ARTICULO ORIGINAL

ISSN 0325-7541
Revista Argentina de Microbiología (2012) 44: 259-265

Rhinovirus detection by real-time RT-PCR in children with acute respiratory infection in Buenos Aires, Argentina

DÉBORA N. MARCONE[1], CRISTINA VIDELA[1], CARMEN RICARTE[1], GUADALUPE CARBALLAL[1], SANTIAGO VIDAURRETA[2], MARCELA ECHAVARRÍA[1*]

*[1]Unidad de Virología y Laboratorio de Virología Clínica y [2]Departamento de Pediatría, Centro de Educación Médica e Investigaciones Clínicas (CEMIC) Hospital Universitario. Av. Galván 4102, Buenos Aires, Argentina. *Correspondence. E-mail: mechavarriaf@hotmail.com*

ABSTRACT

Human rhinoviruses (HRV), the major cause of common colds, have a significant genetic diversity and are classified into 3 species (A, B, C) with more than 100 serotypes. HRV species C, described in 2006, can only be detected using molecular methods. The objectives of this paper were to adapt a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay for HRV detection and to further determine the frequency of HRV in respiratory samples from children under 2 years of age, with acute respiratory infection (ARI), from Buenos Aires, Argentina. Two real-time RT-PCR assays amplifying the 207 base pair of the 5' non-coding region were compared. The original protocol includes locked nucleic acid analogues and a pyrimidine derivative in the forward primer, while the adapted protocol avoided those molecules. Of 67 respiratory samples, 17 (25.4 %) were positive with the original protocol, and 20 (29.9 %) with the adapted one. Discrepant results were confirmed by sequencing analysis. An expanded gold standard was defined to determine the performance of both assays, and was used to describe the clinical characteristics of positive patients. Better sensitivity and specificity were obtained with the adapted protocol. Considering the expanded gold standard, HRV were detected in 23/67 (34.3 %) patients with ARI: 8/18 (44.4%) outpatients and 15/49 (30.6 %) hospitalized. Wheezing episodes were more frequent in HRV positive patients (43.5 %) than in HRV negative patients (18.2 %) (p = 0.041). This study describes the utility and clinical sensitivity of an adapted real-time RT-PCR assay for HRV detection.

Key words: rhinoviruses, real-time RT-PCR, acute respiratory infection, children

RESUMEN

Detección de rinovirus por RT-PCR en tiempo real en muestras respiratorias de niños de Buenos Aires, Argentina. Los rinovirus humanos (RVH) constituyen la principal causa de resfrío común y poseen una gran diversidad genética, con más de 100 serotipos clasificados en tres especies (A, B, C). Los RVH C fueron descritos en 2006 y solo pueden detectarse utilizando métodos moleculares. El objetivo del presente trabajo fue adaptar un protocolo de transcripción reversa seguida de reacción en cadena de polimerasa (RT-PCR) en tiempo real para detectar RVH y posteriormente determinar su frecuencia en muestras de niños menores de 2 años con infección respiratoria aguda (IRA). Se compararon dos protocolos de RT-PCR en tiempo real, que amplifican 207 pares de bases de la región 5' no codificante. El protocolo original incluyó un cebador directo con análogos de nucleótidos bloqueados (LNA) y un derivado pirimidínico en su secuencia, mientras que el protocolo adaptado no los incluyó. De 67 muestras, 17 (25,4 %) fueron positivas con el protocolo original y 20 (29,9 %) con el protocolo adaptado; los resultados discrepantes se confirmaron por secuenciación. Se definió un *gold standard* expandido para determinar el desempeño de ambos ensayos y describir las características clínicas de los pacientes RVH positivos. La mejor sensibilidad y especificidad se obtuvo con el protocolo adaptado. Considerando el *gold standard* expandido, se detectó RVH en 23/67 (34,3 %) pacientes con IRA: 44,4 % (8/18) ambulatorios y 30,6 % (15/49) internados. Los episodios de sibilancias fueron más frecuentes en pacientes RVH positivos (43,5 %) que en RVH negativos (18,2 %) (p = 0,041). El presente estudio describe la utilidad y la sensibilidad clínica de esta RT-PCR en tiempo real adaptada para detectar RVH.

Palabras clave: rinovirus, RT-PCR en tiempo real, infección respiratoria aguda, niños

INTRODUCTION

Human rhinoviruses (HRV) belong to the *Picornaviridae* family, genus *Enterovirus* (25) and were formerly classified in the genus *Rhinovirus*. To date, more than 100 serotypes have been described and classified into 3 species: A, B and C (22, 23). Their genome is a single 7, 2-kb positive RNA strand

with a single open-reading frame (31).

HRV are the most frequent cause of common colds and are also associated with acute otitis media in children and sinusitis in adults (27, 28).

Recent studies have established that HRV can infect the lower airways causing pneumonia and bronchiolitis in children (12, 26), asthma exacerbation in school-aged children and adults, exacerbation of cystic fibrosis (32) and chronic obstructive pulmonary disease in adults (24).

HRV have been identified as an important predictor of recurrent wheezing (14) and asthma development in high-risk children (10). Asymptomatic HRV infection can also occur in infants, children and adults (11, 34, 36).

Isolation of HRV in cell culture is difficult, insensitive and time consuming (36). The development of molecular methods has increased the feasibility of HRV detection. Several reverse transcription-polymerase chain reaction (RT-PCR) assays have been developed for a sensitive detection and differentiation of HRV. Some of them target gene regions that are common for both HRV and enteroviruses (HEV) (1, 7, 13, 15, 30), but other RT-PCR assays are only specific for HRV (8, 16, 33). The 5' non-coding region (5'NCR) of the viral genome contains highly conserved sequences, thus presenting a convenient area for amplification. However, part of this region is shared with HEV.

There is a significant sequence variation between HRV strains due to the imprecise replication of the RNA by the virus-encoded RNA dependent RNA polymerase and frequent intra and interspecies recombination events (25, 31).

The frequency of HRV detected by molecular methods in hospitalized children with acute respiratory infection (ARI) ranges from 6 % to 35 % (2, 5, 6, 17).

Although HRV is frequently detected in coinfection with other respiratory viruses, the role of this simultaneous presence is not yet established: some authors have proposed that viral coinfection increases the severity of disease, while others have not found differences between coinfection and single infections (2).

The goal of this study was to adapt a real-time RT-PCR for HRV detection described by Lu *et al.* in 2008 (16). This method allows detection of all HRV species and showed high sensitivity and specificity. In our study, we avoided the use of locked nucleic acid analogues and a pyrimidine derivative (molecules difficult to obtained in Argentina) in the forward primer, while maintaining a good performance of HRV detection. The frequency of HRV, viral coinfection and clinical features was determined in hospitalized and outpatient children under 2 years of age with ARI in Buenos Aires, Argentina.

MATERIALS AND METHODS

Samples

Nasopharyngeal aspirates (NPAs) from 67 patients under 2 years of age with ARI who attended the emergency room or were hospitalized at CEMIC University Hospital, Buenos Aires, Argentina, from June to November, 2007, were studied. NPAs were sent in viral transport media to the Clinical Virology Laboratory at CEMIC for viral diagnosis. Remaining NPAs were anonymized and stored at -70 ºC until the HRV studies were performed. This study was approved by the Institutional Review Board from CEMIC.

Stored data included: age, clinical characteristics such as upper respiratory tract infection (URTI), recurrent wheezing episodes, bronchiolitis, pneumonia, hospitalization days, oxygen therapy, mechanical ventilation requirement, and admission to intensive care unit (ICU). In addition, the results of other viral tests such as respiratory syncytial virus (RSV), influenza (Flu), adenovirus (AdV) parainfluenza (PIV) by indirect immunofluorescence and human bocavirus (hBoV) and human metapneumovirus (hMPV) by PCR were included (3).

PCR controls

Serial 10-fold dilutions of a positive control (HRV 31, ATCC® VR-506), kindly provided by Dr. Freymuth (Caen University Hospital, France), were used in two different runs to determine the limit of detection of each RT-PCR protocol. Positive HEV controls: serotype 1, 2 and 3 poliovirus 2006 vaccine strains and HEV 68 (highly similar to HRV) were used to test the specificity of both RT-PCR assays to detect HRV.

RNA extraction

Viral RNA was manually extracted from 140 ul of the NPA using the QIAamp® Viral RNA kit (Qiagen GmbH, Hilden, Germany), following manufacturer's instructions.

RT-PCR assays

Two real-time RT-PCR assays amplifying 207 base pair (bp) of the 5' non-coding region (5' NCR) of HRV were compared.

Protocol A: the real-time RT-PCR protocol recommended by Lu *et al.* in 2008 (16). Primers (forward: 5'-CPX GCC ZGC GTG GC-3'; reverse: 5'-GAA ACA CGG ACA CCC AAA GTA-3') and probe (5'-FAM-TCC TCC GGC CCC TGA ATG YGG C-BHQ1-3') were kindly provided by Dr. Erdman (CDC, Atlanta, USA). The forward primer includes locked nucleic acid (LNA) analogues (X = LNA-dA; Z = LNA-dT) and a pyrimidine derivative (P is a degenerate base mimicking a C/T mix). This protocol was performed using iScript One-Step RT-PCR Kit for probes (Bio-Rad, CA, USA): each 25 µl reaction mixture containing 12.5 µl of 2X reaction mix, 0.25 µl of 100 µM forward and reverse primers, 0.25 µl of 10 µM probe, 0.5 µl of iScript reverse transcriptase, 6.25 µl of nuclease free water, and 5 µl of nucleic acid extract. RT-PCR cycling conditions were the following: an initial reverse transcription at 48 ºC for 10 min, 95 ºC for 5 min for polymerase activation, and then 45 cycles of 95 ºC for 15 s and 55 ºC for 1 min, on a SmartCycler II (Cepheid, CA, USA).

Protocol B: was an adaptation of protocol A. The sequence of the forward primer was: 5'-CYA GCC TGC GTG GC-3', avoiding the use of LNA analogues and the pyrimidine derivative. The reverse primer and probe were the same as in protocol A. This protocol was performed using the One-step RT-PCR kit (Qiagen) that includes two enzymes (Omniscript and Sensiscript) for the RT and a HotStart Taq DNA polymerase for amplification. The RT-PCR cycling conditions were the following: an initial reverse transcription at 50 °C for 30 min, 95 °C for 15 min for polymerase activation, and then 45 cycles of 95 °C for 15 s and 55 °C for 1 min, on a SmartCycler II (20).

In addition, protocol B was evaluated with the iScript One-Step RT-PCR Kit for probes (Bio-Rad) and SuperScrip III platinum one-step quantitative RT-PCR kit (Invitrogen, Brazil) to test the performance of different commercial kits. Both kits include an MMLV reverse transcriptase for the RT step and a Hot-Start Taq polymerase for the amplification.

Protocol for HEV: a nested RT-PCR for HEV detection was performed in samples with discrepant results with protocol A and B (4). This RT-PCR amplifies a region of 306 bp to 311 bp of the 5'NCR. The RT was performed using an MMLV reverse transcriptase and a recombinant RNasin ribonuclease inhibitor (Promega, WI, USA), and a Taq DNA polymerase recombinant (Invitrogen) for PCR amplification.

Sequencing

Sequencing was performed in samples that were positive with only one protocol in order to confirm the presence of HRV. The 207 bp product (obtained with protocol A or B) was purified using ethanol precipitation and sequenced in both senses using Authomatic Sequencer 3730XL (Macrogen, Seoul, Korea). Alignment and analysis of the sequences were performed using Blast 2.2.24 (37).

Expanded Gold Standard

A true positive for HRV was defined as a sample that was positive with both, protocol A and B, or with only one protocol, but further confirmed as HRV by sequencing. A true negative for HRV was defined as a sample that was negative with both protocols or with a positive test for HRV with only one protocol but further confirmed as HEV by PCR. This expanded gold standard was used to determine the performance of protocols A and B and for the evaluation of the clinical characteristics in children with ARI.

Statistical analysis

Performance of protocols A and B, including sensitivity (SE), specificity (SP), positive (PPV), negative predictive value (NPV) and receiver operating curves (ROC) with their respective 95 % confidence interval (CI95 %) were calculated using the expanded gold standard. Rocgold was used to independently test the equality of the ROC area of each method against a gold standard curve. For each comparison, Rocgold reports the raw and the Bonferroni adjusted significance probability. Fisher's Exact Test was used to analyze patients' clinical and epidemiological data. Mann-Whitney test was used to compare medians. Statistical significance was assumed for p values less than 0.05. Statistical analyses were performed using STATA 7.0 (StataCorp).

RESULTS

Both RT-PCR protocols (A and B) were able to detect HRV in respiratory samples. Of 67 samples, 17 (25.4 %) were positive with protocol A, and 20 (29.9 %) with protocol B. All three commercial RT-PCR reagent kits (Qiagen one-step RT-PCR kit, iScript One-Step RT-PCR Kit for probes and SuperScrip III platinum one-step quantitative RT-PCR kit) proved to be adequate for protocol B.

The limit of detection for HRV was the same with both protocols, and a dilution of 10^{-5} was achieved of the HRV control. Protocols A and B did not detect poliovirus serotypes 1-3; however, both protocols detected the HEV 68 control.

When testing clinical samples, 1 of 17 HRV positive samples detected with protocol A was negative for HRV with protocol B, and was later confirmed as HEV by a specific RT-PCR. Of the 17 positive samples detected with protocol A, 3 were negative for HRV with protocol B, but were later confirmed as HRV by sequencing. Seven of 20 HRV positive samples detected with protocol B were negative for HRV with protocol A. These 7 samples were later confirmed as HRV by sequencing (Table 1).

Table 1. Comparison of two real-time RT-PCR vs. the Expanded Gold Standard for HRV detection, in 67 NPAs from children with ARI. Buenos Aires, Argentina

Protocol		Expanded Gold Standard		Total
		Positive (n = 23)	Negative (n = 44)	(n = 67)
A	Positive	16	1	17
	Negative	7	43	50
B	Positive	20	0	20
	Negative	3	44	47

A true positive for HRV was defined as a sample that was positive with both, protocol A and B, or with only one protocol, but further confirmed as HRV by sequencing. A true negative for HRV was defined as a sample that was negative with both protocols or with a positive test for HRV with only one protocol but further confirmed as HEV by PCR. This expanded gold standard was used to determine the performance of protocols A and B and for the evaluation of clinical characteristics in children with ARI.

When the expanded gold standard was applied, protocol A missed 7 samples and protocol B 3 samples. Therefore, sensitivity (SE) was 70 % and 87 %, respectively. Specificity (SP) was 98 % for protocol A and 100 % for protocol B. The performance

of both protocols including SE, SP, PPV and NPV are shown in Table 2. Protocol B performed better in the detection of HRV. However, no statistically significant differences were observed in the ROC Area between both protocols.

The clinical characteristics of children with ARI and true positive HRV cases (expanded gold standard) are shown in Table 3. The overall frequency of HRV was 23/67 (34.3 %): 8/18 (44.4 %) outpatients and 15/49 (30.6 %) hospitalized children were HRV positive. The median length of stay was 5 days. All 15 children received oxygen therapy with a median of 4 days. Of 49 hospitalized patients, 1 (2.0 %) was admitted to the ICU due to respiratory illness for 4 days and received mechanical ventilation during 3 days; he was only diagnosed HRV.

Table 2. Performance parameters of two real-time RT-PCR for HRV detection

Protocol	SE (CI95%)	SP (CI95%)	PPV (CI95%)	NPV (CI95%)	ROC Area (CI95%)	X^2 (1gl)	$p\ X^2$	p Bonferroni
A	69.6 (58.6-80.6)	97.7 (94.2-100.0)	94.1 (88.5-99.8)	86.0 (77.7-94.3)	0.837 (0.7-0.9)	2.05	0.153	0.458
B	87.0 (78.9-95.0)	100.0 (100.0-100.0)	100.0 (100.0-100.0)	93.6 (87.8-99.5)	0.935 (0.9-1)			

SE: Sensitivity; SP: Specificity; PPV: positive predictive value; NPV: negative predictive value; CI95%: confidence interval 95%

Median age was 10 months (range 1-48 months) for HRV positive patients, and 8 months (range 1-36 months) for HRV negative patients. Clinical characteristics associated with HRV infection include: URTI, wheezing, bronchiolitis and pneumonia. All HRV positive patients had rhinitis, 70 % had difficulty breathing, and 30 % fever.

HRV was statistically associated with recurrent episodes of wheezing, which was observed in 10 of 23 children (43.5 %), compared to 8 of 44 (18.2 %) children with wheezing and without HRV (p = 0.041). Fever and bronchiolitis were statistically more frequent in HRV negative patients.

HRV were detected throughout the studied period (June to November 2007).

Of 67 patients studied, 32 (47.7 %) were negative for HRV but positive for other respiratory viruses, and 12 (17.9 %) patients were negative for any respiratory viruses studied. The frequency for each respiratory virus is shown in Table 4.

DISCUSSION

Over the last years, HRV have gained wider recognition as clinically relevant pathogens causing not only mild respiratory infections, but also severe respiratory disease. Association with recurrent episodes of wheezing, asthma and severe lower respiratory disease has also been reported (10, 12, 14, 24, 26).

The diagnosis of the classical respiratory viruses was usually done by IF using specific monoclonal antibodies. However, given the high diversity of HRV, there are no specific monoclonal antibodies available for their diagnosis. The development and use of molecular methods for all HRV have improved the diagnosis.

Many molecular assays have been designed for HRV detection; RT-PCR protocols usually target the conserved 5'NCR region (a region that shares common areas for HRV and HEV). However, it has been difficult to obtain a sensitive RT-PCR protocol capable of detecting all HRV strains. Protocols designed before 2006 may be less sensitive since they did not include the sequences from HRV species C in their design.

In 2008, Lu *et al*. (USA) described a real-time RT-PCR assay (herein known as protocol A) that targets a conserved region in the 5'NCR and is able to detect all HRV species (A, B, C) (16). These authors tested 48 HEV laboratory strains. Of these, 34 HEV were non-reactive, and 14 (including echovirus 1, 3, 5, 6, 13, 21; poliovirus types 1 and 2; enterovirus types 68 and 71; coxsackievirus types A4, A6, A24 and B1) showed slightly positive reactions. Lu *et al.* suggested that these reactions with HEV appeared to be related to the virus titer, rather than to any particular virus type. Using the RT-PCR reported by these authors, Faux *et al.* (Australia) using the RT-PCR published by Lu *et al.*, also confirmed that all HRV species were detected, but it was negative for several HEV tested (9).

In order to improve the performance, protocol A included LNA analogues and a pyrimidine derivative in the forward primer. Specifically, these LNA analogues were able to increase the melting temperature of the primer. Therefore, a balanced midpoint temperature was achieved for both primers. We have adapted the protocol described by Lu *et al.*, avoiding the use of LNA analogues and pyrimidine derivative in the forward primer (protocol B). These reagents are only produced by one company, and are therefore difficult to obtain outside the USA.

Protocol B showed higher SE, SP, PPV and NPV compared to protocol A, although this difference was not statistically significant. Another advantage of protocol B is that it can be run with different commercial enzymes kits. On the contrary, protocol A failed entirely

Table 3. Clinical characteristics of 67 children under 2 years of age with ARI

	HRV Positive (n = 23)	HRV Negative (n = 44)	
	n (%)	n (%)	*p*
Median age. month (range)	10 (1-48)	8 (1-36)	0.074[1]
Gender, Male	15 (65.2)	29 (65.9)	0.999[2]
Hospitalized	15 (65.2)	34 (77.3)	0.385[2]
Outpatient	8 (34.8)	10 (22.7)	
Clinical diagnosis			
Pneumonia	3 (13.0)	6 (13.6)	0.999[2]
Bronchiolitis	5 (21.7)	22 (50.0)	0.036[2]
Wheezing episodes	10 (43.5)	8 (18.2)	0.041[2]
URTI	5 (21.7)	8 (18.2)	0.753[2]
Clinical findings			
Rhinitis	23 (100.0)	44 (100.0)	0.999[2]
Fever	7 (30.4)	26 (59.1)	0.039[2]
Difficulty breathing	16 (69.6)	35 (79.5)	0.381[2]
Acute otitis media	2 (8.7)	6 (13.6)	0.705[2]
Clinical severity of Hospitalization			
Median Days	5	6	0.034[1]
ICU admissions	1 (4.3)	7 (15.9)	0.406[2]
Median Days in ICU	4	14	0.149[1]
Requiring mechanical ventilation	1 (4.3)	5 (11.4)	0.652[2]
Median Days under mechanical ventilation	3	9.5	0.218[1]
Requiring oxygen	15 (65.2)	30 (68.2)	0.298[2]
Median Days of oxygen requirement	4	4	0.244[1]

URTI: Upper respiratory tract infection; ICU: Intensive care unit.
[1] Mann-Whitney test was used.
[2] Fisher bilateral test was used.

Table 4. Detection of respiratory viruses in 67 children with ARI and HRV diagnosis

Respiratory virus	HRV positive n (%)	HRV negative n (%)	Total n (%)
RSV	0 -	14 (31.8)	14 (28.4)
hBoV	6 (26.1)	3 (6.8)	9 (20.9)
hMPV	3 (13.0)	4 (0.9)	7 (13.4)
AdV	0 -	3 (6.8)	3 (4.5)
Flu	0 -	2 (4.5)	2 (3.0)
PIV	0 -	1 (2.3)	1 (1.5)
RSV + hBoV	1 (4.3)	4 (0.9)	5 (1.5)
hBoV + hMPV	0 -	1 (2.3)	1 (3.0)
Negative	13 (56.5)	12 (27.3)	25 (17.9)
Total (n)	23	44	67

HRV: human rhinovirus; RSV: respiratory syncytial virus; hBoV: human bocavirus; hMPV: human metapneumovirus; AdV: adenovirus; Flu: influenza; PIV: parainfluenza.

when using the SuperScript III platinum kit. We chose the Qiagen OneStep RT-PCR kit to perform protocol B because it includes two Reverse Transcriptases: an Omniscript and a Sensiscrip, which are optimized for high and low amounts of RNA, respectively. Both enzymes exhibit high affinity for RNA, thus facilitating transcription through secondary structures, such as IRES, present in the 5'NCR of HRV.

In our study, the overall frequency of HRV in children with ARI was 34.3 %: 30.6 % in hospitalized patients and 44.4 % in outpatients. These results are similar to those previously reported in other countries (2, 5).

Two previous publications on HRV in hospitalized children with recurrent wheezing from Argentina reported frequencies of 23 % - 25 % (18, 19), with a conventional RT-PCR. The higher frequency detected in our study may be due to differences in the studied population, or to the use of a more sensitive RT-PCR assay. Some RT-PCR may be less sensitive in detecting certain HRV strains; the use of more specific and sensitive real-time RT-PCR protocols may be necessary to better establish the frequency and epidemiology of these viruses.

Protocol B has been recently performed in our laboratory to study 347 children under 6 years of age with ARI, and enrolled throughout a whole year (June 2008 to May 2009). Results showed an HRV frequency of 43 % in hospitalized and 26 % in outpatient children (21, 35).

In the present study, the only sign statistically associated with HRV was recurrent wheezing episodes (p = 0.041). Likewise, Piotrowska *et al.* (29) conclude that HRV are the major cause of wheezing among children under 2 years of age.

Of the 23 patients with HRV, 43.5 % were diagnosed to be coinfected with other respiratory virus, and 7 (30.4 %) of them were hospitalized. HRV coinfection was most frequently with hBoV, followed by hMPV and RSV. One hospitalized patient, who required oxygen therapy, had a triple coinfection: HRV, RSV and hBoV.

In conclusion, the adapted protocol B proved to properly detect HRV in respiratory samples. Further studies including a larger series of patients with ARI and sequencing HRV samples to identify HRV types are needed to better determine the epidemiology and impact of these viruses in Argentina.

Acknowledgments: the authors would like to thank David Veyer for his work in HRV detection; pediatricians, residents and nurses from CEMIC University Hospital for patients' recruitment and sample collection; Natalia Del Rio for technical assistance; Fernando Poletta and Juan A. Gili for statistical assistance, and Valeria Melia for language revision of the manuscript. This work was granted by PICT 2006-650 from National Scientific and Technologic Agency (ANPCYT). DNM is a PhD student and CR a technician, both from the National Research Council of Argentina (CONICET); ME is a researcher from both CONICET and CEMIC.

Conflict of interest
Competing interest: None declared
Ethical approval: Yes

REFERENCES

1. Bellau-Pujol S, Vabret A, Legrand L, Dina J, Gouarin S, Petitjean-Lecherb J, Pozzetto B, Ginevra C, Freymuth F. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J Virol Methods 2005; 126: 53-63.
2. Calvo C, Garcia-Garcia M, Blanco C, Pozo F, Casas Flecha I, Perez-Breña P. Role of rhinovirus in hospitalized infants with respiratory tract infections in Spain. Pediatr Infect Dis J 2007; 26: 904-8.
3. Cartamil S, Suarez A, Pascutto M, Ricarte C, Ebekian B, Vidaurreta S, Carballal G, Videla C. Estudio de dos nuevos virus respiratorios en población pediátrica con infección respiratoria aguda: el metapneumovirus (hMPV) y el bocavirus (hBoV). IX Congreso Argentino de Virología, Abstract 145. Rev Argent Microbiol 2008; 40 Supl: 78.
4. Casas I, Klapper PE, Cleator GM, Echevarria JE, Tenorio A, Echevarria JM. Two different PCR assays to detect enteroviral RNA in CSF samples from patients with acute aseptic meningitis. J Med Virol 2005; 47: 378-85.
5. Cheuk D, Tang I, Chan K, Woo P, Peiris M, Chiu S. Rhinovirus infection in hospitalized children in Hong Kong: a prospective study. Pediatr Infect Dis J 2007; 26: 995-1000.
6. Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, Lee JH, Song EK, Kim SH, Park JY, Sung JY. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000-2005. Clin Infect Dis 2006; 43: 585-92.
7. Dagher H, Donninger H, Hutchinson P, Ghildyal R, Bardin P. Rhinovirus detection: comparison of real-time and conventional PCR. J Virol Meth 2004, 117: 113-21.
8. Elkan M, Joshi SB, Joshi YB, Hodinka RL. Real-time PCR for detection of human rhinoviruses in children. XXIII Annual Clinical Virology Symposium, 2007, Abstract TP-57, Clearwater, Florida, USA.
9. Faux CE, Arden KE, Lambert S, Nissen MD, Nolan TM, Chang AB, Sloots TP, Mackay IM. Usefulness of published PCR primers in detecting human rhinovirus infection. Emerg Infect Dis 2011; 17: 296-8.
10. Jackson DJ, Gangnon RE, Evans MD, Roberg KA, Anderson EL, Pappas TE, Printz MC, Lee WM, Shult PA, Reisdorf E, Carlson-Dakes KT, Salazar LP, DaSilva DF, Tisler CJ, Gern JE, Lemanske RF Jr. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am J Respir Crit Care Med 2008; 178: 667-72.
11. Johnston SL, Sanderson G, Pattemore PK, Smith S, Bardin PG, Bruce CB, Lambden PR, Tyrrell DA, Holgate ST. Use of polymerase chain reaction for diagnosis of picornavirus

infection in subjects with and without respiratory symptoms. J Clin Microbiol 1993; 31: 111-7.
12. Juvén T, Mertsola J, Waris M, Leinonen M, Meurman O, Roivainen M, Eskola J, Saikku P, Ruuskanen O. Etiology of community-acquired pneumonia in 254 hospitalized children. Pediatr Infect Dis J 2000; 19: 293-8.
13. Kares S, Lonnrot M, Vuorinen P, Oikarinen S, Taurianen S, Hyoty H. Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using LightCycler. J Clin Virol 2004; 29: 99-104.
14. Lemanske RF Jr, Jackson DJ, Gangnon RE, Evans MD, Li Z, Shult PA, Kirk CJ, Reisdorf E, Roberg KA, Anderson EL, Carlson-Dakes KT, Adler KJ, Gilbertson-White S, Pappas TE, Dasilva DF, Tisler CJ, Gern JE. Rhinovirus illnesses during infancy predict subsequent childhood wheezing. J Allergy Clin Immunol 2005; 116: 571-7.
15. Lonnrot M, Sjoroos M, Salminen K, Maaronen M, Hyypia T, Hyoty H. Diagnosis of enterovirus and rhinovirus infections by RT-PCR and time-resolved fluorometry with lanthanide chelate labeled probes. J Med Virol 1999; 59: 378-84.
16. Lu X, Holloway B, Dare RK, Kuypers J, Yagi S, Williams JV, Hall CB, Erdman DD. Real-time reverse transcription-PCR assay for comprehensive detection of human rhinoviruses. J Clin Microbiol 2008; 46: 533-9.
17. Maffey A, Venialgo C, Barrero P, Fuse V, Marques ML, Saia M, Villalba A, Teper AM, Mistchenko AS. New respiratory viruses in children 2 months to 3 years old with recurrent wheeze. Arch Argent Pediatr 2008; 106: 302-9.
18. Maffey A, Barrero P, Venialgo C, Fernandez F, Fuse V, Saia M, Villalba A, Fermepin MR, Teper AM, Mistchenko AS. Viruses and atypical bacteria associated with asthma exacerbations in hospitalized children. Pediatr Pulmonol 2010; 45: 619-25.
19. Manoha C, Espinosa S, Aho S, Huet F, Pothier P. Epidemiological and clinical features of hMPV, RSV and RVs infections in young children. J Clin Virol 2007; 38: 221-6.
20. Marcone DN, Veyer D, Videla C, Ricarte C, Ebekian B, Vidaurreta S, Carballal G, Echavarria M. Comparison of four different RT-PCR for rhinovirus detection. III International Clinical Virology Symposium and Advances in Vaccines, 2010, Abstract P38, p. 89, Ciudad Autónoma de Buenos Aires, Argentina.
21. Marcone DN, Ricarte C, Videla C, Ekstrom J, Carballal G, Vidaurreta S, Echavarría M. Rinovirus: frecuencia en niños ambulatorios con infección respiratoria aguda. Medicina (Buenos Aires) 2012; 72: 28-32.
22. McErlean P, Shackelton L, Andrews E, Webster DR, Lambert SB, Nissen MD, Sloots TP, Mackay IM. Distinguishing molecular features and clinical characteristics of a putative new rhinovirus species, human rhinovirus C (HRV C). Plos One 2008; 3: e1847.
23. Miller E, Edwards K, Weinberg G, Iwane M, Griffin M, Hall C, Zhu Y, Szilagyi PG, Morin LL, Heil LH, Lu X, Williams JV; A novel group of rhinoviruses is associated with asthma hospitalizations. J Allergy Clin Immunol 2009; 123: 98-104.
24. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. BMJ 1993; 307: 982-6.
25. Palmenberg AC, Rathe JA, Liggett SB. Analysis of the complete genome sequences of human rhinovirus. J Allergy Clin Immunol 2010; 125: 1190-9.
26. Papadopoulos NG, Moustaki M, Tsolia M, Bossios A, Astra E, Prezerakou A, Gourgiotis D, Kafetzis D. Association of rhinovirus infection with increased disease severity in acute bronchiolitis. Am J Respir Crit Care Med 2002; 165: 1285-9.
27. Piotrowska B, Vazquez M, Shapiro E, Weibel C, Ferguson D, Landry ML, Kahn JS. Rhinoviruses are a major cause of wheezing and hospitalization in children less than 2 years of age. Pediatr Infect Dis J 2009; 28: 25-9.
28. Pitkaranta A, Arruda E, Malmberg H, Hayden FG. Detection of rhinovirus in sinus brushings of patients with acute community-acquired sinusitis by reverse transcription-PCR. J Clin Microbiol 1997; 35: 1791-3.
29. Pitkaranta A, Virolainen A, Jero J, Arruda E, Hayden FG. Detection of rhinovirus, respiratory syncytial virus, and coronavirus infections in acute otitis media by reverse transcriptase polymerase chain reaction. Pediatrics 1998; 102: 291-5
30. Savolainen C, Mulders M, Hovi T. Phylogenetic analysis of rhinovirus isolates collected during successive epidemic seasons. Virus Res 2002; 85: 41-6.
31. Savolainen C, Blomqvist S, Hovi T. Human rhinoviruses. Pediatr Respir Rev 2003; 4: 91-8.
32. Smyth AR, Smyth RL, Tong CY, Hart CA, Heaf DP. Effect of respiratory virus infections including rhinovirus on clinical status in cystic fibrosis. Arch Dis Child 1995; 73: 117-20.
33. Steininger C, Aberle SW, Popow-Kraupp T. Early Detection of acute rhinovirus infections by a rapid reverse transcription-PCR assay. J Clin Microbiol 2001; 39: 129-33.
34. van Benten I, Koopman L, Niesters B, Hop W, van Middelkoop B, de Waal L, van Drunen K, Osterhaus A, Neijens H, Fokkens W. Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants. Pediatr Allergy Immunol 2003; 14: 363-70.
35. Vidaurreta SM, Marcone DN, Ellis A, EkstromaJ Cukier D, Videla C, Carballal G, Echavarría M. Infección respiratoria aguda viral en niños menores de 5 años. Estudio epidemiológico en dos centros de Buenos Aires, Argentina. Arch Arg Ped 2011; 109: 296-304.
36. Wright PF, Deatly AM, Karron RA, Belshe RB, Shi JR, Gruber WC, Zhu Y, Randolph VB. Comparison of results of detection of rhinovirus by PCR and viral culture in human nasal wash specimens from subjects with and without clinical symptoms of respiratory illness. J Clin Microbiol 2007; 45: 2126-9.
37. Zheng Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol 2000; 7: 203-14.

Recibido: 24/1/2012- Aceptado: 12/7/2012