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Molecular epidemiology of rhinoviruses in Cyprus over three consecutive seasons

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SUMMARY

Human rhinoviruses (HRVs) are widespread respiratory pathogens and a major cause of acute respiratory tract infections. The aim of this study was to investigate the molecular epidemiology of rhinovirus infections in children in Cyprus over three consecutive winter seasons. From a total of 116 rhinovirus-positive samples, 68 were sequenced in the 5'-UTR and VP4/VP2 regions. Thirty-six (52·9%) samples were identified as HRV-A and 27 (39·7%) as HRV-C, with only five (7·4%) samples belonging to the HRV-B species. Of these, a total of 46 different genotypes were identified. In the VP2/VP4 phylogenetic tree all strains clustered in three different well-defined clades, whereas the 5'-UTR tree exhibited clades with a mixed clustering of HRV-A and HRV-C strains reflecting the evolutionary history of recombination between HRV-A and HRV-C that has been observed previously. In summary, a high intra- and inter-season diversity of HRV types was observed. Despite its geographical isolation the frequency of HRV species in Cyprus is comparable to that reported in other regions of the world supporting the concept of an unrestricted global circulation. This study assesses, for the first time, the epidemiology of rhinovirus infections in Cypriot children and will be helpful to clinicians and researchers interested in the treatment and control of viral respiratory tract infections.

Key words: Cyprus, epidemiology, rhinoviruses.

INTRODUCTION

Human rhinoviruses (HRVs) are the most common cause of the upper respiratory tract infections (URTIs) worldwide. Their nomenclature reflects the primary site of infection in the respiratory tract, the nose [1].

Rhinoviruses are members of the family Picornaviridae within the genus *Enterovirus* and are non-enveloped viruses a with single-stranded, positive-sense RNA genome of ~7·2 kb in length [2, 3].

Rhinoviruses were originally classified into two distinct species, designated as HRV-A and HRV-B [3–6]; however, the development of new molecular methods led to the discovery of a novel species, designated HRV-C (http://www.ictvonline.org/virusTaxonomy.asp), which could not be cultured *in vitro* and was therefore undetectable with conventional methods [7, 8]. Currently, more than 100 HRV strains have been identified to circulate worldwide: 80 strains of HRV-A, 32 HRV-B and 54 HRV-C (http://www.picornaviridae.com). Although HRV-C has only

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recently been identified, this species is highly prevalent [9–11]. All rhinovirus species share common characteristics but form distinct phylogenetic clades [8, 12] with a high genetic and antigenic heterogeneity within each species explaining thus the large number of serotypes [13]. Sequence divergence in the VP1 of HRV-C and other external capsid regions is greater than between HRV-A or HRV-B indicating thus, the existence of major antigenic differences [14]. According to sequence data, HRV-C has greater genetic heterogeneity than HRV-A and HRV-B [13].

HRVs are generally associated with mild URTIs [15], but have been increasingly implicated in the pathogenesis of lower respiratory tract infections (LRTIs) and asthma exacerbations [16, 17]. Previous reports have suggested that rhinovirus isolates have been recovered from the lower respiratory tract of patients suffering from virus-induced respiratory illnesses [18]. It has been reported that HRV is the second most frequent aetiological agent associated with recurrent wheezing in hospitalized children aged <2 years [19].

Further reports suggest that HRVs may be associated or may induce exacerbation of wheezing and/or asthma [9, 16], and are responsible for more severe manifestations such as, bronchiolitis in young children and the immunosuppressed, pneumonia and chronic obstructive disease [3, 20]. HRV-C has been shown to be associated with severe asthma attacks more frequently than other HRV species [21, 22].

HRVs are transmitted through the respiratory–salivary route by person-to-person contact and airborne transmission, even though lately the possibility of a faecal–oral route has been proposed [23]. Infections are prevalent throughout the year with peaks in spring and autumn [24]. In countries with temperate climate, two peaks of HRV infection rates are usually observed between April–May and September–October [3].

Rhinoviruses are the most prevalent respiratory viruses in children [25], with HRV-C infections occurring year round and targeting mainly infants aged <2 years [26, 27].

The aim of the study was to investigate the molecular epidemiology of rhinovirus infections in children in Cyprus over three consecutive winter seasons.

MATERIALS AND METHODS

Clinical samples

During the 3-year period November 2010 until October 2013, 485 clinical samples were collected from paediatric patients (age range 17 days to 12 years, median age 15 months, male/female ratio 1·35) presenting with acute respiratory tract infection at the Archbishop Makarios III Children's Hospital, Nicosia, and subsequently sent to the Molecular Virology Department of the Cyprus Institute of Neurology and Genetics. All samples were routinely analysed for a panel of respiratory viruses including: enterovirus, respiratory syncytial virus, influenza viruses A and B, parainfluenzaviruses 1, 2, 3, and 4, adenovirus, coronaviruses E229, NL-63 and OC43, human metapneumovirus, human bocavirus and rhinovirus, using four in-house TaqMan probe-based multiplex real-time reverse transcription–polymerase chain reaction (RT–PCR) assays. A total of 116 samples out of the 485 analysed were found to contain a rhinovirus.

Viral RNA extraction and RT–PCR amplification

Viral RNA was extracted from 400 µl of clinical specimen by the iPrep™ PureLink® Virus kit (Invitrogen, USA) according to manufacturer's instructions. For reverse transcription, 10 µl of the extracted viral RNA was used as a template for cDNA synthesis using random hexamer primers and Superscript III (Invitrogen) according to the manufacturer's instructions. The cDNA was then used in two different PCR amplifications targeting two HRV genomic regions. The primers used amplified parts the 5'-non-coding (NC) region and the VP4/VP2 gene region, and were based on published protocols [12, 28]. Out of the 116 samples that were found positive for rhinovirus a total of 68 HRV-positive samples yielded an amplicon in both regions suitable for further typing.

Sequencing

The PCR products were analysed by gel electrophoresis on a 1·2% agarose gel and the amplicons then cut and purified using the Montage DNA Gel Extraction kit (Millipore, USA). DNA was subjected to Sanger sequencing employing the Dye Terminator Cycle Sequencing with Quick Start kit (Beckman Coulter, USA) on a CEQ 8000 sequencer (Beckman Coulter) using the respective forward and reverse primers. All obtained sequences were deposited at GenBank (accession numbers KP068521–KP068654).

Phylogenetic analysis

The forward and reverse sequences were aligned with CLUSTAL W (http://www.clustal.org/clustal2/) and then manually edited using BioEdit Sequence

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Alignment Editor v. 7.1.7 (bioedit.software.informer.com/7·1/). A GenBank search was then performed for each of the sample consensus sequences. Two datasets containing the 68 sequence alignments were generated for the 5'-UTR and the VP4/VP2 regions, respectively. The phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 [29]. For the phylogenetic analysis of the VP4/VP2 region, a 481 nt segment internal to the sequenced region was used in the final multiple alignment dataset, while for 5'-UTR a 386 nt segment was used. The best-fitting model of nucleotide substitution for each region was selected based on the lowest Bayesian Information Criterion (BIC) scores. All positions with less than 95% site coverage were eliminated. The phylogenies for the two examined regions were then estimated with the maximum likelihood method using the previously selected models, the Kimura two-parameter model and the General Time Reversible model with discrete Gamma distribution for the 5'-UTR and VP4/VP2 regions, respectively. Statistical robustness and reliability of the branching order within each phylogenetic tree were estimated by a bootstrap analysis using 500 replicates. Genetic distances between sequences were computed using the maximum composite likelihood method. Gaps or missing data were treated using pairwise deletion.

RESULTS

HRV types

In total, 116 rhinovirus-positive samples were identified between November 2010 and October 2013. Extracted RNA of these samples was subjected to two different RT–PCR amplifications for genotyping. Out of the 116 HRV-positive samples, 68 (50·0%) had a sufficiently high viral load to produce amplicons and sequences of sufficient quality for analysis and phylogenetic comparison. The remaining 68 (50·0%) HRV-positive samples either did not produce a specific amplicon due to the low viral amount and/or the sequence was of low quality, probably due to a co-infection with either another rhinovirus or an enterovirus.

Based on the results of the GenBank comparison of the VP4/VP2 region, 36 (52·9%) samples were typed as HRV-A, 27 (39·7%) were identified as belonging to the HRV-C species, while only five (7·4%) samples belonged to HRV-B. NCBI Blast comparison with reference strains revealed a total of 46 different genotypes: 24 different HRV-A, 4 HRV-B and 18 HRV-C

Table 1. *Rhinovirus types identified during three epidemic seasons in Cyprus. Each season ranges from November to October of the following year. Both sequences marked with an asterisk (*) had a divergence of 13% from the closest reference strain, which corresponds exactly to the divergence threshold of 13% for HRV-C rhinoviruses [1]*

HRV type	2010/2011	2011/2012	2012/2013	Total
A01		1		1
A09	1	1		2
A10		1		1
A12	1	1	1	3
A18	1			1
A20			2	2
A21		1	1	2
A22		2		2
A28			1	1
A32	1			1
A33			1	1
A40			1	1
A49			2	2
A55			1	1
A56		3		3
A58			1	1
A59	1	2		3
A60	1			1
A71		1		1
A78			1	1
A80			1	1
A82			1	1
A101			2	2
A102	1			1
B06			1	1
B27		1		1
B42			2	2
B83			1	1
C01*	1			1
C02		1		1
C05		1		1
C06		4		4
C11			1	1
C13		2		2
C15		1		1
C22		1		1
C23			1	1
C27			3	3
C28		2	1	3
C32			1	1
C36*			1	1
C41	1			1
C43			2	2
C45			1	1
C47			1	1
C50		1		1
Total	9	27	32	68

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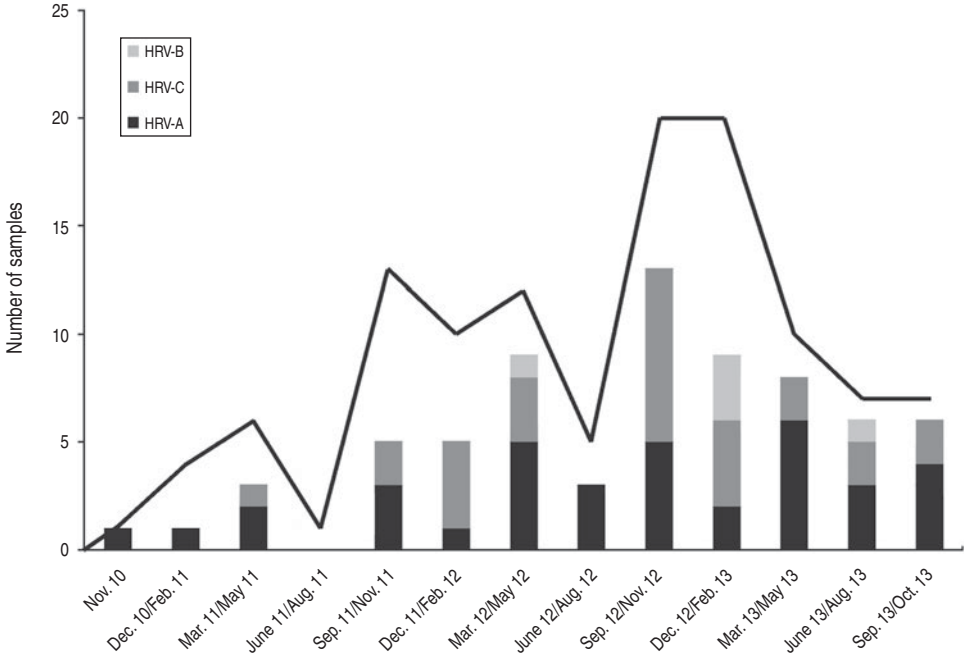

Fig. 1. Three-monthly distribution of HRV species over a 3-year period in children admitted with acute respiratory tract infection to Archbishop Makarios III Children's Hospital, Nicosia, Cyprus. The curve above the bars indicates the total number of HRV-positive samples.

(Table 1). Table 1 illustrates the high intra- and inter-season variability of rhinovirus circulation observed.

Only 3/18 genotypes identified in 2011/2012 were also observed in 2010/2011 and again only 3/25 genotypes circulating in 2012/2013 had been circulating in the previous season.

Considering each season separately, it can be seen that in 2010/11 the nine HRV-positive samples were representative of nine different genotypes, the 27 samples in 2011/2012 belonged to 18 genotypes, and in 2012/2013, 25 genotypes were identified in the 32 positive samples.

Seasonal distribution of HRV infections

The prevalence of rhinovirus infections during a 3-year period in Cyprus is shown in Figure 1. Peaks in incidence of rhinovirus were observed in the spring months of 2011 and 2012 and again in autumn 2012, with a generally increased detection between the end of 2012 and February 2013 (see Fig. 1). If the temporal distribution of each HRV species is examined individually, a higher incidence of HRV-C during the months of autumn and winter is observed with a decline in spring and/or summer. Rhinovirus A species was detected mainly during the autumn and spring months and at lower rates during winter and summer. The small number of HRV-B-positive samples unfortunately does not allow for an assessment of possible seasonality.

Mixed infections with other viruses were observed frequently in 46% of all rhinovirus-positive samples. The most common viruses that were detected along with rhinovirus were respiratory syncytial virus, which was found in 16/116 HRV-positive samples and enteroviruses in 12 cases. With regard to HRV species, one-third (12/36) of the HRV-A positive samples were co-infected with an additional respiratory virus, while 4/5 HRV-B-positive samples were also co-infected. On the other hand, only 7/27 HRV-C samples revealed a co-infection.

Relationship to age and gender

Analysing HRV species distribution with regard to age and gender, it was seen that all three HRV types were proportionally dispersed within all age groups and between genders (data not shown).

Phylogenetic analysis

In order to confirm the classification of HRV types of the 68 sequences, phylogenetic analysis of the 5'-UTR and VP4/VP2 region sequences was performed. The resultant phylogenetic trees of the 5'-UTR and

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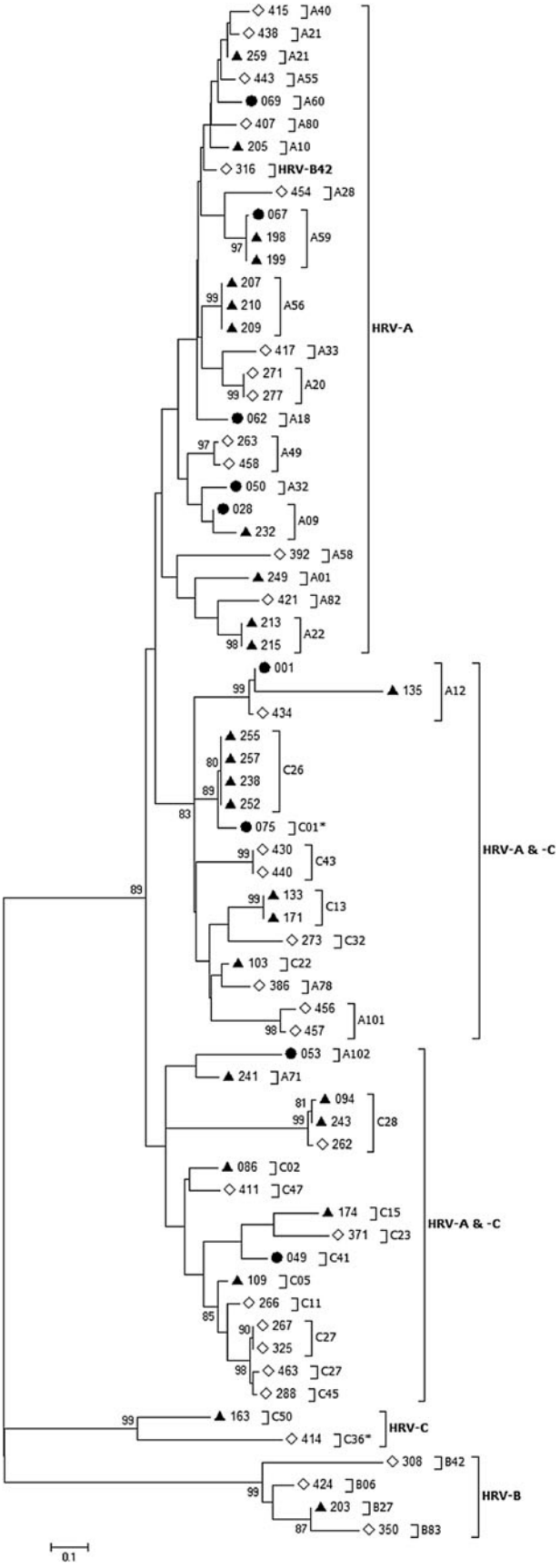

VP4/VP2 regions are shown in Figures 2 and 3. In the VP4/VP2 phylogenetic tree the three HRV types are clearly clustered into three distinct clades confirming the suitability of this region for typing of rhinoviruses, while in the 5'-UTR tree HRV strains are partially intermixed. In particular, the 5'-UTR tree formed a clade that includes intermixed HRV-A and HRV-C sequences, whereas the majority of HRV-A sequences formed a distinct clade. The HRV-B sequences form a phylogenetically separate group distinct from HRV-A and HRV-C, with the exception of a single sequence that clusters in the HRV-A clade. While this could be an indication for a historic recombination event it cannot be excluded that it may be a case of co-infection.

Additionally the nucleotide variability between the 68 obtained rhinovirus sequences was analysed separately for the 5'-UTR and VP4/VP2 regions. The average genetic distance was 0·204 (~20% genetic variation) in the 5'-UTR region and 0·379 (~38% genetic variation) in the VP4/VP2 region, suggesting considerable strain diversity in the population. When assessing the genetic variation within each rhinovirus species in the VP4/VP2 region, HRV-B sequences exhibit the lowest genetic diversity (17·3% genetic variation), whereas HRV-C sequences show the highest genetic variability (32·6% genetic variation) with HRV-A falling in between (23·9% genetic variation).

The high degree of variability between the HRV genotypes can also be observed in the assessment of genetic distance between the three HRV types, A, B and C. The mean genetic distance between HRV-B and HRV-C sequences (50·3% genetic variation) was slightly higher compared to the distance between HRV-A and HRV-C (46·7% genetic variation). The lowest genetic distance was observed between HRV-A and HRV-B sequences (42·0% genetic variation).

Fig. 2. The 5'-UTR phylogenetic tree was inferred using the maximum likelihood method based on the Kimura two-parameter model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Only bootstrap values >80% are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 nucleotide sequences. The HRV type and species are indicated with brackets. The three consecutive seasons are indicated in the tree as follows: ●, 2010/2011, ▲, 2011/2012, and ◊, 2012/2013.

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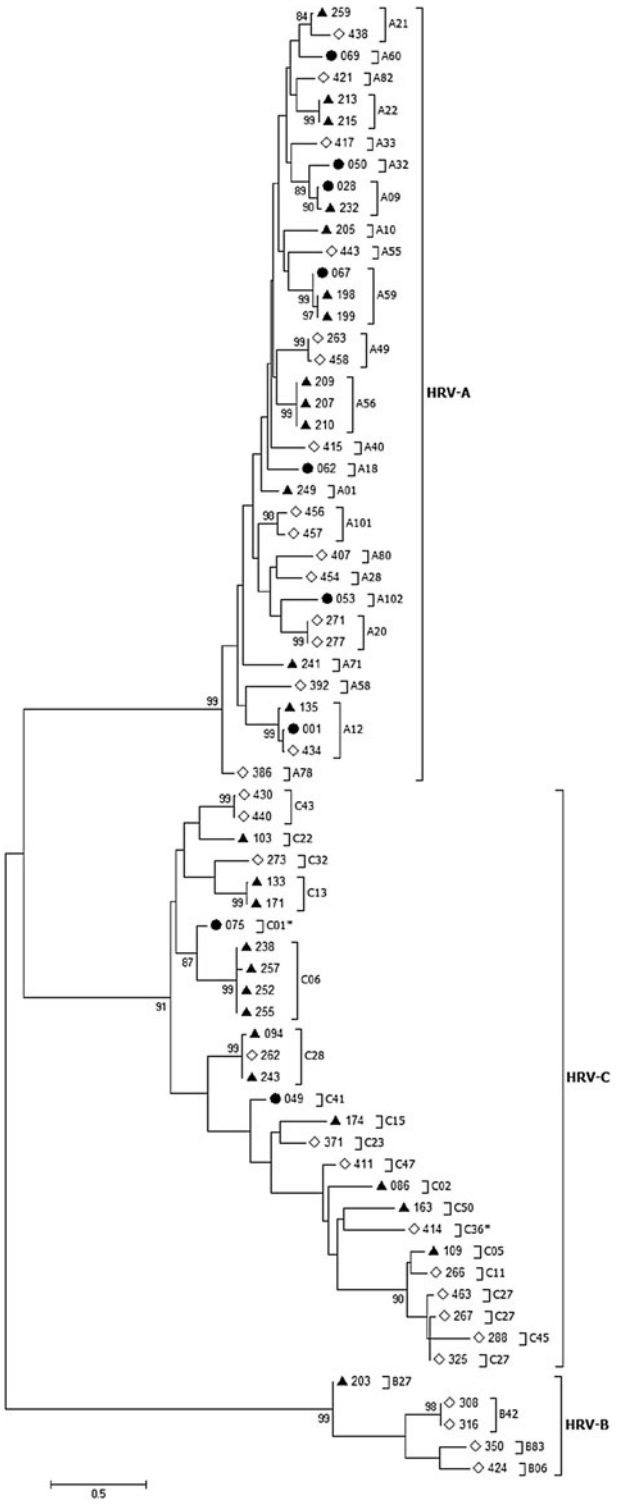

Fig. 3. The VP4/VP2 phylogenetic tree was inferred using the maximum likelihood method based on the General Time Reversible model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Only bootstrap values >80% are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 nucleotide sequences. The HRV type and species are indicated with brackets. The three consecutive seasons are indicated in the tree as follows: ●, 2010/11, ▲, 2011/12, and ◊, 2012/2013.

DISCUSSION

HRVs were previously believed to be associated mainly with mild respiratory symptoms, but have been linked more recently with severe neonatal infections [30, 31] and in addition have been shown to play an important role in the development of bronchiolitis and pneumonia [32, 33].

The aim of the present study was to assess, for the first time, the epidemiology of rhinoviruses causing acute respiratory tract infections in Cypriot children over three epidemic seasons.

Concerning seasonality there was no clearly pronounced pattern; however, some differences were observed with regard to the HRV species. HRV-C incidence was increased during the months of autumn and winter with a decline during the warmer months with rhinovirus A species being detected mainly during the autumn and spring months and at lower rates during winter.

The frequency of HRV species observed in Cyprus (52·9% HRV-A, 7·4% HRV-B, 39·7% HRV-C) is highly similar to that reported in previous studies from around the world with HRV-A being the most prevalent species, closely followed by HRV-C and HRV-B trailing behind [10, 11, 23, 34]. McIntyre *et al.* [1] compared the distribution of HRV types in Europe, USA, Asia, the Middle East, Australia and Africa and found similar detection frequencies in all geographical regions suggesting that most or all HRV types circulate freely worldwide without endemic restrictions.

The spectrum of rhinovirus types that circulated each season was characterized by a high genetic diversity with almost every sample presenting a different genotype. Very rarely were clusters of the same genotype observed. Considering the inter-season variability it can be seen that only 3/18 genotypes identified in 2011/2012 were also observed 2010/2011 and just 3/25 genotypes circulating in 2012/2013 had also circulated in the previous season. Given the geographical isolation of Cyprus and the small population size (~900 000 inhabitants) a less diverse seasonal spectrum of circulating serotypes could have been expected; however, the large number of tourists visiting Cyprus each year may be a significant factor regarding rhinovirus circulation.

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Given the large number of simultaneously circulating rhinovirus types it can be anticipated that longer study periods with significantly larger cohorts will be required to elucidate the epidemiology of individual serotypes.

In our study, phylogenetic analysis was based on sequencing of the VP4/VP2 and 5'-UTR regions. The VP4/VP2 region has been most commonly used for genetic characterization of HRVs, because of its greater sequence conservation and the availability of universal primers amplifying all members of the three rhinovirus species. Previous studies have shown a high phylogenetic congruence between sequencing in the VP1 and the VP4/VP2 regions suggesting the suitability of VP4/VP2 sequencing to predict HRV species and type [1]. Accordingly, the Cypriot strains cluster in three different well-defined clades in the VP2/VP4 phylogenetic tree, whereas the 5'-UTR tree exhibits clades with mixed clustering of HRV-A and HRV-C strains. The mixed clustering in the 5-'UTR region reflects the evolutionary history of recombination between HRV-A and HRV-C that has been observed previously by others [35, 36].

A high mutation rate and intra-species recombination are the driving evolutionary forces in rhinovirus evolution. A recent analysis of complete genomes of rhinoviruses using a model-based population genetics approach identified seven distinct lineages, namely a subdivision of HRV-A into four genetically distinct lineages, a HRV-B lineage and two distinct HRV-C lineages with a high degree of intra-species recombination found within the HRV-A lineages [37]. The emergence of one of the HRV-C lineages could be attributed to interspecies recombination between HRV-A and HRV-C mainly in the 5'-UTR region.

In summary, a high intra- and inter-season diversity of HRV types was observed. The frequency of HRV species in Cyprus is comparable to that reported in other regions of the world supporting the concept of global over local circulation. The study assessed, for the first time, the epidemiology of rhinovirus infections in Cypriot children and will be helpful to clinicians and researchers interested in the treatment and control of viral respiratory tract infections.

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DECLARATION OF INTEREST

None.

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