Molecular genetic characterization of human respiratory sincitial virus in Cuba

✉ Odalys Valdés[1], Clara Savón[1], Ángel Goyenechea[1], Lídice Palerm[1], Grehete González[1], Guelsys González[1], José A Melero[2], Isidoro Martínez[2], Pilar Pérez[2]

[1]Institute of Tropical Medicine "Pedro Kourí"
Autopista Novia del Mediodía Km. 6 / Carretera Central and Autopista Nacional
AP 601, CP 13, Marianao, Havana, Cuba
Fax: 53 -7 204 6051; E-mail: odalys@ipk.sld.cu
[2]Institute of Health "Carlos III", Spain

REPORT

ABSTRACT

The acute respiratory infections (ARI) represent one of the main health problems in children less than 5 years in the developing countries, and they are the fourth cause of morbility by ARI in Cuba. It is thought that viruses are the main causal agents, even though the human respiratory syncytial virus (HRSV) constitutes the main cause of this complaint. ARI cause high morbility and mortality in infants, elderly and immuno-depressed patients. Up to the present, there are no efficacious antiviral vaccines or drugs to control ARI, so it is necessary to go deeper in the natural history of the virus, its evolution and dissemination in the population. The most part of the molecular description studies of HRSV have been performed in developed countries, with different geographic conditions from those of Cuba, where the genotypes circulating or its philogenetic relationship with those from other parts of the world were unknown up to the present time. This is the first research of this kind in Cuba and in The Caribbean. The results will allow making an assessment of the strategy to follow in the design of future vaccines against this pathogen. The genetic variability was studied and the philogenetic analysis of the G glycoprotein of the 64 HRSV strains isolated in clinical samples from infants under a year in 6 provinces of Cuba, from 1994 to 2000 was performed. There were 58 samples from the antigenic A subgroup and 6 samples from the B subgroup. Five genotypes: the old genotype, GA1, GA2, GA3 and GA5 were found in the A subgroup It is important to emphasize that the old genotype strains were philogenetically grouped with an old strain, the prototype Long strain, isolated in 1956. This genotype has not been detected again. The circulation of strains with the old genotype was a particular finding in the Cuban strains. In contrast to what occurred with the A subgroup strains, those of the B subgroup are very far from the old strains and 2 genotypes were detected: SAB1 and SAB3. The strains from both genotypes were grouped with strains that have only circulated in South Africa in the same period.

*I*ntroduction

The Human Respiratory Syncytial Virus (HRSV) is one of the most important pathogens of infections in the respiratory tract of infants. [1-3]. Moreover, it is observed that this virus produces serious respiratory infections in elderly people and in immuno-depressed patients [4-6]. Its distribution is global and the epidemics occur every year, in winter, in the countries with a mild climate and in the rainy seasons in the tropical countries [7]. However, very little is known about the infection caused by that virus in underdeveloped countries, where the epidemiological pattern of the virus could behave differently.

Two antigenic subgroups, A and B, have been identified according to their relationship faced with a panel of monoclonal antibodies [8]. This classification was later corroborated by an analysis of the nucleotidic sequence. It is thought that in an epidemic, both subgroups can simultaneously circulate; however, the A subgroup has been more frequently identified than the B subgroup [9]. In each epidemic, the circulation of multiple genotypes in each subgroup has been also observed, with a replacement of the genotype predominating each year [10].

The genetic variability studies have been focused in the G glycoprotein due to its property of differentiating strains that could be identical in other gene products. Several researchers have expressed that there are strains genetically very similar that can circulate in different regions of the world in the same period of time. Also, scientists have observed that there are viruses isolated in geographically distant locations and in different years that can be more genetically related than other viruses isolated in the same location for two consecutive days [11].

The G glycoprotein is the main component in the protecting immune response of the virus and there are many nucleotidic substitutions present that cause aminoacidic changes. Those changes provoke alterations in the epitopes and, therefore, allow the virus to escape from the previously existing immunity [12].

HRSV presents two important characteristics that should be considered to develop a vaccine: first, it is capable of infecting infants in the presence of maternal antibodies and, second, it causes repeated infections throughout life. The faculty of this virus for causing reinfections could be owed to an inappropriate immune response or to its variability [13].

Because of it, studies of genetic variability in virus detected in 6 provinces of Cuba, from 1994 to 2000 have been performed from the HRSV G glycoprotein. With those studies, we could obtain some information about the genotypes circulating in our country and its philogenetic relationship with those circulating over the world. Moreover, the studies will allow knowing more about that protein, which is one of the antigens inducing the immuno-protecting response, in order to contribute to the development of future vaccines.

1. Fisher RG, Gruber WC, Edwards KM, Reed GW, Tollefson SJ, Thompson JM, Wright PF. Twenty years of outpatient respiratory syncytial virus infection: a framework for vaccine efficacy trials. Pediatrics 1997;99:E7.

2. Boyce TG, Mellen BG, Mitchel EF, Jr Wright PF, Griffin MR. Rates of hospitalization for respiratory syncytial virus infection among children in medicaid. J Pediatr 2000;137:865-70.

3. Shay DK, Holman RC, Roosevelt GE, Clarke MJ, Anderson LJ. Bronchiolitis-associated mortality and estimates of respiratory syncytial virus-associated deaths among US children, 1979-1997. J Infect Dis 2001;183:16-22.

4. Falsey AR. Respiratory syncytial virus infection in older persons. Vaccine 1998; 16:1775-8.

5. McBride JT. Pulmonary function changes in children after respiratory syncytial virus infection in infancy. J Pediatr 1999;135:28-32.

6. Falsey AR, Walsh EE. Respiratory syncytial virus infection in adults. Clin Microbiol Rev 2000;13:371-84.

7. Zlateva KT, Lemey P, Vandamme AM, Van Ranst M. Molecular evolution and circulation patterns of human respiratory syncytial virus subgroup a: positively selected sites in the attachment G glycoprotein. J Virol 2004;78:4675-83.

✉ Corresponding author

Materials and methods

In order to examine the molecular epidemiology and the evolutive patterns of the HRSV G glycoprotein, 64 strains were studied, all of them were isolated in the National Laboratory of Reference of Respiratory Virus of the Institute of Tropical Medicine “Pedro Kouri” (IPK), from 1994 to 2000. Those strains were obtained from clinical samples from infants under a year that were in-patients in the respiratory diseases room with a diagnosis of possibly viral low ARI and that were treated in pediatric hospitals in Havana City (Central Havana, “William Soler” and Cerro Hospitals) and in provincial hospitals from Sancti Spiritus, Cienfuegos, Holguín and Santiago de Cuba). The strains are shown in table 1.

Extraction of ARN

The extraction of RNAv from the control strains (Long y CH18537) and from the strains obtained in the laboratory by the trizole method was carried out according to the manufacturer’s instructions. A starting volume of 500 mL was taken. The mixture was centrifuged at 12 000 rpm for 15 minutes at 4 °C, the supernatant was removed and the precipitate was suspended again in 500 mL trizol; then, it was incubated for 5 minutes at room temperature. Then, 100 mL chloroform were added, the solution was strongly shaken, incubated for 3 minutes at room temperature, centrifuged in similar conditions and the higher part was transferred to other test microtube. Subsequently, 400 mL isopropanole were added, mixed by manual shaking and incubated for 10 minutes at room temperature; the mixture was centrifuged for 15 minutes in the above-mentioned similar conditions. The RNA was washed with 500 mL 75% ethanol, centrifuged for 15 minutes in similar conditions, the supernatant was removed, and the RNA was dried in the safety laboratory and was resuspended in 30 mL ribonuclease-free sterile water (RNase).

Reverse transcription and chain reaction of polymerase

For the reverse transcription (RT) and the first amplification step, 10 mL from the extracted RNA were added to the reaction mixture. In the mixture, 10 mL AMV/Tfl 5X, 1 mL AMV RT (5 U/mL), 1 mL Tfl DNA polymerase (5 U/mL), 6 mL MgSO4 (25 mM), 1 mL dNTP (10 mM), 1 mL from each oligonucleotide I2G/OF139 (100 ng) were used and the mixture was

8. Anderson LJ, Hierholzer JC, Tsou C, Hendry RM, Fernie BF, Stone Y, McIntosh K. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis 1985;151:626-33.

9. Cane PA, Matthews DA, Pringle CR. Identification of variable domains of the attachment (G) protein of subgroup A respiratory syncytial viruses. J Gen Virol 1991;72(9):2091-6.

10. Cane PA, Pringle CR. Molecular epidemiology of respiratory syncytial virus: a review of the use of reverse transcription-polymerase chain reaction in the analysis of genetic variability. Electrophoresis 1995;16:329-33.

11. Melero JA, Garcia-Barreno B, Martinez I, Pringle CR, Cane PA. Antigenic structure, evolution and immunobiology of human respiratory syncytial virus attachment (G) protein. J Gen Virol 1997;78 (10):2411-8.

12. Sullender WM. Respiratory syncytial virus genetic and antigenic diversity [table of contents]. Clin Microbiol Rev 2000; 13:1-15.

Table 1. Nomenclature, isolation date and origin of the strains.

Strains	Isolation date	Origin	Strains	Isolation date	Origin
CHab52/94	1994	Havana	CHab114/97	1997	Havana
CHab54/94	1994	Havana	CHab125/97	1997	Havana
CHab60/94	1994	Havana	Tun135/97	1997	Tunas
CHab67/94	1994	Havana	Tun141/97	1997	Tunas
CHab69/94	1994	Havana	Tun145/97	1997	Tunas
CHab81/94	1994	Havana	CHab223/98	1998	Havana
CHab82/94	1994	Havana	CHab181/98	1998	Havana
CHab83/94	1994	Havana	CHab185/98	1998	Havana
CHab97/94	1994	Havana	CHab197/98	1998	Havana
CHab105/94	1994	Havana	CHab198/98	1998	Havana
CHab106/94	1994	Havana	Cfgo226/98	1998	Cienfuegos
CHab107/94	1994	Havana	Cfgo227/98	1998	Cienfuegos
CHab111/94	1994	Havana	Cfgo228/98	1998	Cienfuegos
CHab115/94	1994	Havana	Cfgo232/98	1998	Cienfuegos
CHab128/94	1994	Havana	CHab236/98	1998	Havana
CHab134/94	1994	Havana	CHab237/98	1998	Havana
CHab140/94	1994	Havana	CHab239/98	1998	Havana
CHab141/94	1994	Havana	CHab240/98	1998	Havana
CHab151/94	1994	Havana	Tun244/98	1998	Tunas
Hol167/95	1995	Holguín	Tun245/98	1998	Tunas
Hol168/95	1995	Holguín	SC93/99	1999	S. de Cuba
CHab5/95	1995	Havana	SC94/99	1999	S. de Cuba
CHab8/95	1995	Havana	CHab33/00	2000	Havana
CHab10/95	1995	Havana	CHab34/00	2000	Havana
CHab11/95	1995	Havana	CHab37/00	2000	Havana
CHab104/96	1996	Havana	CHab42/00	2000	Havana
CHab195/96	1996	Havana	CHab43/00	2000	Havana
CHab201/96	1996	Havana	Tun50/00	2000	Tunas
CHab220/96	1996	Havana	Tun53/00	2000	Tunas
CHab91/97	1997	Havana	Tun57/00	2000	Tunas
CHab102/97	1997	Havana	SS64/00	2000	S. Spíritus
CHab123/97	1997	Havana	SS65/00	2000	S. Spíritus

complete with free bidistillated water and the final volume was 50 mL. The reaction mixture for the RT and the chain reaction of polymerase (RT-PCR) was placed in a thermocycler with the following program to perform the RT: first, the reaction mixture was incubated at 65 °C for 15 minutes and then at 42 °C for 45 minutes. The inactivation of this reaction was performed at 94 °C for 3 minutes. The PCR consisted in 30 amplification cycles (DNA denaturation at 94 °C for 1 minute and 30 seconds, oligonucleotides hybridization with the cast at 50 °C for 1 minute and 30 seconds, and the chain elongation at 68 °C for 1 minute and 30 seconds), followed by a final extension at 68 °C for 5 minutes.

Chain reaction of sheltered polymerase

For the sheltered PCR, 2 mL of the first PCR were added to the reaction mixture composed of 0.25 mL AmpliTaq polymerase (5 U/mL), 5 mL PCR buffer (10 X), 6 mL MgCl2 (2 mM), 0.8 mL dNTP (25 mM), 1mL of each internal oligonucleotide (100 ng) and RNase-free sterile bidistillated water for a final volume of 50 mL. The sheltered PCR mixture was placed in the thermocycler. Before the second reaction, DNA was denatured at 95 °C for 3 minutes and, subsequently, 30 amplification cycles were performed (denaturation at 94 °C for 1 minute, hybridization at 55 °C for 1 minute, polymerization at 72 °C for 1 minute), with a final extension of 72 °C for 5 minutes. The mixture of sheltered PCR was placed in the same thermocycler. Before the second reaction, DNA was denatured at 95 °C for 3 minutes and then 30 amplification cycles were performed (denaturation at 94 °C for 1 minute, hybridization at 55 °C for 1 minute, polymerization at 72 °C for 1 minute), with a final extension of 72 °C for 5 minutes. A second amplification reaction of the G gene was performed to the clinical samples: the used oligonucleotides were I2G-OG316, OG295-OG695 and OG448-G/OF139.

Detection of the extended product

Once the PCR is finished, 8 mL from each product from the amplification reaction were taken and mixed with 2 mL sample buffer 6 X (EDTA 500 mM, glycerol al 10%, bromophenol blue 0.01%). Those products were detected in agarose gels 1 and 2% in TBE 1 X (Tris 0.089 M, boric acid 0.089 M, EDTA 0.002 M) through ethidium bromide dyeing (10 mg/mL). The run was performed at 90 V for 1 hour, by the use of Marker VIII (Promega) as a molecular weight marker, with a rank between 100 and 1 500 pairs of bases (pb). The visualization of the bands was carried out by exposure of the gel to ultraviolet light in a transilluminator. The size of the extended fragment for the partial G gene was 316 pb, 400 pb, 489 pb, and for the complete G gene was 1 104 pb.

Purification of the extended product

The ADN extended by the G gene sheltered PCR was purified to perform the nucleotidic sequencing, according to the protocol described in the sequencing commercial kit, For the purification, 40 mL of the extended product of the sheltered PCR were mixed with 40 mL isopropanol and 8 mL ammonia acetate 5 M. This mixture is centrifuged at 14 000 rpm for 10 minutes at 4 °C, the supernatant was carefully centrifuged and the precipitate was washed with 200 mL ethanol 70%. It was centrifuged again in similar conditions. The precipitate was dried and suspended again in 10 mL RNase-free sterile distilled water.

Automated nucleotidic sequencing

The nucleotidic sequencing was carried out with the commercial kit (Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit, Promega). To do that, first, 4 phials marked A, C, G and T was prepared. To each phial, the mixture 1, composed of 2 mL Cy5-dNTP was added (ddATP, ddCTP, ddGTP, ddTTP in correspondence with the marked vials), 4 mL dNTP (1.1 mM) and RNase-free sterile bidistillated water were added. The final volume was 22 mL. It was strongly shaken, centrifuged and ice stored to be used later. For each sequence reaction, a phial was prepared with the 2 reaction mixture, composed of 3.5 mL of the PCR purified product, 2 mL de *primer* 4 pmol, 3.5 mL reaction buffer, 1 mL Termo Secuenasa I DNA polymerase 10 U/mL and RNase free-sterile bidistilled water. The final volume was 27 mL). The mixture was softly shaken to be mixed. New phials were marked A, C, G y T and placed in ice. To each phial, 2 mL of the mixture 1 were added, according to the A marked phial. Subsequently, 6 mL of the mixture 2 were added. The phials were shaken and centrifuged. The reaction mixture was placed in the above described thermocycler. Thirty amplification cycles were performed (denatu-ration at 95 °C for 30 seconds, hybridization at 60 °C for 30 seconds and extension at 72 °C for 1 minute and 20 seconds). For sequencing, the I2G, OG 448, OG 555, G/F20 and OF 139A oligonucleotides were used.

Purification of the sequence product

The sequence product was purified, according to the sequencing kit instructions. Then, 2 mL ammonia acetate 7.5 M, 2 mL glycogen and 30 mL cold ethanol 100% were added. The sequence product was strongly shaken and incubated overnight at -20 °C to precipitate DNA. Later, it was centrifuged at 13 000 rpm for 30 minutes at 4 °C. The supernatant was carefully removed by using pipettes. The precipitate was washed with 200 mL cold ethanol 70% and centrifuged under the same conditions described. The supernatant was carefully removed, the precipitate was vacuum-dried for 2-3 min, then 6 mL formamide were added and it was vigorously resuspended with shaking. Just before applying in the gel, the samples were heated in a thermal block at 70 °C for 3 minutes and immediately placed in ice. The products of the sequencing were separated in polyacrylamide denaturant gel 6 % and urea 7 M. The gel polymerization was performed by UV light for 10 minutes, the running buffer was TBE 0.5 X. The run was performed at 1 500 V for 8 hours. The reading of the sequence was performed by a computer.

Methods used to analyze and compare sequences

The data from each sequence were analyzed with the computer program Chromas (version 1.3; C. McCarthy, 1996, Griffith University, Brisbane, Queensland, Australia). The results from the positive and negative

chain sequencing of each sample were connected with the program MegAlign (DNASTAR, Madison, Wisconsin), to obtain the final consensus sequences. The nucleotidic and aminoacidic sequences of the HRSV A and B subgroups were separately aligned through Clustal X 1.64b [14]. The philogenetic analysis was performed by the Tamura-Nei nucleotidic substitution model, gamma 0.5. The reconstruction of the philogenetic tree was performed by the neighbor joining method. The philogenetic tree was assessed from the *bootstrap* calculation with 1 000 replicas (programs package contained in the MEGA program, Version 2.3) [15].

Sequences of the HRSV strains

1. From the GenBank database, 32 sequences of the HRSV were obtained and are shown in table 2.

Table 2. Nomenclature, isolation date, classification in subgroups and origin of the strains used for this study, obtained from the GenBank database.

Strains	Isolation Year	Group	Origin
Long	1956	A	United States
WV2780	1979	A	United States
WV19983	1987	A	United States
Mon/2/88	1988	A	Uruguay
Mon/3/88	1988	A	Uruguay
RSB642/89	1889	A	United Kingdom
RSB1734/89	1989	A	United Kingdom
Mon/5/90	1990	A	Uruguay
Mon/1/90	1990	A	Uruguay
Mon4/90	1990	A	Uruguay
ad/4/90	1990	A	Spain
CH57	1990-1995	A	United States
Mon/5/91	1991	A	Uruguay
Mad6/93	1993	A	Spain
AL19376/94-5	1994-1995	A	United States
NY103/94-5	1994-1995	A	United States
MO01/94-5	1994-1995	A	United States
Tx68532	1994-1995	A	United States
AgA48/99	1999	A	South Africa
AgK28/00	2000	A	South Africa
AgK23/00	2000	A	South Africa
AbJ81/00	2000	A	South Africa
Ab03/00	2000	A	South Africa
SAPT56/00	2000	A	South Africa
8/60	1960	B	Suiza
18537	1962	B	United States
B1/85	1985	B	United States
CH10/90-4	1990-1994	B	United States
NY01/94-5	1994-1995	B	United States
NY97/94-5	1994-1995	B	United States
SA934D/97	1997	B	South Africa
SA800V/99	1999	B	South Africa
SA439V99	1999	B	South Africa
Moz/198/99	1999	B	Mozambique
SA25/00	2000	B	South Africa
Ab27CT/00	2000	B	South Africa
Ab5078P/01	2001	B	South Africa
SA3064C/01	2001	B	South Africa

Results and discussion

The HRSV detection was performed by the nucleotidic sequencing of the extended product, obtained from an RT-PCR of 64 strains, 58 of them belonged to the A subgroup, and 6 to the B subgroup. The most frequent changes were the nucleotidic substitutions and transitions were more common than transversions. The nucleotidic substitutions were of three types: substitutions of nucleotides that did not induce changes of aa (synonymous substitutions), substitutions of nucleotides that induced changes of aa (non synonymous substitutions) and substitutions of nucleotides that induced changes in the position of the ending codon. The substitutions of nucleotides inducing changes of aa in the G glycoprotein, are the most frequently found genetic changes in the majority of the strains studied in other areas of the world [12, 16]. In those studies, the authors observed that the C end of the G glycoprotein is one of the areas where the aminoacidic changes are accumulated, and that it contains multiple variable epitopes, recognized by monoclonal antibodies (MsAb). This suggests that the choice of new variants by antibodies (Abs) could be one of the factors contributing to generate the HRSV diversity [11, 17, 18].

The substitutions of nucleotides inducing alterations in the position of the ending codon give raise to molecules with different length. The G glycoprotein of the viruses belonging to the B subgroup and detected in Cuba, presented a protein of 295 aa with two different stopping codons (UAG o UAA). However, among the sequences of the A subgroup, two proteins with different sizes (297 aa and 298 aa) with only one stopping codon (UAG) were observed. The changes in the stopping codon have been associated to important antigenic variations of escaping mutant genes of HRSV [11].

In the results of the G gene sequencing of the strains circulating in Cuba among the strains of the 1994 and 1996 period, a high identity was found between them and the reference Long strain. All the strains were identical and very similar to Long, with only 6 changes of nucleotides in the gene (58, 260, 640, 659, 746 and 888 positions). The CHab220/96 strain also showed a change in the 824 position and 6 were aminoacidic changes (15 K-Q, 82 Q-L, 209 K-E, 215 H-L, 244 I-T and 270 S-F positions); the change in the 888 position was silent.

The homogeneity of the HRSV strains that circulated in Havana City in 1994-1996 and an old strain (Long strain) is a unique characteristic when they are compared to the HRSV strains that circulated in countries with a mild climate and with different socioeconomic status, since in former epidemics, strains with old sequences had not been found. The seasonal nature of the infections by that virus differs between the tropical regions and those with a mild climate. While the epidemics occur in winter in the countries with a mild climate, in Cuba, in spite of being a tropical country, the infections induced by that virus are distributed from September to February. The influence of this season nature in the evolution of the virus is unknown, but the geographic features of Cuba could be influencing this unusual behavior.

The strains found in Cuba that belong to the A subgroup, were philogenetically distributed in 5 genotypes, according to the rate of nucleotidic similarity with the

13. Morrison WI, Taylor G, Gaddum RM, Ellis SA. Contribution of advances in immunology to vaccine development. Adv Vet Med 1999;41:181-95.

14. Thompso J,TG, FP, FJ, DH. The CLUSTAL X windows interface: flexible strategies for multiple secuence alignment aided by quality analysis tools. Nucleic Acids Res 1997;25:4876-82.

15. Kumar S, Tamura K, Jakobsen I, Nei M. MEGA: molecular evolutionary genetics analyses software., Vol. 109., University Park: Pennsylvania State University;2001.

16. Cane PA. Molecular epidemiology of respiratory syncytial virus. Rev Med Virol 2001;11:103-16.

reference genotypes (old genotype, GA1, GA2, GA3 and GA5). The results are shown in figure 1.

All the strains that circulated in Havana City from 1994 to 1996 a strain, also from Havana City, isolated in 1998 and another strain that circulated in Las Tunas in 2000 were grouped within the old genotype. Those strains were grouped with a 99% nucleotidic similarity to an old strain, the prototype Long strain, isolated in 1956. This genotype has not been detected anymore, since the moment it was isolated. The circulation of strains with the old genotype was a unique characteristic in the Cuban strains. All the strains grouped into this genotype presented identical or semi-identical nucleotidic sequences.

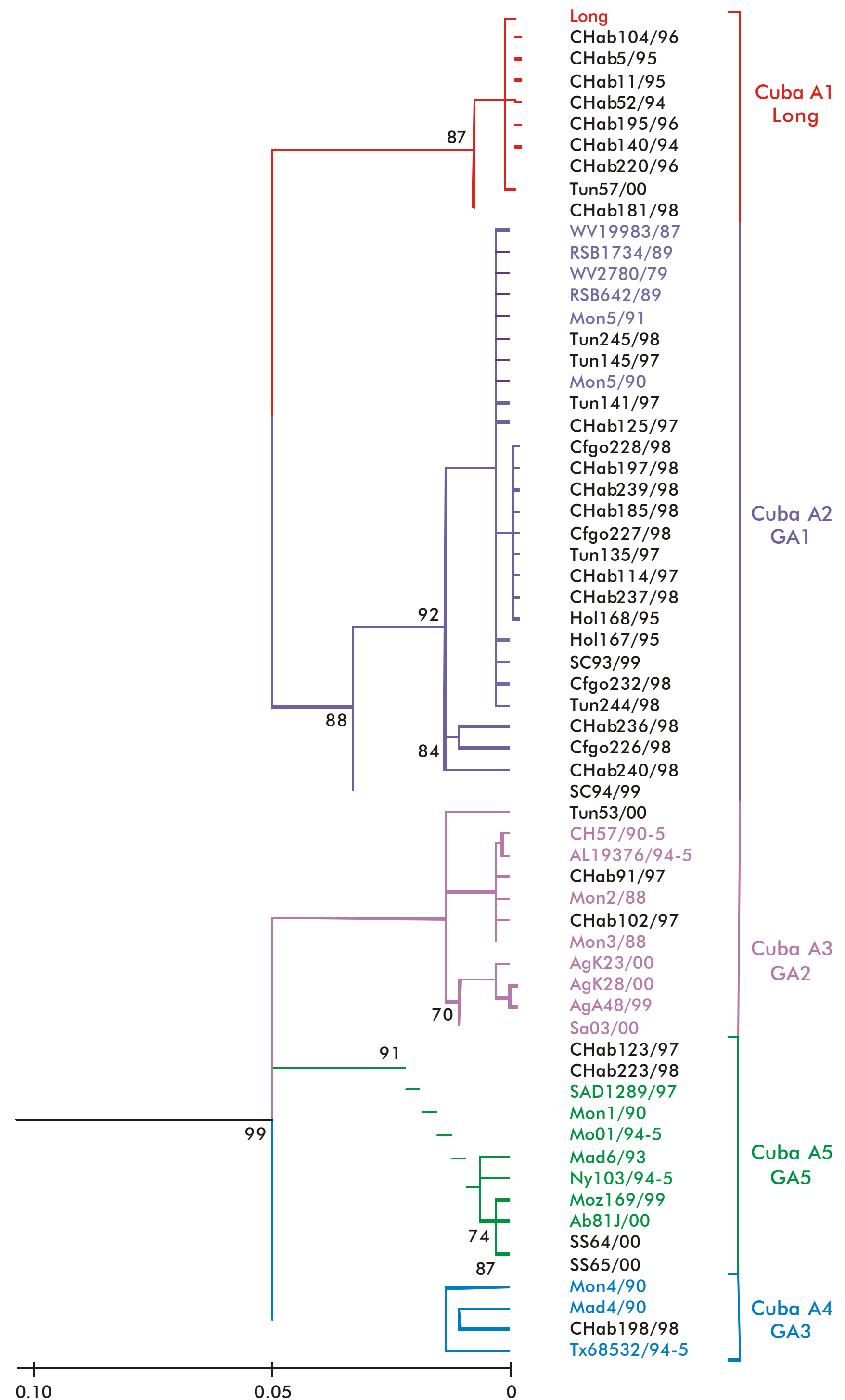
Similar results were previously reported in Denmark, during several consecutive epidemics (from 1993 to 1995). The predominating genotype showed a restriction pattern identical to the A2 prototype strain, isolated in 1961 [19]. Strains with similar restriction pattern were found in subsequent studies from different regions in Denmark, and two of them showed this genotype as the dominant one. The authors express that certain genotypes that have not been detected during many epidemics in other parts of the world, can continue circulating in an endemically stable way in a population for years, with sporadic manifestations [20].

The majority of the viruses that circulated from 1995 to 1999 in the different Cuban provinces were philogenetically grouped with strains of the GA1 strains, with 97% nucleotidic similarity. The viruses belonging to this genotype found in Cuba were grouped with strains that circulated for several years in Uruguay, the United States and the United Kingdom from 1979 to 1991. This genotype was common in the 80s and at the present, it has been detected with a very low frequency. Again, the circulation of strains with this genotype with almost identical nucleotidic sequences was observed.

Previously, in other locations, similar results were observed. In 1993, in Gambia, isolated viruses were philogenetically grouped with a strain circulating in Madrid, detected in 1984 [21]. This variant was relatively common in Europe in the 80s and has not been detected since that moment [10, 22].

In Denmark, during several consecutive epidemics (from 1992 to 1998) viruses were described, with restriction patterns similar to those of the virus isolated in the United States from 1982 to 1986 [19, 20]. Those descriptions suggest that the temporal fluctuation of a predominant genotype in a geographically restricted area could be owed to a certain immunological condition in the host that favors certain strains of the circulating population, more than by the molecular evolution induced by the selective immune pressure. Those papers express that certain genotypes not recognized for several winters could become endemic in a population for years and appear occasionally. A short time ago, the genetic variability of HRSV strains of the A subgroup, isolated in Buenos Aires, Argentina in 1996 and 1998 was studied. In this period, the circulation of the virus with the GA1 genotype was detected with a very low frequency [23].

The minority of the strains that circulated in the period of 1997 and 1998 and in 2000 were philogenetically grouped in the GA2, GA3 and GA5 genoty-



Figures 1. HRSV philogenetic tree of A subgroup that circulated in some cuban provinces and other geographic areas. The viruses from Cuba are shown in black.

pes with strains that circulated later in other regions of the world, in the same period or in different periods.

The GA2 genotype contained strains that circulated in different periods in South Africa, Montevideo (Uruguay) and North America. Two viruses detected in Havana City, that circulated in 1997 and a virus

17. Garcia-Barreno B, Portela A, Delgado T, Lopez JA, Melero JA. Frame shift mutations as a novel mechanism for the generation of neutralization resistant mutants of human respiratory syncytial virus. Embo J 1990;9:4181-7.

that circulated in Las Tunas in 2000 were grouped with that genotype. The viruses detected in 1997 were related to strains from North America and Montevideo that circulated in the early 90s and in 1998, respectively. The CHab198/98 virus was grouped with the GA3 genotype with strains of the virus that circulated from 1990 to 1995 in Montevideo, Madrid and the United States. Two viruses detected in Havana City in 1997 and 1998 and two viruses that circulated in Sancti Spiritus in 200 were grouped with the GA5 genotype. Strains that circulated at the same time in geographically distant areas and strains isolated in periods and geographically different areas were grouped with the same genotype. The viruses found in Sancti Spiritus were much related to the strains of the viruses that circulated in South Africa and Mozambique at the same epidemic period.

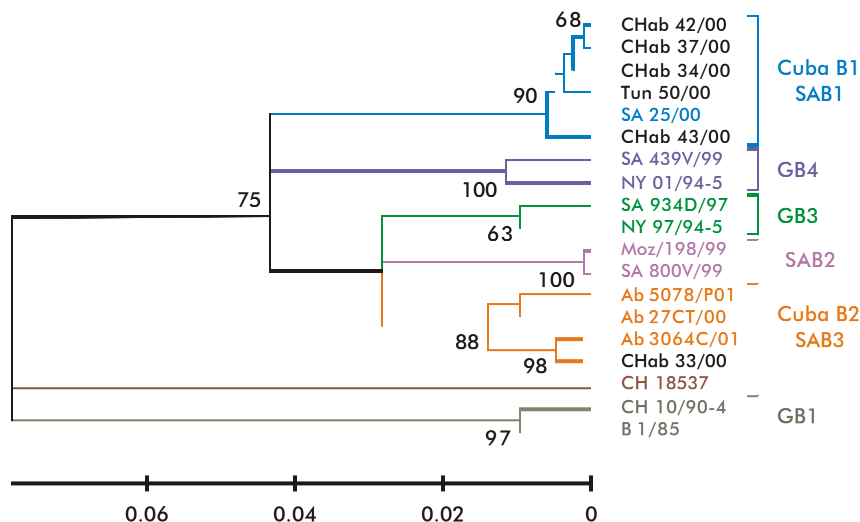
The analysis by RFLP and the partial sequencing of a segment of the SH, N y G genes revealed that very similar viruses simultaneously appear in very distant countries [24, 25]. The HRSV genotypes show a global distribution. Isolated viruses have been detected in distant locations and in different times and they can be more related to each other that other viruses isolated during two consecutive epidemics 11]. Viruses with the same genotype have also been identified in the same season indifferent areas of the world [22, 26].

The viruses of the B subgroup detected in Cuba were philogenetically grouped in two main evolutive lines. The results are shown in figure 2.

The viruses with both genotypes were grouped with those of the genotyped previously identified in South Africa [27]. Almost all the viruses identified in Cuba were grouped with the SAB1 or Cuba B1 genotypes. Viruses from that subgroup, with identical or semi-identical sequences, were observed. In South Africa, from 1998 to 2000, this genotype was detected with a low frequency and could not be grouped with any of the strains published in the GenBank. However, only one strain detected in Cuba (CHab 33/00) was grouped with the SAB3 o Cuba B2 genotypes. This genotype was dominating in South Africa from 1997 to 2000 [27]. The viruses circulating in Cuba were philogenetically very distant from the strain prototype CH18537.

In 1991, Sullender *et al.*, studied the sequence of the G glycoprotein of a group of strains of the B subgroup, isolated from 1960 to 1989. In this study, the circulation of multiple genotypes was identified. Strains belonging to the B subgroup, isolated in three epidemic periods (from 1993 to 1996) in Alabama were studied Different genotypes of that subgroup were observed and the old strains were grouped very far from the most recent strains [28]. In a community of The United States, a group of strains, isolated in five academia periods were tested; four genotypes (GB1-GB4) were also identified. This study was extended to five communities of the United States, for a same academic period and only the circulation of the GB3 and GB4 genotypes was detected [29, 30].

In 2001, Venter *et al.*, identified the circulation of multiple genotypes of the B subgroup during three consecutive epidemics in South Africa. They detected 5 genotypes, two of them were previously identified (GB3 and GB4), and three new genotypes (SAB1, SAB2 and SAB3). In Uruguay, during the periods from 1989 to 1996 and from 1999 to 2001, the genotypes GB1, GB2, GB4 and SAB2 circulated and, moreover, two new genotypes were identified URU1 and URU2. Those two genotypes were composed of strains that circulated in Uruguay in 1990, 1991, 1999 and 2001 [31].



Figures 2. HRSV philogenetic tree of B subgroup that circulated in some Cuban provinces and other geographic areas. The viruses from Cuba are shown in black.

Differently from the A subgroup, the strains from the B subgroup were very distant from the old strains. The viruses of the same period were philogenetically grouped in two genotypes, with strains that circulated at the same time in South Africa. This high similarity to the strains from Africa could be tightly related to the increase of the exchange of Cuban people traveling to Africa in the late 90s.

The epidemiological pattern described for HRSV in Cuba has been discussed in previous laboratory works and it has been established that this virus circulates from September to February- The geographic and socioeconomic conditions of Cuba could influence its dissemination and evolution.

Conclusions

1. The substitutions of aminoacids and the alternative use of the ending codons were the most frequently changes found in the C end of the G glycoprotein of the viruses studied in Cuba. Those are antigenic and immunogenic mechanisms involved in the generation of the HRSV diversity.

2. The sequences of the HRSV strains of the A subgroup were philogenetically grouped with sequences of different genotypes reported in different geographic regions of all the continents, what corroborates that the HRSV variants are disseminated all around the world.

3. The circulation of the strains of the A subgroup with nucleotidic sequences, similar to those of the prototype Long strain was a particular finding of the strains detected in Cuba.

4. The nucleotidic sequences of the HRSV, found in Cuba, belonging to de B subgroup, were philogene-

18. Rueda P, Delgado T, Portela A, Melero JA, Garcia-Barreno B. Premature stop codons in the G glycoprotein of human respiratory syncytial viruses resistant to neutralization by monoclonal antibodies. J Virol 1991;65:3374-8.

19. Johansen J, Christensen LS, Horns-leth A, Klug B, Hansen KS, Nir M. Restriction pattern variability of respiratory syncytial virus during three consecutive epidemics in Denmark. Apmis 1997;105:303-8.

20. Christensen LS, Larsen LB, Johansen J, Andersen EA, Wejse C, Klug B, Hornsleth A. The fluctuating pattern of various genome types of respiratory syncytial virus in Copenhagen and some other locations in Denmark. Apmis 1999;107:843-50.

21. Cane PA, Weber M, Sanneh M, Dackour R, Pringle CR, Whittle H. Molecular epidemiology of respiratory syncytial virus in The Gambia. Epidemiol Infect 1999; 122:155-60.

22. Garcia O, Martin M, Dopazo J, Arbiza J, Frabasile S, Russi J, Hortal M, Perez-Brena P, Martinez I, Garcia-Barreno B, *et al.* Evolutionary pattern of human respiratory syncytial virus (subgroup A): cocirculating lineages and correlation of genetic and antigenic changes in the G glycoprotein. J Virol 1994;68:5448-59.

23. Frabasile S, Delfraro A, Facal L, Videla C, Galiano M, de Sierra MJ, Ruchansky D, Vitureira N, Berois M, Carballal G, Russi J, Arbiza J. Antigenic and genetic variability of human respiratory syncytial viruses (group A) isolated in Uruguay and Argentina: 1993-2001. J Med Virol 2003;71: 305-12.

24. Cane PA, Pringle CR. Molecular epidemiology of respiratory syncytial virus: rapid identification of subgroup A lineages. J Virol Methods 1992;40:297-306.

25. Lukic-Grlic A, Cane PA, Bace A, Pringle CR, Mlinaric-Galinovic G, Popow-Kraupp T. Antigenic and genomic diversity of central European respiratory syncytial virus strains. Arch Virol 1998; 143:1441-7.

tically related with the sequences of the strains reported in South Africa in the same epidemic season.

5. The presence of semi-identical nucleotidic sequences of the G glycoprotein was a characteristic predominating in the HRSV strains detected in the provinces where the studies were performed, what suggests that the virus was disseminated from a common source.

26. Sanz MC, Kew OM, Anderson LJ. Gene-tic heterogeneity of the attachment glycoprotein G among group A respiratory syncytial viruses. Virus Res 1994;33: 203-17.

27. Venter M, Madhi SA, Tiemessen CT, Schoub BD. Genetic diversity and molecular epidemiology of respiratory syncytial virus over four consecutive seasons in South Africa: identification of new subgroup A and B genotypes. J Gen Virol 2001;82:2117-24.

28. Coggins WB, Lefkowitz EJ, Sullender WM. Genetic variability among group A and group B respiratory syncytial viruses in a children's hospital. J Clin Microbiol 1998; 36:3552-7.

29. Peret TC, Hall CB, Schnabel KC, Golub JA, Anderson LJ. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J Gen Virol 1998;79(9):2221-9.

30. Peret TC, Hall CB, Hammond GW, Pie-dra PA, Storch GA, Sullender WM, Tsou C, Anderson LJ. Circulation patterns of group A and B human respiratory syncytial virus genotypes in 5 communities in North America. J Infect Dis 2000;181:1891-6.

31. Blanc A, Delfraro A, Frabasile S, Arbiza J. Genotypes of respiratory syncytial virus group B identified in Uruguay [editorial]. Arch Virol 2004.