulations require that patients with suspected meningitis be referred to this site. In support of the surveillance protocol's validity, the hospital reports more than 98 percent of *H. influenzae* meningitis case notifications from Salvador. Furthermore, differential case ascertainment for *H. influenzae* type a and b meningitis cases is unlikely given the similarity between patient groups and clinical presentations (Table 2). Review of vaccination cards could only be performed in 52 percent of cases that were eligible to receive Hib immunization. Nevertheless, administration of 2-3 conjugate vaccine doses was documented in four of the 5 type a meningitis cases in the group eligible for vaccination (Table 3). Since surveillance was limited to meningitis cases; the study's findings may not apply to the other forms of *H. influenzae* invasive disease.

Serotype replacement in *H. influenzae* has not been previously observed for nasopharyngeal carriage [5, 7] or in population-based surveillance studies of invasive disease. [2, 23] Identification of serotype replacement in meningitis cases from Salvador

may be due, in part, to the presence of circulating virulent type a clones not found in other locations. Type a strains from geographically disparate regions of Brazil had PFGE patterns identical to those in Salvador, indicating that dissemination of these clonal groups is not a local phenomenon, restricted to our surveillance region. With reports of virulent type a [11, 12, 24] and f [21, 22] strains isolated from different regions of the world and the expanding global use of Hib conjugate vaccines, serotype replacement may become an emerging and more widespread possibility.

Clinically, virulence of type a strains was indistinguishable from that of Hib: type a meningitis cases had a case fatality rate of 23 percent and among survivors, 20 percent developed neurological sequelae (Table 2). Increased virulence among non-type b capsulated strains, generally considered to be less common causes of invasive disease, appears to be associated with partial deletion of one of two *bexA* copies within duplicated *cap* loci [11] and/or amplification of *cap* loci. [24] Virulent type a strains identified in this study were responsible for sporadic meningitis cases in the pre-vaccine period. However, it was only following introduction of the Hib immunization that these strains emerged as a significant cause of disease. We propose that in Salvador, the use of conjugate vaccines provided circulating virulent type a strains an increased opportunity to replace Hib during nasopharyngeal colonization.

Surveillance in Salvador found that the increase in type a meningitis rates due to serotype replacement was, albeit significant, small in comparison to the impact of Hib immunization in preventing *H. influenzae* meningitis. Within the campaign's first year,

overall *H. influenzae* meningitis rates decreased 64 percent and 72 percent among the total population and children less than two years of age, respectively. Without question, the public health priority for *H. influenzae* disease is the implementation of Hib immunization in developing countries where the cost of conjugate vaccines has precluded their use. More than 10 years after introduction of conjugate vaccines, current immunization practices contribute to a less than 2 percent reduction in the global burden of Hib disease. [1] The findings of this study indicate that as efforts progress to reduce vaccine costs, as done recently in Brazil, [25] and expand global Hib immunization coverage, continued surveillance for *H. influenzae* will be needed to monitor potential increases due to serotype replacement in non-type b disease burden.

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Table 1. Annual incidence of *H. influenzae* meningitis in Salvador, Brazil, before and after introduction of routine Hib immunization

Age Group	Pre-vaccine period	Post-vaccine period	Relative risk* (95% CI)	P Value*
	Cases per 100,000 population (No. of cases)			
All *H. influenzae* meningitis cases (N=357)†				
Total	2.88 (324)	1.03 (33)	0.36 (0.25-0.51)	<0.001
<2 years	57.59 (228)	15.91 (18)	0.28 (0.17-0.45)	<0.001
2–4 years	12.74 (80)	6.13 (11)	0.48 (0.26-0.90)	0.022
5–9 years	1.02 (11)	0.98 (3)		
>9 years	0.05 (5)	0.04 (1)		
With *H. influenzae* type b isolates (N=320)				
Total	2.62 (294)	0.81 (26)	0.31 (0.21-0.46)	<0.001
<2 years	53.80 (213)	12.38 (14)	0.23 (0.13-0.40)	<0.001
2–4 years	10.98 (69)	5.57 (10)	0.51 (0.26-0.98)	0.042
5–9 years	0.93 (10)	0.65 (2)		
>9 years	0.02 (2)	0.00 (0)		
With *H. Influenzae* type a isolates (N=7)				
Total	0.02 (2)	0.16 (5)	8.75 (1.70-45.10)	0.008
<2 years	0.00 (0)	1.77 (2)		0.049
2–4 years	0.32 (2)	0.56 (1)		
5–9 years	0.00 (0)	0.33 (1)		
>9 years	0.00 (0)	0.04 (1)		

* Values are not shown for non-significant ($p>0.05$) associations and when they could not be determined

† Includes 2 patients with non-capsulated isolates and 26 with isolates that were not serotyped.

Table 2. Characteristics of *H. influenzae* serotype a and non-type a meningitis cases

Characteristic	Type a cases (N=13)	Non-type a cases (N=459)
	No. of cases (%) or median (range)	
Median age (years)	1 (0-15)	1 (0-53)
Male gender	7 (54)	260 (57)
Underlying disease*	1 (10)	26 (6)
Seizures	4 (31)	127 (28)
Focal neurological signs	4 (31)	110 (24)
Cerebral Spinal Fluid examination†		
CSF leukocyte count (10^3 cells/μl)	7.8 (0.8-10.0)	5.8 (0.03-31.0)
CSF glucose (mg/dl)	20 (20-39)	20 (20-60)
CSF protein (mg/dl)	260 (150-500)	300 (30-500)
Intensive care unit admission	3 (23)	96 (21)
Median days in intensive care unit‡	7 (3-8)	2 (1-33)
Case fatality	3 (23)	75 (16)
Days of hospitalization		
Among those who died	2 (1-3)	2 (1-16)
Among survivors	16 (12-40)	16 (10-75)
Sequelae on discharge§	2 (20)	96 (25)

* Percentages were calculated based on 10 cases with type a isolates and 442 cases with non-type a isolates for whom information on comorbidity was obtained.

† Result of CSF examination are shown for 13 cases with type a isolates and for 454 cases with non-type a isolates.

‡ Median days were calculated for cases admitted to the ICU. There was a significant difference ($P<0.05$) between the two groups for this but not other characteristics.

§ Sequelae among survivors included ataxia (48 of 472), motor deficit (20), auditory deficit (15) and hydrocephalus (15).

Table 3. *Characteristics of the 13 cases of* H. influenzae *serotype a meningitis identified during surveillance in Salvador, Brazil*

Month of Hospitalization	Age	Sex	No. of Hib Vaccine Doses Administered*	Isolate Biotype (PFGE type)	Days in Intensive Care Unit	Outcome (Days of Hospitalization)
07/1996	4 years	Male	0	II (B1)	0	Discharged (21)
11/1996	3 years	Male	0	II (B1)	0	Discharged (12)
12/1997	9 months	Female	0	I (A1)	0	Death (2)
09/1998	2 years	Male	0	II (B1)	0	Discharged (17)
04/1999	4 months	Female	0	I (A1)	3	Death (3)
09/1999	3 years	Female	0	II (B1)	0	Discharged (16)
09/1999	5 months	Female	ND	II (B1)	8	Discharged (40)
10/1999	15 years	Male	0	I (A1)	0	Discharged (13)
12/1999	18 months	Female	2	I (A1)	0	Discharged (12)
04/2000	5 months	Male	2	II (B1)	7	Discharged (31)
06/2000	12 months	Male	3	III (B1)	0	Discharged (16)
07/2000	9 years	Male	0	II (B1)	0	Death (1)
07/2000	8 months	Female	3	II (B2)	0	Discharged (12)

*ND, not determined. Hib immunization campaign was initiated on August 9, 1999.

Figure 1: Monthly distribution of 522 *Haemophilus influenzae* meningitis cases identified during surveillance from March 1996 to August 2000 in Salvador, Brazil. Cases were stratified according to the serotype status of the clinical isolate: type b (*gray bars*), type a (*black bars*), type f (*horizontal hatched bars*), non-capsulated (*cross hatched bars*) and unknown because the isolate was unavailable for serotyping (*open bars*).

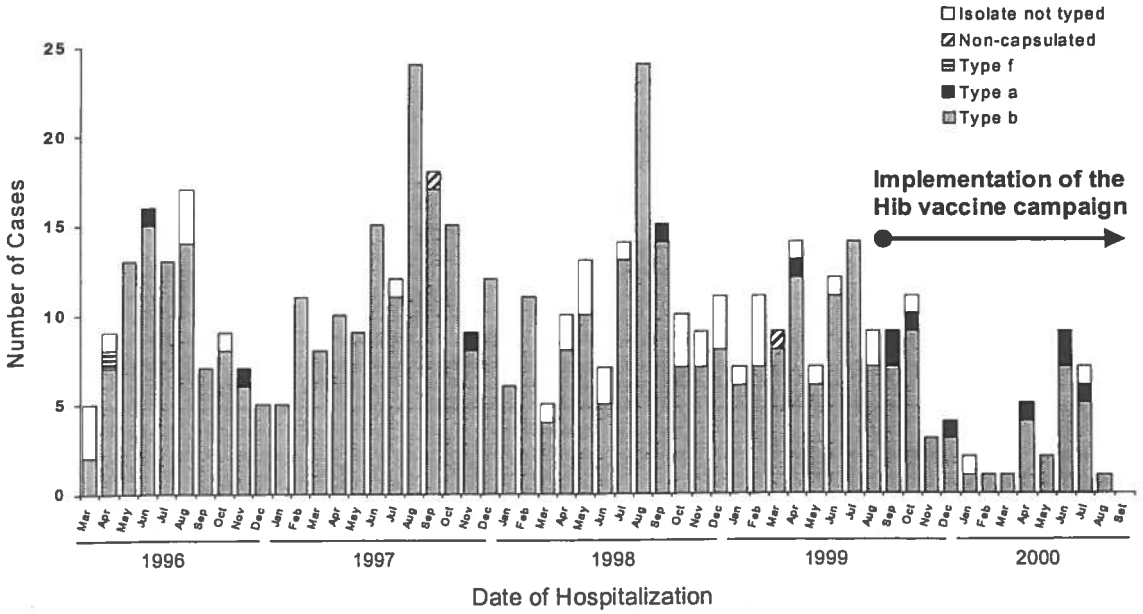


Figure 2: PCR amplification of capsular loci sequences from *Haemophilus influenzae* isolates. Amplification reactions were performed with DNA from type a (ATCC 9006, *lane 2-3*), f (ATCC 9007, *lanes 16-17*) and non-capsulated (*lanes 22-23*) reference strains and 8 isolates identified during surveillance (*lanes 4-15*, *18-19*, and *22-23*) with primers specific for type a (*lanes 2-15*) and type f (*lanes 16-19*) capsular loci and those that recognize conserved regions present in loci of all capsular types (*lanes 20-23*) [17]. Products from the first (*even numbered lanes, 2-22*) and second (*odd numbered lanes, 3-23*) semi-nested reactions were separated during agarose gel electrophoresis. The position and size (bp) of fragments in the molecular mass standards (*lanes 1* and *24*) are shown on the left.

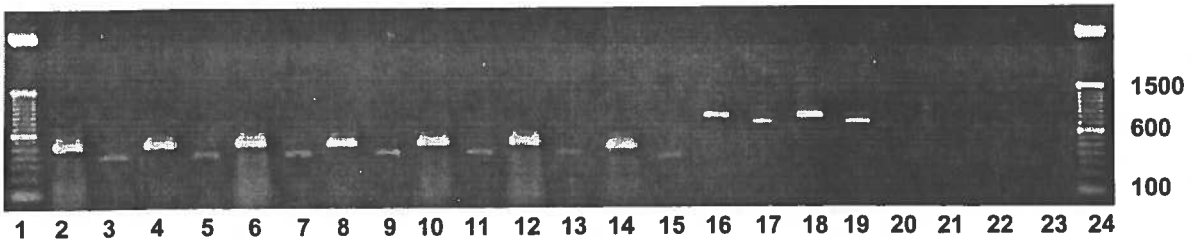
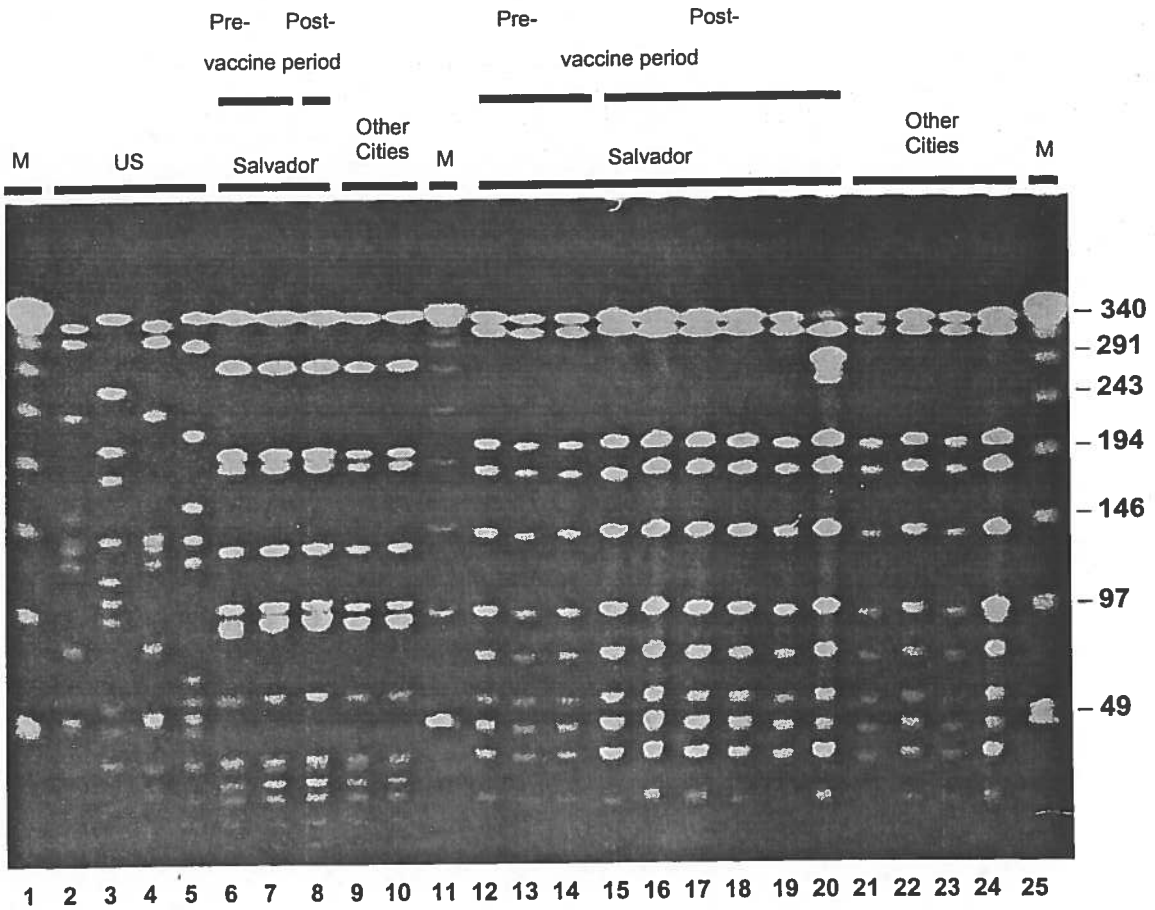


Figure 3: Pulsed-field gel electrophoresis (PFGE) analysis of SmaI digested DNA from *Haemophilus influenzae* type a isolates. Type a isolates from meningitis cases were identified before (*lanes 6-7* and *lanes 12-14*, respectively) and after (*lane 8* and *lanes 15-20*, respectively) introduction of routine Hib immunization and belong to two closely-related PFGE patterns (A1, *lanes 6-8*; B1 and B2, *lanes 12-19* and *20*, respectively). Type a clinical isolates from other Brazilian cities had identical PFGE patterns to the A1 and B1 pattern (A1, Curitiba [*lane 9*] and São Paulo [*lane 10*]; B1, São Paulo [*lanes 21, 23* and *24*] and Recife [*lane 22*]). The two closely-related patterns observed for type a isolates from Salvador were unrelated to those for type b isolates obtained during surveillance in Salvador (not shown) and type a reference strain (ATCC 9006) and isolates from the US (*lanes 2* and *3-5*, respectively). The position and size (Kb) of fragments in the molecular mass standards (*lanes 1, 11* and *25)* are shown on the left.



IV. CONCLUSION

The findings from active hospital-based surveillance reported above contribute to knowledge about the epidemiologic characteristics of *N. meningitidis* and *H. influenzae* meningitis in Salvador, Brazil, with implications for understanding the impact of ongoing and future vaccination campaigns.

During five years of surveillance, 408 cases of meningococcal meningitis were identified, with a mean annual cumulative incidence of 1.89 per 100,000 inhabitants. The incidence of meningococcal meningitis was highest in infants (29.4 per 100,000 infants 4-5 months of age). The incidence increased during the rainy season (between April and September). The case fatality ratio was 8.7%, with a significantly increased risk of death among those less than one year of age (16.4% vs. 7.5%, P=.039). Eighty-two percent (314/382) of *N. meningitidis* isolates were serogroup B and 64% (244/381) were serotype/serosubtype 4,7;P1.19,15. Improved understanding of the epidemiologic features of meningococcal meningitis in this region of Brazil may help guide development of serogroup B vaccines and strategies for their use.

Five hundred twenty two cases of *H. influenzae* were identified during 4.5 years of surveillance. Serotype information was available for 483 (93%) of cases. After introduction of Hib immunization, the proportion of type a strains isolated from cases increased from 1.2 (5 of 431) to 15.4 (8 of 52) percent (P<0.001). Whereas the incidence of Hib meningitis decreased 77% during the period after initiation of the program (2.62 to 0.81 cases per 100,000 person-years, P<0.001), that for type a meningitis increased eight

fold (0.02 to 0.16 cases per 100,000 person-years, P=0.008). Type a isolates belonged to two clonally-related groups, both of which were found prior to the start of Hib immunization program and in other Brazilian cities. Acquisition of type a meningitis was associated with increasing number of Hib conjugate vaccine doses administered (P=0.003). Hib immunization was associated with increased incidence of type a meningitis and appears to have selected for two major clonal groups of type a strains circulating in Brazil before the introduction of the vaccine. While the risk of type a meningitis is small, these findings highlight the need to maintain surveillance as the use of conjugate vaccines expands worldwide.

As these findings illustrate, ongoing surveillance for bacterial meningitis in Brazil supplements scarce data on the epidemiological characteristics of bacterial meningitis in Latin America and the developing world and provides baseline information against which the impact of public health interventions can be measured. Brazil has mounted a successful Hib vaccination campaign against *H. influenzae,* and is considering distribution of new PorA vaccines against *N. meningitidis.* Continued surveillance for bacterial meningitis in Salvador, Brazil will guide design and implementation of these and future vaccine strategies.