Genetic Diversity of Respiratory Syncytial Virus Isolated During an Epidemic Period From Children of Northeastern Brazil

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Outbreaks of human respiratory syncytial virus (HRSV) are the leading cause of serious acute lower respiratory viral disease in many countries in different continents. Data on clinical and epidemiological aspects of HRSV infections in this country have been reported, but there is lack of data regarding the molecular epidemiology of this virus in Salvador. The genetic variability of HRSV isolated during an outbreak in Salvador, Brazil (1999) has been analysed. Partial sequences of the G protein gene of 13 isolates from antigenic group A and 4 isolates from antigenic group B of HRSV were determined. Nucleotide sequences of C-terminal G gene were compared to sequences of HRSV isolates from countries of South America and from the rest of the world available at the GenBank. Brazilian group A and B isolates were clustered into previously characterised genotypes: GA5, GA2, GA7, and GB3, SAB3, respectively. This is the first study of GA7 and SAB3 genotypes circulation in South American countries. It is interesting to point out that viruses isolated in Salvador appear to be closer related with those from Montevideo-Uruguay and Buenos Aires, Argentina strains, suggesting circulation of similar strains among different South American countries in different seasons. Moreover, viruses closely related genetically circulated in the same year in Salvador and distant places such as Mozambique, supporting the previous suggestion on the complexity of HRSV strain circulation patterns, and the high capability of HRSV spreading world-wide.

INTRODUCTION

The importance of human respiratory syncytial virus (HRSV) in respiratory infections in infants, young children, the elderly and the immunocompromised has been well documented [Selwyn, 1990; Englund et al., 1991; Falsey et al., 1995]. Infections by HRSV occur as annual epidemics throughout the world, showing different patterns of occurrence associated with different climate regions [Weber et al., 1998]. Sharp outbreaks during the winter months in temperate regions contrast with extended periods related to the rainy season in tropical countries [reviewed by Cane, 2001; Moura et al., 2003a].

Brazil is a large country and this contributes to the climatic diversity observed in its different regions. This characteristic can influence the diverse occurrence of the HRSV infections that has been observed in cities from Northern, Northeastern, Southeastern and Southern regions [Nascimento et al., 1991; Mello and Silva, 1992; Vieira et al., 2001; Checon et al., 2002; Straliotto et al., 2002]. Salvador, the capital of Bahia, Northeastern Brazil, has an estimated population of 2,800,000, at sea level, 13° south of the equator. The climate is tropical with high relative humidity, rainfall

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throughout the year with a peak in April. In this region as in the Northern region, HRSV infections are related to the rainy season, while in the Southern region the association is with the coldest months [Mello and Silva, 1992; Straliotto et al., 2002; Moura et al., 2003a]. The majority of HRSV infections in Northern, Northeastern and Southeastern Brazil occur during the first semester, whereas in cities of the Southern region they extend for some months in the second semester [Nascimento et al., 1991; Mello and Silva, 1992; Checon et al., 2002; Straliotto et al., 2002; Moura et al., 2003a].

Two principal antigenic groups A and B have been identified by their reactivity with monoclonal antibodies [Anderson et al., 1985; Mufson et al., 1985].

Studies on the circulation of the main HRSV group A and B strains in different Brazilian cities have found that both antigenic groups A and B co-circulate in the majority of the epidemic periods, although group A viruses predominated [Siqueira et al., 1991; Cintra et al., 2001; Straliotto et al., 2001; Moura et al., 2003b].

Several lineages within each group have been identified by numerous strains sequenced worldwide [Cane and Pringle, 1991, 1992, 1995a,b; Garcia et al., 1994; Peret et al., 1998, 2000; Martinez et al., 1999; Roca et al., 2001; Venter et al., 2001; Frabasile et al., 2003], focusing the genetic variability of HRSV on the G protein, the viral gene product with the highest degree of antigenic and genetic diversity among viral isolates [reviewed by Melero et al., 1997].

In order to gain information on the genetic diversity of HRSV in Brazil, we evaluated the genetic variability of both group A and B HRSV strains isolated during an epidemic period in a single community (Salvador, Bahia, Brazil) by sequencing the hypervariable C-terminal third of the G protein ectodomain. The co-circulation pattern and genetic variability of both group A and B isolates are described and compared with HRSV isolates from Salvador with genotypes identified in other countries of South America and South Africa as well as HRSV sequences from other countries available at the GenBank.

**MATERIALS AND METHODS**

**Specimen Collection and Virus Isolation**

Nasopharyngeal aspirates were collected by mechanical suction from infants and children under 5 years of age who were attending the emergence room and paediatric wards of the Professor Hosannah de Oliveira Pediatric Center (PHOPC) in Salvador, Brazil with upper- or lower respiratory tract infection from January to December, 1999.

All samples were tested by indirect immunofluorescence for antigen detection of RSV, adenovirus, influenza and parainfluenza virus (Respiratory Viruses Panel I Viral Screening and Identification Kit) according to manufacturer’s instructions (Chemicon International, Inc., Temecula, CA, Catalog no. 3105).

For virus isolation, one aliquot of 100 μl of sample was inoculated onto HEp-2 cells, and the cultures with characteristic cytopathic effect were confirmed by immunofluorescence. Isolates were stored at −70°C until further passages in HEp-2 cells for RNA extraction.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Sequencing**

Total RNA was extracted from infected cells lysates by a Trizol® procedure according to the manufacturer’s recommendations. The extracted viral RNA was used as a template for cDNA synthesis. For this purpose, 2 μl of total RNA, 500 ng of antisense primer LG3 (–) 5’-GGCCGGGAAAGCTTTTTTTTTTTTTTTT-3’; which has an Ava I site (underlined) for cloning purposes, 10 mM dNTPs, in a final reaction volume of 10.5 μl were incubated for 5 min at 65°C. This was added to a mixture containing 200 U/μl of MMLV-RT reverse transcriptase (Gibco-BRL, Gaithersburg, MD), 5 U/μl of RNase inhibitor (Promega, Madison, WI) and 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂, 0.1 M DTT, at 37°C for 50 min, the enzyme was inactivated at 70°C for 5 min.

To obtain the entire G gene, the PCR assay was carried out in a mixture containing 2 μl of cDNA, 5 μl 10× PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 μl MgCl₂ 50 mM, 10 mM dNTPs, 5 U of Taq DNA polymerase (Gibco-BRL), 10 μM of antisense primer LG3 (–) and a sense primer LG5 (+) 5’-GGATCCCCGGGCAAAATGCAAACATGTC-3’, which has an Ava I site (underlined) and the first 20 nucleotides of the G protein from the Long strain. The reaction was carried out in a Thermolyne AmpliSim® I. Amplification conditions consisted of 5 min at 95°C, followed by 35 cycles of 94°C for 1 min 30 sec; 54°C for 2 min; 72°C for 1 min with a final extension at 72°C for 7 min.

A seminested-PCR was performed to amplify the C-terminal region of G protein with group specific primers. The reaction conditions were the same but using as a template the first-round PCR products 1/20 dilution and the inner sense primers GA 480 (+) 5’-GGCCGGGAAAGCTTTTTTTTTTTTTTTT-3’ to group A and B, and GA 496 (+) 5’-GGATCCAGATTTCTTGAGTCTCA-3’ to the isolates of group B.

Ten microliters of seminested RT-PCR products were run on 1.5% agarose gel and visualized under UV light after staining with ethidium bromide.

The PCR products were purified with a QIAquick® gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequencing was carried out in a ABI PRISM™ 377 Sequencer using a fluorescent dye terminator kit (Applied Biosystems, Foster City, CA) with 32.5 ng/μl of primers GA 480 (+) and GB 496 (+) to group A and B, respectively.

**Phylogenetic Analysis**

Nucleotide sequences from the second variable region of the G gene of group A and B viruses were aligned separately with ClustalX 1.8 [Higgins and Sharp, 1988; Thompson et al., 1997] applying the multiple alignment option. P-distances were calculated with MEGA 2.1
Phylogenetic analyses were carried out with Paup* 4.0b10 [Swofford, 2000]. Distances were calculated under Kimura 2 parameter model with a transition/transversion ratio of 3 for group A and 8 for group B. The topological accuracy of the tree was estimated by the bootstrap method, with 500 replicates; only values greater than 65% were considered to be significant.

Forty-one published sequences from group A and 35 from group B were downloaded from GenBank as reference of different lineages and genotypes.

**Nucleotide Sequences Accession Number**

The sequences examined in this study have been submitted to the GenBank with accession numbers: AY472086–AY472102.

**RESULTS**

During 1999, a total of 254 specimens collected from infants and children under 5 years of age with upper- or lower respiratory tract infection at the Professor Hosannah de Oliveira Pediatric Center (PHOPC) in Salvador, Brazil were processed for virus diagnosis. Fifty-two samples were HRSV positive by indirect immunofluorescence assay directly from the nasopharyngeal aspirates. Of these, only 17 samples were isolated in HEp-2 cells. Thirteen isolates were classified as group A and four as group B, by an ELISA with monoclonal antibodies specific for each group kindly supplied by Dr. Melero as described by Garcia et al. [1994] and Frabasile et al. [2003].

To define the genetic variability within and between groups, the 3' terminal third of the G protein gene was sequenced, representing the most variable portion of this protein [reviewed by Melero et al., 1997; Peret et al., 1998; Martínez et al., 1999]. The last 270 nucleotides at the C-terminus region of the G gene were used to establish phylogenetic relationships between the Brazilian isolates and published sequences from different genotypes [Peret et al., 1998, 2000; Sullender, 2000; Venter et al., 2001, 2002; Frabasile et al., 2003].

HRSV group A isolates in Salvador clustered into three genotypes assigned previously as GA2, GA5 and GA7, displaying 67–98% bootstrap values (Fig. 1a). Salvador group B isolates clustered into two previously described genotypes SAB3, supported by 71% bootstrap value, and genotype GB3 which could not be supported by significant bootstrap statistics.

**Fig. 1.** Phylogenetic trees of group A (a) and B (b) of human respiratory syncytial virus (HRSV) isolates constructed under distance criterion. Reference sequences were downloaded from GenBank and included for comparison: From Montevideo, Uruguay (Mon) and Buenos Aires, Argentina (Ba) [Garcia et al., 1994; Frabasile et al., 2003]; Madrid, Spain (MAD), [Garcia et al., 1994]; United States (TX, Texas; AL, Alabama; NY, New York; MO, Missouri; CH, Rochester, NY); Canada (CN) [Peret et al., 1998]; SA, Soweto, South Africa [Venter et al., 2001]; Ag, Agincourt, South Africa [Venter et al., 2002]; Mozambique (Moz) [Roca et al., 2001]; Long and CH18537 strains [Johnson et al., 1987] were used as outgroup sequences in the trees. Inner numbers represent bootstrap proportion in support to the adjacent node. Genotypes are indicated in brackets.
**DISCUSSION**

HRSV is the most prevalent virus associated with respiratory infections in Brazilian children [Nascimento et al., 1991; Straliotto et al., 2002; Moura et al., 2003a]. In the present study we report on the genetic analysis of HRSV isolated from hospitalised children in Salvador, in the Northeastern region of Brazil, occurring during the epidemic period (February to August) in 1999. As seen in previous studies from different countries, simultaneous circulation of both groups A and B of HRSV was observed in one community during an epidemic period [Cane and Pringle, 1991; Peret et al., 1998; Carballal et al., 2000; Venter et al., 2002; Arbiza et al., 2003]. Data from other Brazilian regions have shown the co-circulation of both groups in several epidemic periods [Siqueira et al., 1991; Cintra et al., 2001; Straliotto et al., 2001; Moura et al., 2003b].

Although a small amount of samples was analysed in the present study, it was possible to identify different genotypes from both groups A (GA2, GA5, GA7) and B (GB3, SAB3) co-circulating during an epidemic period as was previously reported [Peret et al., 1998, 2000; Roca et al., 2001; Venter et al., 2001, 2002; Frabasile et al., 2003; Madhi et al., 2003]. Phylogenetic analysis revealed that Salvador HRSV isolates clustered with previously assigned group A and B genotypes, confirming that HRSV variants can spread world-wide [Cane and Pringle, 1995a,b; Melero et al., 1997].

Isolates from Montevideo, Uruguay and Buenos Aires, Argentina appear to be related more closely with the Salvador strains, suggesting circulation of similar strains among different South American countries in different seasons. For instance, within the GA2 genotype, the Salvador strains are in the same branch together with strains from Montevideo and Buenos Aires, but separately with strains from Mozambique, even those isolated in the same year. Thus, the nucleotide p-distances between Salvador isolates ranged from 0.037 to 0.067 and 0.011 to 0.052 for Mozambique and South American isolates, respectively. Therefore, Brazil, Argentina and Uruguay may be sources of epidemic strains for each other, supporting the idea suggested previously where a transfer of viruses between communities may occur [Peret et al., 2000; Venter et al., 2001].

All the genotypes identified in Salvador during 1999 circulated in countries such as South Africa and Mozambique in a distant continent during the same year. However, the results of the phylogenetic analysis and p-distances indicated that the same genotype does not necessarily suggest that identical strains circulated globally. Thus, some isolates from Salvador, Brazil clustered within the same genotype but on separate branches relative to isolates from Africa (e.g., Sal/54/99 and SA99V360 in GA7 genotype); p-distances between Salvador isolates ranged from 0 to 0.019 (data not shown) and between Salvador and South Africa from 0.026 to 0.041. However, in other genotypes as GA5 (e.g., Sal/173/99 and Moz/169/99) the isolates from Salvador and Mozambique that are placed in the same branch, have a p-distance of 0.007 between them, indicating that viruses related closely genetically occurred in the same year in distant places, confirming the complexity of HRSV strain circulation patterns.

The Salvador strains group B clustered into different branches in the same genotype respect to Mozambique strains isolated in the same year. This is supported by the p-distances values shown in Table I and the bootstrap value (Fig. 1b).

**REFERENCES**


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