

Neutralizing Antibodies to Enterovirus 71 in Belém, Brazil

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Non-polio enteroviruses (Coxsackievirus A, Coxsackievirus B, Echovirus and EV 68-72) which belong to the enterovirus (EV) genus, Picornaviridae family, may be responsible for acute flaccid paralysis, aseptic meningitis, myocarditis, hepatitis, pleurodinia, neonatal sepsis, hand, foot and mouth disease (HFMD) even though 50-80% of infections are asymptomatic. EV 71 has been responsible for outbreaks and epidemics of HFMD and acute neurologic disease justifying its study in our country. The aim of this study was to detect neutralizing antibodies (NtAb) to EV 71 in individuals up to 15 years of age living in Belém, State of Pará, northern Brazil. Serum samples from 238 patients attending the Virology Sector of Evandro Chagas Institute in Belém, Brazil, were analyzed using microneutralization tests that included RD cells and BrCr strain. Overall 40.8% (97/238) of tested samples had NtAb to EV 71. Regarding the distribution per age group, 85.2% (92/108) of patients aged 0-3 years had no NtAb to this virus and 69.2% of those 12 to 15 years of age were seropositive. These results confirm that EV 71 infection occurs in the city of Belém; and that a high rate of individuals in this study were infected aged 3 years and over and, when aged 15 years nearly 70% had EV 71 NtAb.

Key words: enterovirus 71- neutralizing antibodies to EV 71 - seroepidemiology study - Belém - Brazil

At the beginning of this century, motor deficiency cases could be related to pathogens such as viruses and particularly to polioviruses, which belong to the enterovirus (EV) genus, *Picornaviridae* family. Besides the poliovirus (3 serotypes), EV includes Coxsackievirus A (23 serotypes: types 1-17, 19-22 and 24), Coxsackievirus B (6 serotypes), Echovirus (30 serotypes): 1-7, 9, 11-27, 29-33), and EV 68-72 (Wiedbrank & Johnston 1993).

Enteroviruses are non-enveloped small viruses (20-30 nm) of icosahedral symmetry. Their RNA genomes have 7.5 kb, positive sense and single strand. They are responsible for an extensive variety of diseases, although 50 to 80% of the infections are asymptomatic. They cause hepatitis, pleurodinia, stomatitis and neonatal sepsis in a significant number of patients every year. In developing countries, the poliovirus is clinically the most significant member of the genus EV causing paralysis diseases in every 4 out of 1,000 children in school age. The non-polio enteroviruses (NPEV) are the main responsible for aseptic meningitis, myocarditis and nonspecific febrile exanthematous illnesses. Approximately 75% of infections by EV occur in children under 15 years of age and the attack rates are highest in children under 1 year of age (Wiedbrank & Johnston 1993).

EV 71, one of the last NPEV studied, was described for the first time by NJ Schimidt et al. (da Silva et al. 1990) in 1974, in California, associated with cases of the central nervous system (CNS) diseases. In the same year Kennett et al. (1974) mentioned the occurrence in Melbourne, Australia, of cases of aseptic meningitis, cutaneous eruption, acute respiratory tract infections and infective polyneuritis caused by EV 71. In 1975, an epidemic was observed in

Bulgaria with clinical symptoms of poliomyelitis, encephalitis, encefalomyocarditis and aseptic meningitis. Ninety-two strains of EV 71 were isolated from the 65 cases with the same symptomatology of the poliomyelitis, including 37 strains from brain and medulla, 1 from cerebrospinal fluid, 10 from mesenteric lymph nodes and tonsils and 44 from feces (Chumakov et al. 1979). An outbreak of infections caused by EV 71 occurred in Australia during the winter of 1986. Of the 114 studied patients, 65 were hospitalized and 33 frequently had CNS involvement associated to severe symptoms (Gilbert et al. 1988). During an outbreak of hand, foot and mouth disease (HFMD) in Malaysia, in 1997, 4 children developed cardiopulmonary collapse and neurological problems. All the children received cardiopulmonary resuscitation but died. Postmortem studies showed infection by EV 71, with extensive damage to the medulla and pons (Lum et al. 1988). More recently the EV 71 was responsible for an epidemic occurred in Taiwan. Most of the patients had HFMD with or without complications. Many enteroviruses were isolated from the 238 cases, approximately half of them were EV 71 (Wang et al. 1998). In Brazil the first evidence of infections by EV 71 was mentioned by da Silva et al. (1990); these authors studied cases of acute flaccid paralysis (AFP) and finding neutralizing antibodies (NtAb) for EV 71 in 32.1% of serum samples. In another study developed by da Silva et al. (1996) positive IgM to EV 71 was detected in 20 (21%) of 92 children living in different areas of Brazil that presented clinical symptoms of AFP over a 3 year-old period (1988 to 1990). Takimoto et al. (1998) detected positivity for EV 71 in 5.6% of the 426 samples of children's feces with symptoms of acute neurological disease (AND), residents in the State of São Paulo and in the city of Brasília. Despite the significant variability of clinical and epidemic manifestations, there is close antigenic similarity among strains of EV 71 isolated in different countries (da Silva et al. 1990), justifying its study in several aspects including seroprevalence. On this subject Hagiwara et al. (1979) in the 70s showed interesting results including reference to circulation of EV 71 in Japan in 1966. It is important to mention

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that the presence of this virus in the community is concerning, considering the evaluation of the poliomyelitis control program (Blal et al. 1998).

The aim of this work was to detect NtAb to the EV 71 in serum samples from patients up to 15 years of age living in the city of Belém, Brazil.

MATERIALS AND METHODS

Patients - Serum samples from 238 patients of both sexes aged up to 15 years and residents in the city of Belém, were included in this study with symptoms of fever and exanthem. Patients received care from the Virology Section of the Evandro Chagas Institute during the year of 1998.

Cell culture - RD cell (rhabdomyosarcoma of human origin) was used, and maintained in Eagle's MEM (Minimum Essential Medium) with Earle's salts, without sodium bicarbonate and without phenol red. The preparation of this medium was as follows: MEM powder was added with (9.7 g, sodium bicarbonate 1.5 g, fetal bovine serum 100 ml, L-glutamine 200 mM 2 ml, HEPES 1 M 10 ml, penicillin 100,000 U/ml, and streptomycin 100,000 µg/ml, 1 ml of each) in 1,000 ml of distilled and purified water. This mixture was filtered in membranes of 0.22 µm, distributed in small volumes and maintained at 4°C. The RD cells were maintained weekly using Trypsin 0.25% plus EDTA.

Virus - An aliquot of EV 71, BrCr strain, provided by EE da Silva, was inoculated in RD cells to obtain a large volume. When the cellular layer presented 75% of cytopathic effect, the cultivation was frozen and thawed three times, centrifuged at 3,000 rpm for 30 min; the supernatant was distributed in aliquots of 500 µl and frozen at -20°C.

Neutralization microtechnique - Aliquots of 100 µl of serum were separated, inactivated at 56°C for 30 min and diluted 1:8 in Eagle's MEM (prepared equal to the growth medium except in the bovine serum concentration that decreased to 2% and the increase of 0.06% of anfotericin B 5,000 µg/ml), which was used as diluent of the test. The protocol utilized in this test was the same used for polioviruses (WHO 1996) with some modifications. Initially 50 µl of the diluent was put into all wells of the microplate (polystyrene, 96 flat bottom wells) except for the first row, then 50 µl of 1:8 dilution serum was put in the first and second rows. With the aid of the multichannel pipette, serum samples were diluted starting from the second row, removing 50 µl and placing it into the third row and so forth until the last dilution. In the following stage, 50 µl of virus contends the challenge dose (100 TCD₅₀ with variations from 31.5 to 315) was added. Two incubation periods were used: 2 h at 37°C in CO₂ incubator, and overnight at 4°C. The next day 100 µl of the suspension of RD cells with 3 days of growth was added, in the concentration of 2x10⁵ cells/ml. They were maintained at 35°C in CO₂ incubator. Microscopic observation was made on the 5th day.

RESULTS

In the analysis of 238 serum samples it was verified that the largest percentage of samples tested against EV 71 were located in the age group up to 3 years, with 45.4%, followed by the 4 to 7 years-old with 24.4%. The rest were divided into the 8 to 11 years-old (19.3%) and 12 to 15 years-old (10.9%) age groups. In terms of gender, 54.2% were female and 45.8% male.

Fig. 1 relates the levels of antibodies less (<) than 1:8 and higher or equal to (≥) 1:8, with the patients' age group. It was observed that in 59.2% the level of antibodies was < 1:8 and in 40.8% ≥ 1:8. In the age group from 0 to 3 years which included 108 cases, 85.2% showed a level of < 1:8 while 14.8% ≥ 1:8. In the 4 to 7 years-old group, an inversion of percentage was noted because in the dilution ≥ 1:8, the percentage increased to 53.4% while at < 1:8 it decreased to 46.6%. The 8 to 11 and 12 to 15 years-old groups showed a pattern similar to the previous one.

Fig. 2 shows the four groups of levels of NtAb detected in the samples of these patients. Of the 238 samples tested, 141 (59.2%) presented level of < 1:8. The two following groups 1:8-1:32 and 1:64-1:256 contained 39 (16.4%) and 36 (15.1%) samples respectively. The last group, > 1:256 included 22 (9.2%) samples.

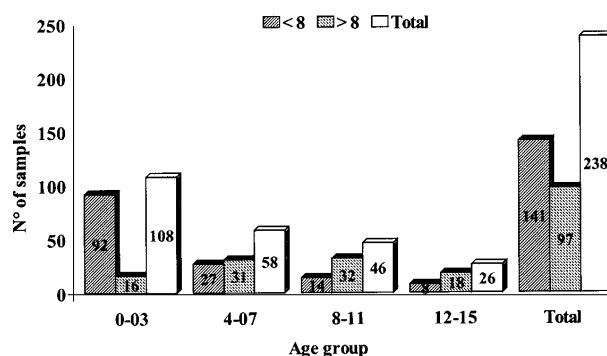


Fig. 1: neutralizing antibodies to enterovirus 71 in sera samples from patients living in Belém, Pará, Brazil according to the age.

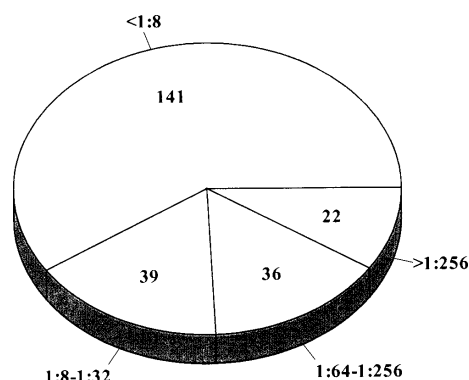


Fig. 2: neutralizing antibodies level to enterovirus 71 in 238 sera samples from patients from 0 to 15 years old living in Belém, Pará, Brazil.

DISCUSSION

Studies involving detection of NtAb to EV 71 are scarce and when existent they are related to the cases of HFMD, AND in general, and AFP in particular. As the main objective of this work was to verify the seroprevalence in a group of individuals with less than 15 years of age presenting different symptomatology, it was difficult to discuss our results.

Hagiwara et al. (1979) analyzing 137 samples of healthy children from 0 to 10 years of age, living in the city of Kawasaki, in Japan detected 6.6% with NtAb to EV 71. In our study the percentage corresponding to the patients up to 11 years of age was 37.3%. The use of different cells lines, Cynomolgus monkey cell line (CMK1-S1) in the Japa-

nese study and RD in our study could have influenced these results. It is also possible that in the period in which the Japanese evaluation was done, circulation of EV 71 was extremely low and perhaps restricted to a determined country. In Brazil studies involving EV 71 were carried out by da Silva et al. (1990) and Takimoto et al. (1998) both relating that this viruses to motor disorder occurred in the states of Bahia, Goiás, Piauí, São Paulo and Federal District, different from our study that involved patients with fever and exanthem. These authors detected positivity to EV 71 in 32.1% (9/28) and 5.6% (24/426) of the cases respectively. Our results show a percentage of 40.8% (97/238). It is evident that a great number of people were infected by EV 71 in Belém in contrast to the low number of individuals infected in São Paulo. Our percentage of infection is closer to the percentage found by da Silva et al. (1990) in the other localities. In relation to clinical symptoms the patients studied by Takimoto et al. (1998) and da Silva et al. (1990) presented disease with motor involvement while our patients presented fever and exanthem. Probably the strain of EV 71 that has circulated in Belém is genetically different from the strain that has circulated in the localities studied by that authors. According to our results the infection by EV 71 in Belém occurred with great frequency after 3 years of age considering that the individuals in the age group 0-3 years with NtAb > 1:8 was 14.8% (16/238). Another point is that a large number of individuals when reaching the age of 15 years posses NtAb to EV 71 considering the percentage of 69.2% (18/26) detected. In relation to the children with AND studied by Takimoto et al. (1998) the majority was infected later because until 5 years of age the percentage of individuals infected was 7.3% (17/231).

Although the importance of EV 71 has already been defined, more studies will be necessary in order to increase the knowledge on the several aspects related to this virus in Brazil. The data obtained in this study will be used for comparison with other studies in different localities of Brazil where this virus can be associated to other clinical disorders.

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