Medical Science Journal for Advance Research

Vol. 6, No. 4, December 2025
e-ISSN: 2774-4892

Molecular detection of Rhinovirus by RT-PCR and Amplicon Sequencing

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ABSTRACT

Background: *It is known that Rhinoviruses belonging to the enterovirus are detected in more than half of the common influenza. However, this virus has not been stressed due to mild cold symptoms caused by it. The fact that many evidences that rhinoviruses are related to chronic respiratory diseases has recently been reported suggests that monitoring of this virus should not be neglected. In this study, we designed a primer capable of detecting all A, B and C subtypes in the 5'UTR region of rhinovirus genome and performed RT-PCR to examine the laryngopharynx tampon sample of the patients with respiratory infection. Of the 264 samples, 52(19.7%) samples were rhinovirus-positive. The sequencing of amplicons by ABI3500 and homology analysis was performed, all were determined as Rhinoviruses and the number of subtypes were ten.*

Keywords: Rhinoviruse, RT-PCR, Amplicon Sequencing.

Article Information
Received: November 11, 2025; Revised: December 12, 2025; Online: September, 2025

1. INTRODUCTION

Rhinoviruses are known to be the most frequent causative agents of mild upper respiratory tract infections, or common colds(1). Their impact on health and disease is often ignored because they are considered to cause the self-limiting common cold alone, and because specific diagnosis has been tedious and expensive. However, in addition to upper respiratory illnesses, rhinoviruses are also associated with more severe diseases such as acute otitis media(2) in children and sinusitis in adults(3). Rhinoviruses can also infect the lower respiratory tract and therefore account for lower respiratory tract symptoms such as pneumonia (4), wheezing in children (5), and exacerbations of asthma and chronic obstructive pulmonary disease (COPD) in adults (6).

The view of overall disease burden caused by rhinovirus infections was changed by the emergence of a rapid and sensitive nucleic-acid test protocol in the late 1980s(7).

RT-PCR was introduced to rhinovirus detection in 1988(8). Subsequently, about 20 different RT-PCR applications for rhinovirus detection have been described(9, 10). These have been used extensively in several laboratories and have proven to be sensitive and convenient methods for both diagnostic and epidemiological studies of rhinovirus infections. Most of the rhinovirus RT-PCR assays take advantage of short, highly conserved stretches in the 5'non-coding region as the binding sites

https://doi.org/10.46966/msjar.v6i4.336

for oligonucleotide primers. These assays are shown to be sensitive but not specific, with the same primer binding sites also being conserved in enteroviruses.

The differentiation of rhinoviruses from enteroviruses needs additional steps, for example, restriction fragment length polymorphism(11), hybridization with rhinovirus-specific probes(12,13,14,15), sequencing of PCR amplicons(16) or (semi)nested-PCR with rhinovirus-specific primers(17,18). The differentiation of rhinoviruses and enteroviruses by the size of the RT-PCR amplicon is also possible, when the RT-PCR is performed from 5'NCR through VP2(19). However, the sensitivity of this application is probably lowered by the mismatches in the VP2 primer binding site but yet it is brilliant in, for example, replacing the acid-resistance test of clinical isolates(20).

Although the greater sensitivity of RT-PCR in the detection of rhinovirus infections compared with virus isolation by tissue culture(21, 22) is well established, there are still major problems. For example, optimization of designing of the detecting primers is difficult mainly due to the insufficiency of sequence information about subtypes of rhinoviruses. Primers and probes used today are based on the limited sequence information. In order to improve the sensitivity and specificity, more sequence information of subtypes must be accumulated.

2. MATERIALS AND METHODS

2.1. Primer design

The accession numbers (GenBank 233.0) of the sequences used in primer design for Rhinovirus detection are shown in **Table 1**.

Table 1. Sequences used in primer design for Rhinovirus detection.

FJ445111(1), EU095989(2), EU095990(3), EU870478(6), EU095994(7), EU095995(8), EU095996(9), EU095997(10), EU095998(11), EU095999(12), EU095000(13), EU095001(14), EU095002(15), EU095003(16), EU095004(17), EU095005(18), EU095006(19), EU095007(20), EU095008(21), EU095009(22), EU095010(23), EU095011(24), EU095012(25), EU095013(26), EU095014(27), EU095015(28), EU095016(29), EU095017(30), EU095027(40), EU095037(50), EU095047(60), EU095057(70), EU095067(80), EU095076(90), KC492755(A), JF285310(A), JQ837724(A), JN798582(A), JX074057(A), JF285309(B), JN798572(B), JX074054(B), KY369900(B), MN306025(B), AB683872(C), KC492762(C), JF317013(C), GQ223228(C), HM581871(C)

The numbers in parentheses indicate serotype and the alphabetic letters indicate genotype.

Sequences were aligned and the forward and reverse primers were set up to detect rhinovirus in conservative region in the 5′ UTR.

Specificity of the set of primers was confirmed by BLAST search and virtual PCR on the genomic database.

The sequence of the designed forward primer was 5'-GCACTTCTGTTTCCGGTC-3', the sequence of the reverse primer was 5'-GCATTCAGGGCCGGAGGA-3', and the expected amplicon size was 292 bp.

2.2. Nucleic acid isolation from specimens

Nucleic acid was isolated from 264 specimens (laryngopharynx tampon) from patients with respiratory symptoms using QIAmp Viral RNA mini Kit) according to the manual.

The isolated nucleic acids were immediately used for RT-PCR or stored at −80 °C.

2.3. RT-PCR

RT-PCR was performed using Superscript II RNase H-reverse transcriptase (Invitrogen) and AmpliTaq Gold DNA polymerase (Applied Biosystems).

The reaction mixture consisted of 5µL of nucleic acid isolate, 4µL of 5×first-strand buffer, 100 U of Superscript II RNase H-reverse transcriptase, 5U AmpliTaq Gold DNA polymerase, 2µL of dNTPs (1.5 mM, Amersham Biosciences) and 20 U of RNase inhibitor (Roche Diagnostics), 1µL of 2.5 mM forward and reverse primers respectively and 2µL of nuclease-free water.

RT-PCR was performed through the steps of reverse transcription at 50 °C for 20 min, reverse transcriptase inactivation and pre-denaturation 95 °C for 10 min, 40 cycles of 95 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s and final extension of 72°C for 5 min.

2.4. Sequence analysis and phylogenetic study of RT-PCR products

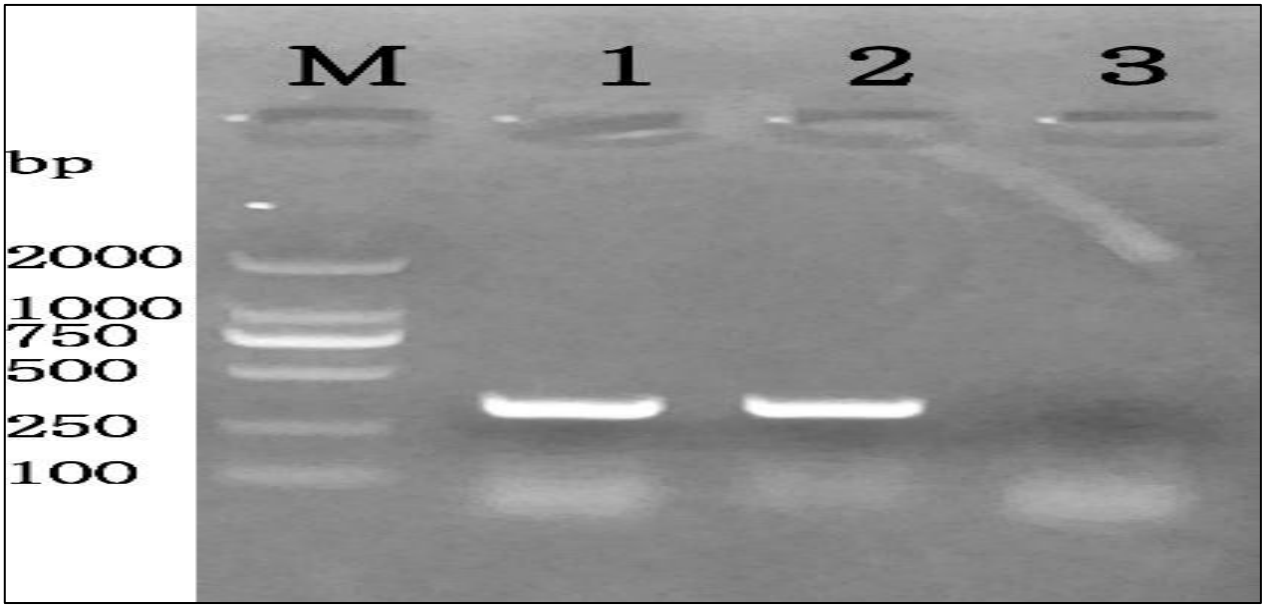
Positive products with the expected amplicons were purified using agarose gel purification kit (UNIQ-10 column Micro DNA Gel Extraction kit, Sangon Biotech), followed by sequencing forward and reverse primers respectively used in RT-PCR with an ABI3500 sequencing machine, to determine the sequence of the complete amplicons.

The sequence of the amplicon was queried to perform BLAST searches on the genomic database to perform molecular identification.

3. RESULT

Nucleic acid was isolated from samples of 264 patients with respiratory tract infections and RT-PCR showed 52 positive results.

The electrophoretic patterns of some RT-PCR products tested positive were as follows.



Picture 1. 1.5% agarose gel electrophoresis of positive specimen RT-PCR products.

M: (DL2000), DNA molecular mass marker: 1: positive specimen 1, 2: positive specimen 2, 3: negative control.

As shown in **Picture 1,** in both samples tested positive, amplification products of expected size (292 bp) were observed. For the identification of the amplification products by sequencing, the amplification products of 10 random samples that were tested as positive were sequenced using forward and reverse primers, respectively and the resulting sequences were aligned and assembled into the full sequence. Sequence analysis of the positive sample 1 amplicon and the complete sequence of the alignment are as follows.

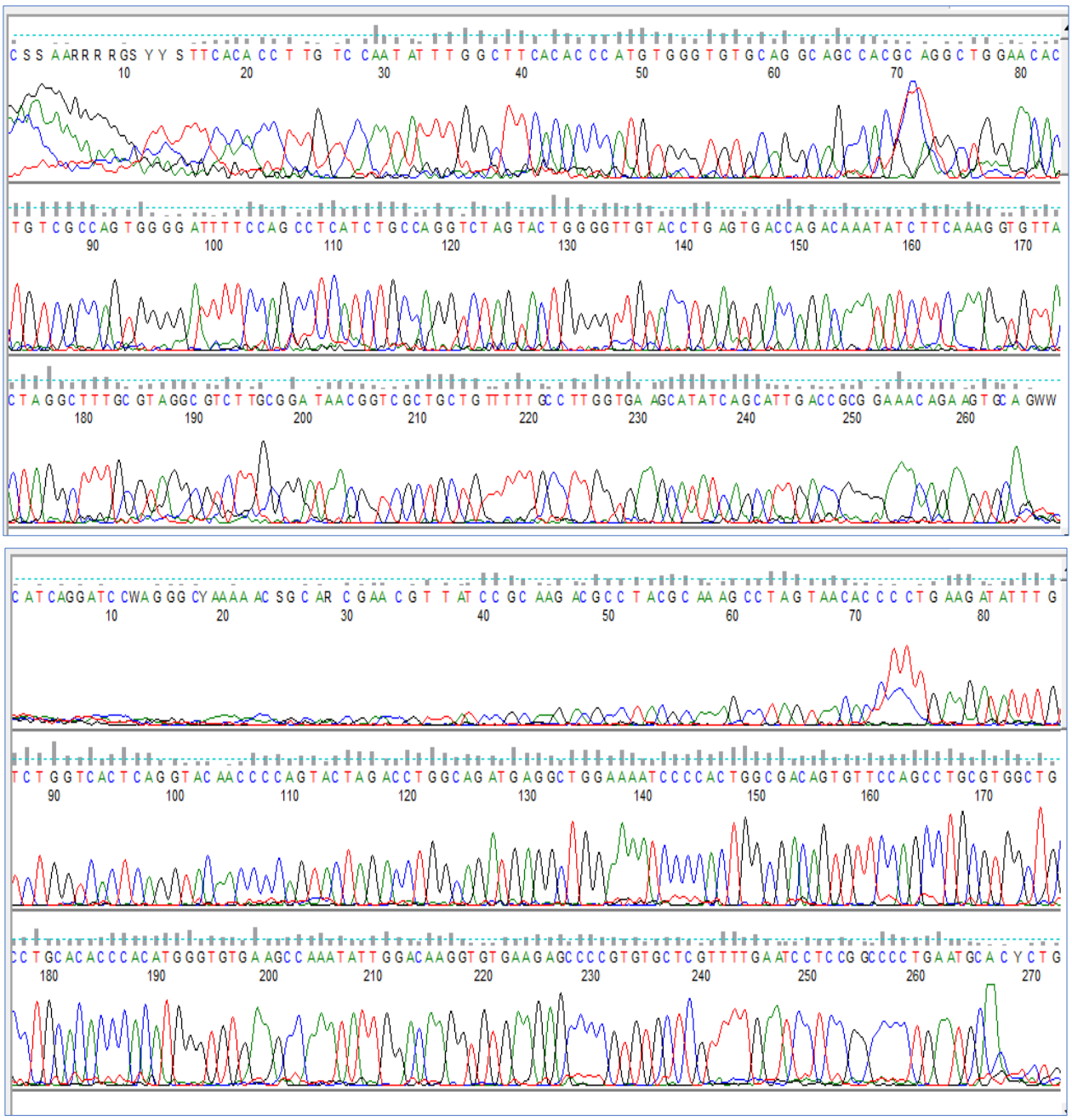


Figure 1. Sequencing image of PCR amplicon using primers for rhinovirus detection

(upper:forward primer, lower:reverse primer) Sequences obtained by both primers were aligned to construct the following 292bp sequence.

GCACTTCTGTTTCCCCGGTCAATGCTGATATGCTTCACCAAGGCAAAAACAG
CAGCGACCGTTATCCGCAAGACGCCTACGCAAAGCCTAGTAACACCTTTGAA
GATATTTGTCTGGTCACTCAGGTACAACCCCAGTACTAGACCTGGCAGATGA
GGCTGGAAAATCCCCACTGGCGACAGTGTTCCAGCCTGCGTGGCTGCCTGCA
CACCCACATGGGTGTGAAGCCAAATATTGGACAAGGTGTGAAGAGCCCCGTG
TGCTCGTTTTGAATCCTCCGGCCCCTGAATGC (292bp)

Figure 2. Sequence analysis of the positive sample 1 amplicon

This sequence was queried to perform homology searches on the genomic database.

MH648088	Human rhinovirus A64 isolate A64/Singapore/2618/2011 pol...	509	1e-141
JF285323	Human rhinovirus A isolate P37_URT_LCT-15d polyprotein g...	501	8e-139
JF285322	Human rhinovirus A isolate P37_URT_LCT-2.9m polyprotein ...	501	8e-139
JF285321	Human rhinovirus A isolate P37_LRT_LCT-7.6m polyprotein ...	501	8e-139
FJ950886	Human rhinovirus sp. isolate PUMCH1343 5' UTR	500	8e-139
FJ950884	Human rhinovirus sp. isolate PUMCH1334 5' UTR	500	8e-139
JF285324	Human rhinovirus A isolate P37_URT_LCT-0d polyprotein ge...	499	3e-138
FJ950877	Human rhinovirus sp. isolate PUMCH1001 5' UTR	496	3e-137
FJ445181	Human rhinovirus 64 strain ATCC VR-1174, complete genome	487	2e-134
EU096051	Human rhinovirus 64 5' UTR	487	2e-134
EF173417	Human rhinovirus 64, complete genome	487	2e-134
EU126727	Human rhinovirus 64 5' UTR	465	5e-128
EU840985	Human rhinovirus sp. strain CL-perLCT080057 5' UTR	456	3e-125
KJ919926	Human rhinovirus sp. isolate KA08-3358 5' UTR	437	8e-120
MK859128	Rhinovirus A isolate ZAM_Control_85 5' UTR	419	2e-114
MH648087	Human rhinovirus A94 isolate A94/Singapore/2609/2011 pol...	419	2e-114
KR054583	Rhinovirus A isolate CU33/B1816/2013 5' UTR	419	2e-114
FJ445185	Human rhinovirus 94 strain ATCC VR-1295, complete genome	419	2e-114
EU096080	Human rhinovirus 94 5' UTR	419	2e-114
EF173419	Human rhinovirus 94, complete genome	419	2e-114
MK858701	Rhinovirus A isolate ZAF_Control_94 5' UTR	411	1e-111
EU126756	Human rhinovirus 94 5' UTR	398	7e-108
MK859007	Rhinovirus A isolate MALI_Control_57 5' UTR	397	2e-107
MK858961	Rhinovirus A isolate MALI_Case_50 5' UTR	397	2e-107
KF498930	Human rhinovirus sp. isolate JN54 5' UTR	397	2e-107
MH648024	Human rhinovirus A82 isolate A82/Singapore/784/2009 poly...	392	3e-106
MF978999	Rhinovirus A isolate ER41169 5' UTR	392	3e-106

Figure 3. BLAST search result of the positive sample amplicon.

As shown in **Figure. 3**, the sequence with the highest homology to the sequence of the positive sample 1 amplicon was the sequence of the rhinovirus A64 virus detected in Singapore in 2011. In this way, all positive sample amplicons were sequenced and identified as shown in the following **Table 2.**

As shown in the **Figure 4**, the ten subtypes detected were observed to differ significantly in the sequence.

Table 2. Serotype of detected Rhnovirus

№	Serotype	Case(n)
1	***Human Rhinovirus* 64**	**25**
2	***Human Rhinovirus* 94**	**10**
3	***Human Rhinovirus* 82**	**7**
4	***Human Rhinovirus* 22**	**3**
5	***Human Rhinovirus* 50**	**2**
6	***Human Rhinovirus* 19**	**1**
7	***Human Rhinovirus* 31**	**1**
8	***Human Rhinovirus* 38**	**1**
9	***Human Rhinovirus* 54**	**1**
10	***Human Rhinovirus* 57**	**1**

To observe sequence homology of the ten subtypes detected, the aligned results are as follows.

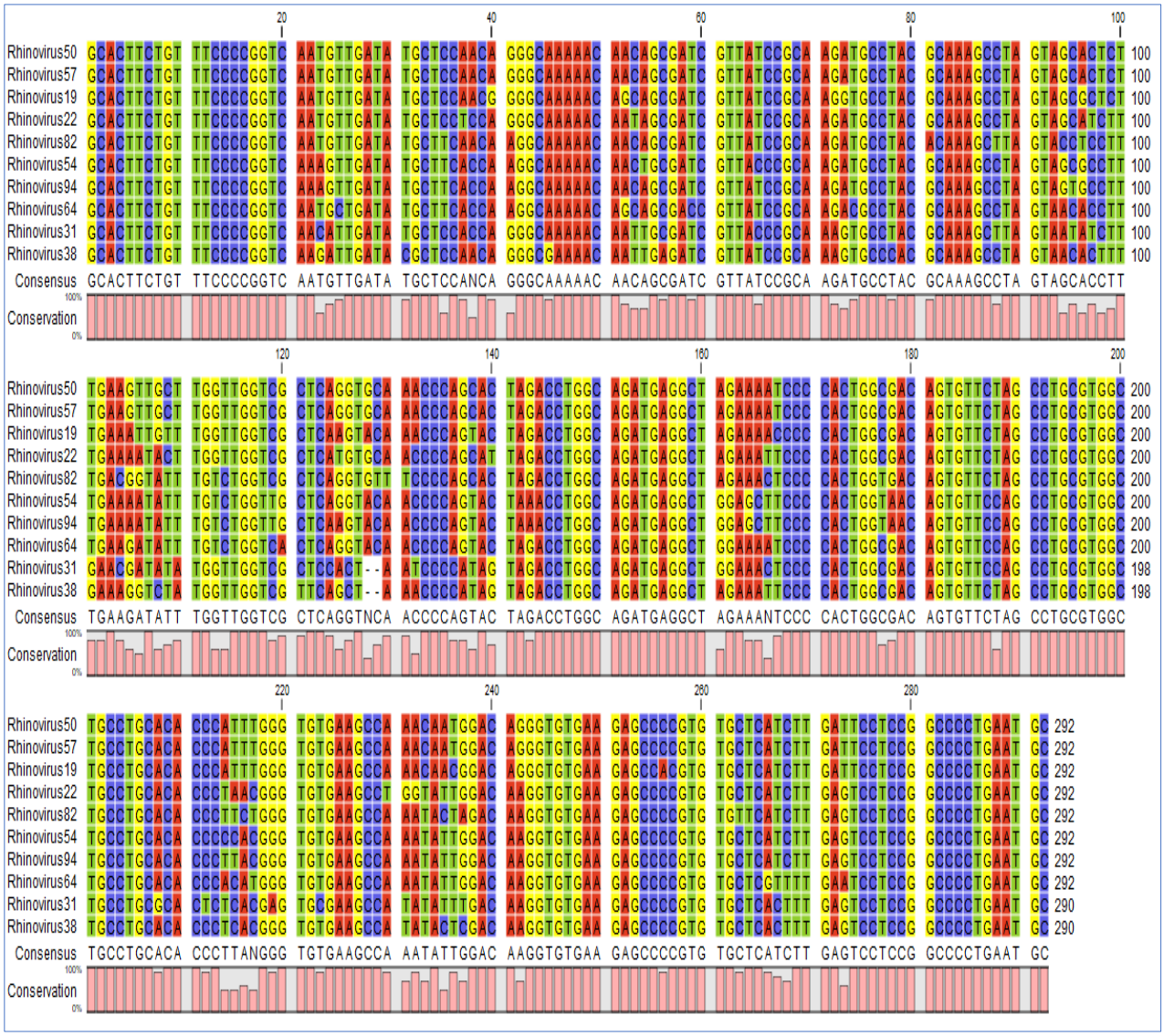


Figure 4. Homology analysis image of detected sequences of rhinoviruses.

4. DISCUSSION

Rhinovirus is a main pathogen of the common cold, but it was of little account for mild symptoms. However, in recent years, it has been shown that acute otitis media in children and severe diseases such as sinusitis in adults are also associated with this disease, and it has become an important virus in the detection of respiratory pathogens.

We designed primers capable of detecting rhinovirus to perform RT-PCR and performed sequencing of the amplicons to verify their specificity and to study phylogenetic relationships. We collected 264 specimens from the patients with respiratory infections and performed RT-PCR, and as a result 52 were positive.

We purified each amplicon by fractionation and performed sequencing using them as template by forward and reverse primers used for amplification, aligned and assembled to obtain the complete amplicon sequence, and the obtained sequence was queried to determine subtypes by BLAST search on the genomic database. 52 positive specimens detected had 10 rhinovirus subtypes, with the most serotypes being 64, 94, and 82. We aligned to observe sequence homology of the detected subtype sequences and a large number of base

differences were observed in other regions except the primer design region.

CONCLOSION

This study suggests that different rhinovirus subtypes infect human at a certain time and it is necessary to improve the specificity of the detection reagent continuously and to continue to increase the number of sequencing to give variety to database and to modify the sequence and species of the detection primers in order to enhance control of this virus.

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