

Development of a Collection of Bacteria Causing Meningitis in Rio de Janeiro from 1990 to 1991

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From March 1990 to December 1992, the National Institute for Quality Control of Health-INCQS Research Collection received 1476 bacterial samples isolated from human cerebrospinal fluid of patients suspect of meningitis in Rio de Janeiro, from the São Sebastião State Institute of Infectious Diseases (IEISS).

Neisseria meningitidis was found in most of these materials, followed in smaller number by Haemophilus sp. and Streptococcus pneumoniae. The great majority of N. meningitidis strains was serogroup B, followed by serogroup C and a few strains of serogroup W135. More than 50% of the isolated bacterial agents came from the predominant 0-4 years age group. The majority of the strains were from patients in the region known as "Baixada Fluminense" (Low Lands).

The aim of the work presented here is to obtain samples of meningitis cases in at least 70% of the State of Rio de Janeiro and develop a collaborative research between INCQS-FIOCRUZ and the IEISS, in order to set up a collection of strains for future studies. However, despite work being carried out in a rather satisfactory way, difficulties still arise and have to be overcome, to survey data.

Key words: bacterial meningitis - culture collection - frequency of isolates

The National Institute of Quality Control for Health (INCQS-FIOCRUZ) and the São Sebastião State Institute of Infectious Diseases (IEISS), both in Rio de Janeiro, are carrying out since March 1990 a joint study intending to isolate and characterize meningitis bacterial agents, with lyophilization and maintenance of the isolated strains.

The main purpose of this study is to provide a general view of the meningitis situation in Rio de Janeiro and most important, a collection of the strains causing meningitis in the State. Along these years, isolation of certain microorganisms has considerably increased, specially *Neisseria meningitidis*, *Haemophilus sp.* and *Streptococcus pneumoniae*, main etiologic agents of bacterial meningitis in Brazil and elsewhere in the world (de Moraes et al. 1974, Peltola 1983, Caugant et al. 1987, Achtman 1991, Sacchi et al. 1992). The strains are isolated at the IEISS and freeze-dried at the INCQS. Efforts have been accomplished for more than two years, to establish a collection of the bacterial strains causing meningitis in the State, in order to supply important information for epidemiology, microbiology, molecular biology and biochemistry, among other current scientific issues. These microorganisms shall provide data for later studies, such as the development,

improvement and control of local (Brazil and Latin America) biologics, as vaccines, diagnostic reagents and patterns of susceptibility to antimicrobial agents.

MATERIALS AND METHODS

The Laboratory of Reference Biological Substances and Culture Collections (SBRCC) located at the INCQS, holds a collection of reference bacteria used in official assays (Gherna et al. 1992, de Almeida et al. 1992) and is now developing a research collection with microorganisms recovered from different sources such as human and animal clinical material, contaminants from biological materials and others.

The SBRCC laboratory supplies part of the culture media used for the isolation of microorganisms at the IEISS: chocolate agar slants (Columbia agar base and 5% sterile defibrinated rabbit blood) and Eugon medium slants. The microorganisms are recovered from cerebrospinal fluid and placed in duplicate on both above mentioned media (two chocolate agar and two Eugon agar slants): two samples remain at the IEISS for laboratory routine, while other two are sent to the SBRCC/INCQS and incubated in a 5% CO₂ environment and high moisture level for 24-48 hr.

At the SBRCC laboratory, tubes not presenting growth after 48 additional hours incubation at 35°C in a 5% CO₂ chamber with high moisture level were discarded, and those presenting significant growth of one of the three referred organisms associated with identification of IEISS, were

inoculated into trypticase soy broth with Levinthal extract (Koneman et al. 1983) and incubated in a shaking water-bath (250 rpm) at 35°C for 4-6 hr. Broths presenting turbidity corresponding to approximately 10⁸ CFU/ml were then plated into chocolate agar and left 24 hr at 35°C in a moisturized CO₂ chamber (Koneman et al. 1983, Krieg & Holt 1984, Lennette & Edwin 1985, Wentworth 1987).

The growth was then homogenized into an aqueous sterile solution of 20% skim milk (DIFCO Labs), dispensing 0.2 to 0.5 ml of this suspension in ampoules for lyophilization, which were frozen in a pure ethanol bath with dry ice (-75°C) and then kept in a -70°C freezer, waiting to be freeze-dried (Mellor 1978, Hatt 1980, de Almeida et al. 1992).

Part of the growth obtained from chocolate agar plates was used to check the primary identification of the microorganisms received from IEISS (pre-lyophilization test). Colony morphology was always observed in all three cases as an initial selective character. For a final identification, the following set of tests was performed:

Neisseria meningitidis - Gram stain, for diplococci screening (Koneman et al. 1983, Krieg & Holt 1984, Lennette & Edwin 1985, Wentworth 1987); oxidase test in filter paper strips impregnated with N,N,N',N'-tetramethyl-1,4-phenylenediamine (Koneman et al. 1983, Krieg & Holt 1984, Lennette et al. 1985, Mc Faddin 1985, de Almeida et al. 1992); transfer to Mueller-Hinton agar and incubation in CO₂ chamber at 35°C for 24 hr to differentiate *N. meningitidis* from *Haemophilus* sp., which doesn't grow on Mueller-Hinton agar without supplements (Koneman et al. 1983, Lennette & Edwin 1985); biochemical characterization by rapid carbohydrate fermentation tests (Brown 1974); specific serology assays to identify the serogroup (anti-A, anti-B, anti-C and anti-W135 sera- DIFCO Labs); Y strains were only tested at IEISS. Identification tests were first performed at IEISS and then confirmed at SBRCC laboratory.

Haemophilus sp. - Strains not growing in Mueller-Hinton agar, but growing primarily in Eugon or chocolate agar and presenting on microscopy as tiny Gram-negative coccobacilli were considered *Haemophilus* sp. (Konemann et al. 1983, Krieg & Holt 1984, Lennette & Edwin 1985, Wentworth 1987). Species distinction was performed by the growth requirements test using disks impregnated with factors X and V (DIFCO Labs). The strains were streaked on a Mueller-Hinton plate and the disks were then applied on the inoculated agar. The growth pattern obtained indicated the probable species. Further specific antisera tests were performed to identify *H. influenzae* type b. However, species and serotype of a limited number of strains were confirmed by our laboratory. Therefore, these organisms were all referred to as *Haemophilus* sp.

Streptococcus pneumoniae - Strains presenting on microscopy as Gram-positive streptococci, with a negative catalase reaction and umbilicated colonies with yellowish zones, after 48 hr of incubation on chocolate agar and bile soluble on Todd-Hewitt broth, were considered *S. pneumoniae* (Koneman et al. 1983, Krieg & Holt 1984, Lennette & Edwin 1985, Mc Faddin 1985, Wentworth 1987).

After the strains under study had been frozen and their identity and purity confirmed, lyophilization was performed (Mellor 1978, Hatt 1980, de Almeida et al. 1992).

Once lyophilized, the strains were stored at -25°C, in ampoules properly identified with labels indicating month, year and batch number (Hatt 1980, de Almeida et al. 1992).

RESULTS

This work began in March 1990, and, until 31 December 1992, INCQS had received from IEISS a total of 1476 samples with chance of isolating microorganisms, among the three groups quoted above. Most samples (50.6%) contained *N. meningitidis* (Tables I, II) of which 84% were serogroup B (Table III). *Haemophilus* sp. accounted for 14% of the isolates, while only 8.3% of recovered strains were *S. pneumoniae*.

Table II shows the annual distribution of these isolates, presenting a slight decrease in the incidence of *N. meningitidis* and a rise in *Haemophilus* sp.

The great majority of *N. meningitidis* strains were received at the SBRCC laboratory with the serogroup already identified, and were confirmed later. At present, about 7% of the strains have to be serogrouped again and 9% need to be lyophilized. As much as 40% of the strains sent from IEISS arrived without apparent growth, which suggest problems in the collection of sample or unsuitable transport of this demanding group of microorganisms.

TABLE I
Total distribution of the isolates

<i>Neisseria meningitidis</i>	747 (50.6%)
<i>Haemophilus</i> sp.	207 (14%)
<i>Streptococcus pneumoniae</i>	123 (8.3%)
Other microorganisms ^a	399 (27%)
Total	1476

^a : recovery of other microorganisms, including *Pseudomonas* sp. (3), *S. aureus* (6), *Streptococcus* sp. (1), *C. freundii* (1), *Cryptococcus* sp. (7), *E. agglomerans* (1), *E. coli* (6), *Enterobacter* sp. (1), *Klebsiella* sp. (2), *P. mirabilis* (1), *S. epidermidis* (9), contaminants (9), not confirmed (47), negatives (305).

TABLE II
Total yearly distribution

	1990	1991	1992
<i>N. meningitidis</i>	199(84.3%)	313(69.8)	235(59.8%)
<i>Haemophilus</i> sp. ^a	37(15.7%)	78(17.5%)	92(23.4%)
<i>S. pneumoniae</i> ^a	0	57(12.7%)	66(16.8%)
Total	236	448	393

a : remittance of *Haemophilus* sp. strains started in August 1990, and of *Streptococcus pneumoniae* in May 1991.

TABLE III
Yearly distribution of *Neisseria meningitidis* serogroups

	1990	1991	1992	Total
B	176(88.4%)	265(84.6%)	187(79.6%)	628(84%)
C	13(6.5%)	33(10.5%)	22(9.3%)	68(9.1%)
W135	2 (1%)	3(0.9%)	0	5(0.7%)
NS ^a	8 (4.1%)	12(4%)	26(11.1%)	46(6.2%)
Total	199	313	235	747

a : non-determined serogroup

Other epidemiologically important bacterial species were also rarely received, and depending on the organism may be freeze-dried and kept in our collection. Among these were many enterobacteria, *Pseudomonas* sp. and *Staphylococcus* sp., as well as a strain of beta-hemolytic *Streptococ-*

cus sp., recovered from the cerebrospinal fluid of a newborn child in July 1992.

Table III shows that the average prevalence of serogroup B among *N. meningitidis* strains isolated in Rio de Janeiro remained high (84%), indicating the urgent need for a greater protection of the population against serogroup B. The geographic distribution of the isolates in the State of Rio de Janeiro is displayed in Table IV.

DISCUSSION

From the data herewith presented, some important points may be promptly derived: bacterial meningitis in Rio de Janeiro (specially by *N. meningitidis*) are not yet under control mainly due to lack of a quick and accurate clinical and laboratorial diagnosis, long with the difficulties of applying effective antimicrobial therapy.

The absence of a satisfactory vaccine against meningococcal serogroup B points to a serious public health problem in the State and in the whole country.

Among the surveyed meningitis agents in Rio de Janeiro *N. meningitidis* group B occurred more oftenly considering that our laboratory received 70-80% of the samples from CSF of patients with meningitis of the State of Rio de Janeiro.

A service for collection and recovery of these agents as well as processing the microorganisms should be set up and improved in the hospitals, in order to obtain material for future microbiologic, serologic, epidemiologic, and molecular studies for the production of national vaccines able to protect the population against the strains circulating in Brazil.

TABLE IV
Geographic distribution of isolates

	Borth Region	Paraíba Valley	Mountain Region	Low Lands	South Region	Lakes Region	ND ^d
Nm ^a	0	7 (0.9%)	1 (0.1%)	620 (83%)	24 (3%)	43 (6%)	52 (7%)
H.sp. ^b	0	0	1 (0.5%)	174 (84%)	5 (2.5%)	21 (10%)	6 (3%)
Sp. ^c	0	0	1 (0.8%)	93 (76%)	4 (3.2%)	10 (8%)	15 (12%)
Total	0	7 (0.6%)	3 (0.3%)	887 (82%)	33 (3%)	74 (6.9%)	73 (7.2%)

a : *Neisseria meningitidis*

b : *Haemophilus* sp.

c : *Streptococcus pneumoniae*

d : not determined

Some of these studies are already being developed at the INCQS-FIOCRUZ Culture Collection laboratory, such as the "Isoenzymes analysis of *N. meningitidis*", to determine the most frequent "Electrophoretic Types (ET)" in Rio de Janeiro and compare it with the ETs leading to meningococcal meningitis epidemic outbreaks in other countries (Caugant et al. 1986, 1987, Achtman 1991, Sacchi et al. 1992).

Another study on the "epidemiology of *N. meningitidis* infection through close contacts" is also being carried out at the Department of Bacteriology-Instituto Oswaldo Cruz, based on meningococcal disease cases from the IEISS.

Considering many different variables presented in this investigation, conclusive and accurate results may be reached within forthcoming years, collaborating directly to public health development and improvement of the quality control of biologic products in Brazil.

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