RESEARCH NOTE

Characterization of *Haemophilus influenzae* Obtained from Invasive Diseases in Cuban Children under Five Years of Age

Isis Tamargo[+], Gilda Toraño, Oxandra Rodriguez, Miriam Perez, Alina Llop

Centro Nacional de Referencia para Haemophilus, Instituto de Medicina Tropical Pedro Kourí, Apartado Postal 601, Marianao 13, La Habana, Cuba

Key words: *Haemophilus influenzae* - serotyping - subtyping - outer membrane protein patterns - lipopolysaccharide serotyping

Meningitis caused by *Haemophilus influenzae* (Hi) is a serious disease that has high morbidity and mortality rates in many countries. Approximately 99% of Hi invasive infections are caused by *H. influenzae* b (Hib). Its worldwide incidence in infants varies widely according to different regions of the world (AC Moloney et al. 1997 *Infect Dis 16*: 123-126).

In Cuba the annual incidence rate of bacterial meningoencephalitis caused by Hi in children less than 5 years of age is 3,2 x 100,000. *H. influenzae* has been the most frequently isolated bacterial in cerebrospinal fluid nation-wide since 1992 and infants are usually affected. Several Hib-conjugated vaccine trials in different countries shown satisfactory results in preventing disease (EM Mulholland et al. 1996 *Vaccine 9*: 905-909). Hib vaccination could be an adequate solution for Cuba, but there data serotype prevalence is little regarding.

Serotyping, biotyping, outer membrane protein (OMP) subtyping and lipopolysaccharide (LPS) serotyping are valuable tools for characterizing Hib (L Van Alphen et al. 1996 *Pediatrics 85*: 636-642). Analysis of OMP profiles by SDS-PAGE has been used previously for the bacterial epidemiology of Hib (S Barenkamp et al. 1990 *J Infect Dis 143*: 667-668). Twenty-one different OMP subtypes have been described in Hib isolates from the United States of America (USA), but 89% of the strains are grouped in six subtypes (1H, 1L, 2H, 2L, 3L, 4H). In contrast L Van Alphen et al. (1983 *J Infect Dis 148*: 75-81) described a single subtype, subtype 1 (subtype 3L by The Barenkamp's system), in 80% of the strains isolated in Western Europe (WE).

In this paper, we show data supporting the hypothesis that only a few clones of Hib are predominant in Cuba and we compare the distribution found with those previously reported by others. This is the first study to analyse such a large number of Hib isolates characterized by OMP and LPS typing in Central and South America.

A total of 765 Hib strain isolated from children under 5 years with meningitis were received during the 1992-1997 period in the National Reference Center for *Haemophilus* at the Tropical Medicine Institute "Pedro Kourí" as a result of a national surveillance of Hi.

Each patient was represented by a single isolate. The strains were transported in a special medium elaborated in our laboratory (patent no. 22 325) containing Proteosa Peptone (Difco), Lam Lemco (Oxoid), Tryptone (Oxoid), salts, and supplemented with NAD (Sigma) and lysed horse blood. This medium supports the conservation of *Haemophilus* strains for at least 16-20 weeks.

Once received in the laboratory, the strains were subcultured and stored at -70^{o}C in BHI broth supplemented with 15% glycerol until further processing. The confirmation was carried out according to the morphological and cultural characteristics of the strains. Serotyping of isolates was done by using a latex agglutination test (Pasteur Merieux)(Dirks-Go Sis, 1978 *J Clin Pathol 31*: 1167-1171). The ability of the isolates to produce indole, urease, and/or ornitine decarboxylase was tested as described by MA Kilian (1976 *J Gen Microbiol 93*: 59-62). OMP subtyping and LPS serotyping was performed as described by L Van Alphen et al. (1983 *loc. cit.*).

Each individual strain was tested by two independent observers, and aliquots of predominant subtypes were sent to Loek Van Alphen for confirmation.

Biotyping of the Hib isolates revealed that biotype I was the predominant biotype among the invasive strains (76%). Biotype II accounted for 18% of Hib invasive strains. Others biotypes were rarely encountered.

The distribution of serotypes and biotypes of Cuban strains did not differ significantly from in

[+]Corresponding author. Fax: +53-7-24.6051. E-mail: tamargoi@sld.ipk.cu
Received 9 July 1998
Accepted 3 March 1999

other reports where invasive Hib disease, biotypes I and II, were also predominating (V Agarwae et al. 1996 *J Med Res 103*: 296-298, A Adegbola et al. 1996 *Ann Trop Pediatric 16*: 103-111).

Regarding OMP subtyping, we have found that OMP subtypes most frequently encountered were: 18L (37%) and 2L (30%). Other subtypes were found but less frequently: 12U (9%); 1L (6%); 13L (6%). In the 11.7% of the total number of strains tested, the subtype was undetermined. There are reports in the USA where various subtypes appeared more or less randomly distributed over the country (S Barenkamp et al. 1990 *J Infect Dis 143*: 665-668). Nevertheless, other studies have found that the pattern may change with time. In addition, new subtypes appear and are designated as a new numbers in the OMP subtype description system (HA Bijlmer et al. 1992 *J Clin Microbiol 30*: 386-390, DA Clements et al. 1992 *J Clin Microbiol 30*: 1879-1881).

In our study, the subtype most frequently encountered (18L) has not been commonly reported elsewhere. Van Alphen found the subtype 18L in France and the United Kingdom (UK), but less frequently, and it has not been found in other countries of Europe. Strains with SDS-PAGE subtype 2L (the second most commonly detected in Cuba) had frequently been found in Europe and the USA (Moloney 1996 *loc. cit.*). Several studies have described that a single OMP subtype may be dominant in one area, and it has been reported that certain OMP subtypes cause different types of diseases or that they were rarely isolated among carriers (L Van Alphen 1991 *J Med Microbiol 34:* 313-316).

Putting together these data with data on the distribution of subtypes of Hib in Cuba, we may conclude that a single or a small number of subtypes are present in most countries but that their frequency varies according to geographic area.

Serotyping based on the LPS different antigenic response was determined: LPS 1 and LPS 2 were the serotypes encountered in 70% of studied strains. In WE, LPS serotype 1 is the most prevalent type in invasive disease (Van Alphen 1983 *loc. cit.*). Moreover, in Gambia, this serotype was infrequently found (4.2%) and seven of eight LPS 1 strain were noninvasive Hi (Bijlmer 1992 *loc. cit.*).

Generally, in the Hib invasive disease, the different characteristics occur in defined combinations and they have been employed by different authors to explain the clonal diversity in different geographical areas. Most of the invasive disease worldwide is caused by Hib belonging to nine clones (Van Alphen 1996 *loc. cit.*).

The results obtained in this investigation (Table) suggest that three main clones are present in 46% of invasive Cuban strains: 18L-1-I, 2L-2-I and 18L-2-I. Other clones were represented in less than 12% of the invasive strains. L Van Alphen et al. (1987 *J Infect Dis 156*: 216-218) found that clone 18L-1-I was present in only 11% of the strains in UK and the others two (2L-2-I and 18L-2-I) were not reported in invasive strains at all.

TABLE

Characterization of the isolates by SDS-PAGE, lipopolysaccharide serotyping (LPS) and biotyping

Number (%)	SDS-PAGE[a]	LPS	Biotype
137 (18%)	18L	1	I
84 (11%)	18L	2	I
39 (5%)	18L	2	II
23 (3%)	18L	1	II
130 (17%)	2L	2	I
62 (8%)	2L	1	I
39 (5%)	2L	2	II
69 (9%)	12U	1	I
46 (6%)	1L	1	I
46 (6%)	13L	1	I
38	ND	1	I
12	ND	1	II
27	ND	2	I
13	ND	2	II

a: these designations refers to the nomenclature described by S Barenkamp et al. (1990 *J Infect Dis 143*: 667-668); ND: not determined.

On the basis of our results, we may conclude that invasive isolates of Hib in Cuba differ in some characteristics from those studied from other countries, this implying that Hib clones are distributed randomly throughout the world.

The strains of Hib from patients with invasive disease in Cuba are not quite homogeneous, as is the case in Europe or Iceland. In our strains, we found different characteristics referring to OPM subtyping and LPS serotyping in approximately 70% of all studied strains. Nevertheless, further studies are necessary for knowing more about the molecular epidemiology of Hib in Cuba.

In this paper we described for the first time some aspects about the characterization of invasive isolates of Hi in Cuba on the basis of capsular polysaccharide serotyping, biotyping, OMP subtyping and LPS serotyping which could aid in a better understanding of the epidemiology of Hi invasive disease in our country.