Brazilian Journal of Microbiology (2012): 739-743
ISSN 1517-8382

RHINOVIRUS DETECTION USING DIFFERENT PCR-BASED STRATEGIES

Silva, E.R.M.; Watanabe, A.S.A.; Carraro, E.; Perosa, A.H.S.; Granato, C.F.H.; Bellei, N.C.J.

Laboratório de Virologia Clínica, Unidade de Doenças Infecciosas, Departamento de Medicina, Universidade Federal de Sao Paulo, São Paulo, SP, Brasil.

Submitted: July 13, 2011; Approved: June 07, 2012.

ABSTRACT

Human rhinoviruses (HRVs) are the major cause of the common cold. HRVs were recently reclassified into the *Enterovirus* genus (HEV) in the *Picornaviridae* family. HRVs and other members of the HEV genus share many common features, including sense RNA genomes and partial nucleotide sequence identity. The aim of this study was to evaluate different HRV detection strategies. Samples from adults with acute respiratory infection (n = 291) who were treated in Sao Paulo Hospital (2001–2003) were tested using three assays. The first assay detected picornaviruses by RT-PCR and hybridization, the second detected rhinoviruses using RT-PCR/sequencing, and the third differentiated HRV from HEV using duplex semi-nested-RT-PCR. Analysis of the results obtained from the first two strategies revealed 83% concordance. Discordant samples were then evaluated by the third protocol, and 82% were negative. The picornavirus detection protocol was more sensitive but less specific than the rhinovirus detection protocols. The semi-nested protocol utilized in the present study was less sensitive and was not useful in differentiating HRV from HEV. Sequencing assays examining different genes would address the best strategy of confirming rhinovirus and enterovirus infections.

Key words: Rhinovirus; Enterovirus; Detection, Differentiation.

INTRODUCTION

Human rhinoviruses (HRVs) are the major cause of common cold symptoms, and they are the most frequent causative agent of upper respiratory tract infections (13). HRVs were discovered in 1956 (16) and are now classified, along with enterovirus species, as members of the *Enterovirus* genus (22) in the *Picornaviridae* family. These viruses share many common features, including a non-enveloped icosahedral capsid, a sense RNA genome and partial nucleotide and amino acid sequence identity (19).

Virus diagnostic procedures using cell culture are limited by the expertise of the diagnostic laboratory and the time required to obtain a result. As such, viral culture has a limited place in routine diagnostic microbiology, although these assays must still be considered in reference or research laboratories. For rhinoviruses, there are also additional limitations related to the number of serotypes circulating and the lack of broad

***Corresponding Author. Mailing address:** Clinical Virology Laboratory-Infectious Disease Unit, Department of Medicine, Federal University of Sao Paulo, Sao Paulo, SP, Brazil. Rua Pedro de Toledo, 781, 15° andar. Vila Clementino CEP: 04039-032 São Paulo – SP - Brasil.; Fax.: 55 11 50815394.; E-mail: nbellei@uol.com.br

detection tools, as each serotype is only recognized by specific antibodies. Most PCR protocols that detect rhinovirus amplify a fragment of the 5' UTR (Untraslated region) of the viral genome. This region is highly conserved among picornaviruses. These assays provide high sensitivity, but because the gene sequences are similar between rhinovirus and other enterovirus species, the assays also necessitate an additional step to differentiate the two. Examples of other methods to detect these viruses include hybridization with specific probes (4, 17), nested-PCR with specific HRV primers (1), and duplex nested-PCR with enterovirus and rhinovirus primers that distinguish between enterovirus- and rhinovirus-positive samples in only one reaction (2). Moreover, sequencing can distinguish these viruses following their detection (14).

In the present study, we aimed to compare three strategies for HRV detection in respiratory samples. The first strategy was a picornavirus RT-PCR hybridization assay, the second strategy was a rhinovirus RT-PCR sequencing approach and the third strategy was a semi-nested-RT-PCR for rhinovirus and enterovirus.

METHODS

Two different protocols for rhinovirus detection were applied to samples from nasal washes from 291 adults 18 or older who presented with acute respiratory infection at Hospital Sao Paulo–Federal University of Sao Paulo (HSP–UNIFESP) during 2001 to 2003. Samples were also collected from adults who were categorized as healthcare workers, members of the general population seen in the emergency room, and renal transplant outpatients. This study was

approved by the Ethics Committee of Sao Paulo Federal University and written consent was obtained from all patients or those responsible for the individual patient.

The first protocol used RT-PCR to amplify the 5'UTR gene of viruses from the *Picornavirus* family (17), followed by hybridization to increase the picornavirus detection. These picornavirus-positive samples were then hybridized with enterovirus-specific probes to differentiate enterovirus- from non-enterovirus-positive samples (4, 17). Those samples which did not hybridize to the enterovirus probe were considered positive for rhinovirus by exclusion.

The second protocol used RT-PCR with rhinovirus-specific primers that target a hypervariable fragment of the 5'UTR, the entire VP4 gene and the 5' terminus of the VP2 gene, followed by gene sequencing (20).

The samples tested using these two protocols, which showed discordant results, were then tested using another protocol to discriminate rhinovirus and enterovirus.

The third protocol was a duplex semi-nested-RT-PCR involving two PCR steps with 5'UTR target primers. The first step detects picornaviruses (EV2 and EV3 primers), and the second step distinguishes human rhinovirus from human enterovirus with specific primers (CCRV3/CCRV4 to HRV and EV3/EVNC1 to HEV) (2). The amplified products were detected by 2% agarose gel electrophoresis to identify a 93 base pair (bp) fragment for rhinovirus or a 154 bp fragment for enterovirus.

RESULTS

In total, 291 samples were tested with the first RT-PCR/hybridization protocol. Those samples which did not hybridize with enterovirus were considered positive for rhinovirus; 95 were classified as rhinovirus, while 9 were classified as enterovirus. All 291 samples were tested by the second RT-PCR/sequencing protocol, and 76 were positive for rhinovirus. Analysis of the results obtained after the first two strategies revealed 83% concordance involving 60 positive results and 180 negative results. Fifty-one samples had discordant detection between these protocols. Of these samples, 45 could be evaluated by a third protocol. These selected samples included 29 samples that were rhinovirus-

positive by RT-PCR hybridization but negative by RT-PCR sequencing and 16 samples that were rhinovirus-negative on RT-PCR hybridization but positive on RT-PCR sequencing. Duplex semi-nested RT-PCR on these 45 samples indicated that 6 were rhinovirus, 2 were enterovirus, and 37 were negative. Of the 37 negative samples, 28 were previously negative by RT-PCR sequencing, and the other nine were previously negative with RT-PCR hybridization. All results obtained in 291 tested samples are shown in Table 1, including the results of rhinovirus type-specific nucleotide sequencing analysis by RT-PCR/sequencing (second protocol). Table 1 shows the patterns of the different protocol comparisons.

Table 1. Rhinovirus and Enterovirus detection according different protocols for the 291 tested samples.

N	RT-PCR/hybridization	RT-PCR/sequencing	Types[a]	Semi-nested-RT-PCR
60	Rhinovirus	Rhinovirus	ND	-
180	Negative	Negative	-	-
28	Rhinovirus	Negative	-	Negative
7[b]	Rhinovirus	Negative	-	-
9	Negative	Rhinovirus	4A/2B/2C/1ND	Negative
1	Rhinovirus	Negative	-	Rhinovirus
1[c]	Negative	Rhinovirus	1B	Rhinovirus
4	Negative	Rhinovirus	2A/2C	Rhinovirus
2	Negative	Rhinovirus	1A/1C	Enterovírus

[a]Rhinovirus types determined by nucleotide sequencing only the RT-PCR/sequencing-positive samples.
[b]Seven samples were not tested using the duplex semi-nested-RT-PCR protocol.
[c]An enterovirus-positive sample in RT-PCR/Hybridization was considered negative for rhinovirus and positive in RT-PCR/sequencing; it was a discordant sample and thus selected for further analysis.
ND = Not determined

DISCUSSION

The present study is one of a small number that have compared rhinovirus molecular detection protocols. Several studies have discussed the HRV and HEV similarity and identity at the genetic and amino acid levels (3, 8, 23). Recently, these two different viral species were grouped into the same *Enterovirus* genus (22). Discrimination between these two viruses using diagnostic tools has been attempted, but no one protocol has been completely successful (7). Sequencing protocols can distinguish these two species, but these protocols are laborious and expensive. A recent study accomplished by Faux *et al*. (6) tested 10 different specific HRV primer pair in a panel of 57 clinical specimens from preschool children with colds and influenza-like illness in Melborne, Australia. None of the used primer pairs alone detect all the HRV species. The authors concluded that the best strategy to detect HRV was to use ≥ 2 primer pairs.

Palmenberg *et al.* (15) published the complete genome sequence of all known HRV species. Complete genome sequencing apparently provide more information about HRV epidemiology, could sustain rational evolutionary molecular studies and also evaluate possible association between disease clinical presentations with specific genome regions.

The concern about the viral agent causing individual respiratory infections has led researchers to seek new conventional PCR-based strategies to distinguish these viruses. In this context, we tested three different protocols, including one that differentiated HRV form HEV by nested-PCR (2), in samples that were discordant when tested with two other protocols (17, 20).

The picornavirus RT-PCR and hybridization protocol had a higher detection ability compared with the RT-PCR/sequencing protocol, but 28 picornavirus/rhinovirus positive-samples were exclusively detected by this assay (Table 1). One limitation of this protocol is that rhinovirus-

positivity was detected by exclusion as samples that did not hybridize to enterovirus, which is a non-specific result. These infections could be caused by other picornaviruses, such as echovirus, that are neither enterovirus nor rhinovirus. In fact, the other two protocols using with specific HRV primers demonstrated negative results for these same samples.

Sixteen other discordant samples were positive via RT-PCR/sequencing for rhinovirus, but only 6 of them were considered positive by our duplex semi-nested-RT-PCR protocol. These data suggest a lower sensitivity for the duplex semi-nested-RT-PCR protocol, but only some of our discordant samples were compared among the three different protocols. Moreover, the fact that only 1 out of 16 samples was not detected and confirmed by the RT-PCR/sequencing protocol (Table 1) suggests a higher specificity for the sequencing protocol.

Seven samples demonstrated a positive result from both the duplex semi-nested-RT-PCR and the sequencing protocol, but 2 other samples had discordant results and were confirmed as rhinoviruses (1 HRV A and 1 HRV C) by the RT-PCR/sequencing protocol after having been classified as enterovirus by the duplex semi-nested-RT-PCR assay. These data suggest that the duplex semi-nested-RT-PCR protocol lacks specificity for discrimination between rhinovirus and enterovirus. This may be due to the genetic similarity (23) between these members of the *Enterovirus* genus.

Finally, some authors have suggested that inclusion of capsid coding sequences (21) in the PCR target region may slightly decrease an assay's screening sensitivity compared with targeting the 5'UTR alone (10) However, authors using a capsid coding sequence protocol have pointed out that this strategy is more robust for genetic typing and has been used in other studies (5, 9, 11, 12, 18, 20, 21, 24). In the present study the sequencing protocol, including capsid sequences, confirmed the previous data and was specific for detecting and confirming HRV-positive samples.

In conclusion, RT-PCR/hybridization was more sensitive but less specific for rhinovirus detection. The duplex semi-nested-RT-PCR protocol assessed in the present study was less sensitive and not useful in differentiating HRV from HEV. A sequencing assay using a different gene or genomic approach could determine which protocol is the best strategy to confirm rhinovirus and enterovirus infections.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP Project number: 07/01166-9) and Coordenação de aperfeiçoamento de pessoal de nível superior (CAPES).

REFERENCES

1. Arruda, E.; Hayden, F.G. (1993). Detection of human rhinovirus RNA in nasal washings by PCR. *Mol. Cell Probes*. 7, 373-9.
2. Billaud, G.; Peny, S.; Legay, V.; *et al*. (2003). Detection of rhinovirus and enterovirus in upper respiratory tract samples using a multiplex nested PCR. *J. Virol. Methods*. 108, 223-8.
3. Blomqvist, S.; Savolainen, C.; Råman, L.; *et al*. (2002). Human rhinovirus 87 and enterovirus 68 represent a unique serotype with rhinovirus and enterovirus features. *J. Clin. Microbiol*. 40, 4218-23.
4. Blomqvist, S.; Skytta, A.; Roivainen, M.; Hovi, T. (1999). Rapid detection of rhinoviruses in nasopharyngeal aspirates by microwell reverse transcription-PCR-hybridization assay. *J. Clin. Micrbiol*. 37, 2813-6.
5. Coiras, M.T.; Aguilar, J.C.; García, M.L.; *et al*. (2004). Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. *J. Med. Virol*. 72, 484-95.
6. Faux, C.E.; Arden, K.E.; Lambert, S.B.; Nissen, M.D.; Nolan, T.M.; Chang, A.B.; Sloots, T.P.; Mackay, I.M. (2011). Usefulness of published PCR primers in detecting human rhinovirus infection. *Emerg Infect Dis*. 17(2):296-8.
7. Halonen, P.; Rocha, E.; Hierholzer, J.; *et al.* (1995). Detection of enteroviruses and rhinoviruses in clinical specimens by PCR and liquid-phase hybridization. *J. Clin. Microbiol*. 33, 648-53.
8. Ishiko, H.; Miura, R.; Shimada, Y.; *et al*. (2002). Human Rhinovirus 87 identified as Human Enterovirus 68 by VP4-Based Molecular Diagnosis. *Intervirology* 45, 136–141.

9. Jin, Y.; Yuan, X.H.; Xie, Z.P.; *et al*. (2009). Prevalence and clinical characterization of a newly identified human rhinovirus C species in children with acute respiratory tract infections. *J. Clin. Microbiol*. 47, 2895-900.
10. Kiang, D.; Kalra, I.; Yagi, S.; *et al*. (2008). Assay for 5' noncoding region analysis of all human rhinovirus prototype strains. *J. Clin. Microbiol*. 46, 3736-45.
11. Kistler, A.; Avila, P.C.; Rouskin, S.; *et al*. (2007). Pan-viral screening of respiratory tract infections in adults with and without asthma reveals unexpected human coronavirus and human rhinovirus diversity. *J. Infect. Dis*. 196, 817-25.
12. Lau, S.K.; Yip, C.C.; Tsoi, H.W.; *et al.* (2007). Clinical features and complete genome characterization of a distinct human rhinovirus (HRV) genetic cluster, probably representing a previously undetected HRV species, HRV-C, associated with acute respiratory illness in children. *J. Clin. Microbiol*. 45, 3655-64.
13. Mäkelä, M.J.; Puhakka, T.; Ruuskanen, O.; *et al*. (1998). Viruses and bacteria in the etiology of the common cold. *J. Clin. Microbiol*. 36, 539–542.
14. Mori, J.; Clewley, J.P. (1994). Polymerase chain reaction and sequencing for typing rhinovirus RNA. *J. Med. Virol*. 44, 323-9.
15. Palmenberg, A.C.; Spiro, D.; Kuzmickas, R.; Wang, S.; Djikeng, A.; Rathe, J.A.; Fraser-Liggett, C.M.; Liggett, S.B. (2009). Sequencing and analyses of all known human rhinovirus genomes reveal structure and evolution. *Science*. 324(5923):55-9.
16. Pelon, W.; Mogabgab, W.J.; Phillips, I.A.; Pierce, W.E. (1957). A cytopathogenic agent isolated from naval recruits with mild respiratory illnesses. *Proc Soc Exp Biol. Med*. 94, 262-7.
17. Pitkäranta, A.; Arruda, E.; Malmberg, H.; Hayden, F.G. (1997). Detection of rhinovirus in sinus brushings of patients with acute community-acquired sinusitis by reverse transcription-PCR. *J. Clin. Microbiol*. 35, 1791–1793.
18. Renwick, N.; Schweiger, B.; Kapoor, V.; *et al*. (2007). A recently identified rhinovirus genotype is associated with severe respiratory-tract infection in children in Germany. *J. Infect. Dis*. 196, 1754-60.
19. Rueckert, R.R. (1996). Picornaviridae: The virus and their replication. In: Fields, B.N.; Knipe, D. M.; Howley, P.M.; *et al*. Fields virology. Lippencott-Raven Publishers: Philadelphia. p. 609-654.
20. Savolainen, C.; Blomqvist, S.; Mulders, M.N.; Hovi, T. (2002). Genetic clustering of all 102 human rhinovirus prototype strains: serotype 87 is close to human enterovirus 70. *J. Gen. Virol*. 83, 333–340.
21. Savolainen-Kopra, C.; Blomqvist, S.; Smura, T.; *et al*. (2009). 5' noncoding region alone does not unequivocally determine genetic type of human rhinovirus strains. *J. Clin. Microbiol*. 47, 1278-80.
22. Stanway, G.; Brown, F.; Christian, P.; *et al*. (2005). Family Picornaviridae. In: Fauquet, C.M.; Mayo, M.A.; Maniloff, J.; Desselberger, U.; Ball, L.A. eds. Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. London: Elsevier/Academic Press. 757–778.
23. Tapparel, C.; Junier, T.; Gerlach, D.; *et al*. (2007). New complete genome sequences of human rhinoviruses shed light on their phylogeny and genomic features. *BMC Genomics*. 8:224.
24. Xiang, Z.; Gonzalez, R.; Xie, Z.; *et al*. (2008). Human rhinovirus group C infection in children with lower respiratory tract infection. *Emerg. Infect. Dis*. 14, 1665-7.