Journal of Clinical Virology 52 (2011) 317–320

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Highly frequent infections with human rhinovirus in healthy young children: A longitudinal cohort study

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ARTICLE INFO

Article history:
Received 16 June 2011
Received in revised form 14 July 2011
Accepted 6 September 2011

Keywords:
Rhinovirus
Genetic diversity
Respiratory virus infections
Children

ABSTRACT

Background: Human rhinoviruses (HRVs) are an important cause of respiratory tract infections.
Objectives: We questioned whether the high prevalence rates of HRVs found in epidemiological studies is due to long-term individual continuity or a result of frequent infections with different HRV subtypes.
Study design: In a 6-month winter period 18 healthy controls, aged 0–7 years, were at least sampled every two weeks for HRV-PCR, irrespective of respiratory symptoms. All HRV positive samples were genotyped to determine HRV diversity.
Results: In total 272 samples were collected. HRV was found in 101/272 (37%) samples. Genotyping revealed 27 different HRV subtypes. A median of 3.0 different HRV subtypes was found per child. Re-infections and continuity with identical HRV sequences were observed. The number of HRVs were higher in the youngest age group ($p = 0.01$) and they had more different HRV subtypes ($p = 0.05$) compared to oldest age group.
Conclusions: We found a high HRV exposition with a considerable diverse population of HRV subtypes in young children. These results have major implications for future research into the pathogenic role of HRV in respiratory diseases. Characterisation of subtypes will be necessary to discriminate between prolonged continuity and re-infections in patients with respiratory diseases.

1. Background

During the last decade human rhinoviruses (HRVs) have raised increasing interest as they seem to be responsible for a wide range of respiratory illnesses. HRVs are frequently found in asymptomatic children and adults,[1,2] but are also detected in patients with symptoms ranging from mild common colds[3] to serious lower respiratory tract disease.[4,5] Since the development of molecular assays for the detection of HRVs, the detection rate of HRV in patients with respiratory infections has increased to up to 50%.[6,7]

Besides an increasing awareness from epidemiological studies regarding the high prevalence of HRV there is growing evidence for the importance of different HRV subtypes. HRV is a member of the *Picornaviridae* family and more than 100 genetically and serologically different HRV subtypes have been described.[8] HRV subtypes can be classified according to several parameters, including receptor specificity, antiviral susceptibility and nucleotide sequence homologies.[9] Taxonomically HRV subtypes can be distinguished accurately by sequencing the 5′NCR[10] and divided in clades, like HRV A, HRV B and, HRV C. It has been suggested that some HRV subtypes might be associated with more severe or different respiratory disease patterns than others.[11–13]

Despite this increasing knowledge from epidemiologic and basic studies, longitudinal data on the diversity of HRVs in individuals are lacking.

2. Objectives

We questioned whether the high prevalence rates of HRVs found in epidemiological studies is due to long-term individual continuity with the same subtype[14] or whether it is a result of highly frequent subsequent infections with different HRVs subtypes. Therefore, we performed a prospective longitudinal cohort studying young children to closely monitor the occurrence of HRV subtypes over time.

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1386-6532
doi:10.1016/j.jcv.2011.09.003

3. Study design

3.1. Study population

We conducted a prospective longitudinal cohort study during a 6-month period (from November 2004 through April 2005) in 19 healthy children aged 0–7 years. One of them (male, almost 3 years old) failed to complete the study after the ninth week and was excluded from the analysis. None of the children had a history of asthma or recurrent respiratory complaints.

At the beginning of the study, parents were instructed to take samples for virus detection by rubbing one of the nostrils and posterior oropharynx of their child using separate cotton-tipped swabs. The two swabs were collected into a single vial containing GLY medium containing 0.1 mg/ml pimaricine as viral transport medium and sent to our laboratory via regular mail.[15] Samples were stored at −20 °C until analysis. Samples were taken every two weeks regardless of any respiratory symptoms and additional samples were taken when respiratory symptoms were present for more than two days. Sampling of respiratory pathogens by the parents using nose and throat swabs has been shown to be feasible and reliable. Both the sampling frequency and the viral recovery rate in parental samples are higher compared to sampling by a dedicated research nurse.[16]

The study was approved by the local Medical Ethics Committee (University Medical Center, Utrecht) and all parents gave written informed consent.

3.2. PCR, sequencing and phylogenetic analysis

Viral RNA was isolated from 200 μl of the original sample using the High pure RNA isolation kit (Roche, Germany). cDNA synthesis, nested PCR and Southern blotting were carried out to detect HRV.[17] In case of a positive PCR for HRV, the amplicon was extracted from gel and purified with Qiaquick gel extraction kit (Qiagen® Germany). These amplicons from the inner primer set (approximately 310 nucleotides of the 5′NCR region) were sequenced using capillary DNA sequencer (ABI model 3700). When sequencing failed initially, nucleic acid isolation, PCR and sequencing were repeated once on the original sample. Sequence data were blasted against Genbank and analyzed with BioNumerics 4.6 (Applied Matths, Gent, Belgium) with a maximum parsimony algorithm performing 100 bootstraps. Subtypes were defined as different when sequence homologies were <90%.

3.3. Statistical analysis

Statistical analysis was performed using SPSS Inc., 2001, Chicago, USA, version 12.0. Comparisons of the distributions of categorical variables between groups were examined using a two-tailed Chi-square and the medians of continuous variables using the nonparametric Mann Whitney *U* test. A significance level of $p \leq 0.05$ was used throughout.

4. Results

Eighteen children were longitudinally followed during a 6-month study period. There were 3 male and 15 female children. The median age of the children was 3.6 years. A median of 15.5 samples was taken (interquartile range IQR 13.8–17.0) and a median of 3.0 (2.0–4.3) different HRV types was found.

In total 272 samples, regardless of symptoms, were collected and tested for the presence of HRV. We observed a high prevalence of HRV in our population, HRV was found in 101/272 (37%) samples. All children had at least one HRV positive sample during the study period with a maximum of thirteen (median number of HRV positive samples 5.0).

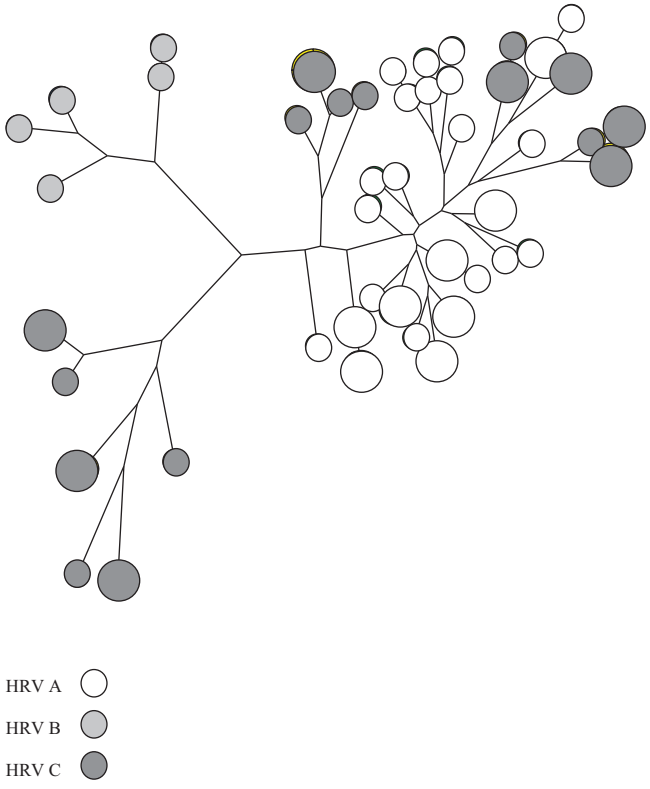

Fig. 1. Phylogenetic analysis of HRV positive samples. Circles refer to HRV clades described in literature (HRV A, white; B, light grey and C, dark grey). Sequence data were analyzed with maximum parsimony algorithm performing 100 bootstraps.

To investigate whether this high frequency of HRV positive samples in our study is due to frequent infections with different HRV subtypes or continuity of the same subtype, we performed sequencing on HRV positive samples. Sequencing was successful in 71/101 (70%) of the HRV positive samples. In total 27 different HRV subtypes were found. A median of 3.0 different HRV subtypes was found per child (range 1–6). A maximum of 6 different HRV subtypes was found in 2 children.

To study the distribution and diversity of HRV subtypes a dendrogram of the HRV subtypes was constructed (Fig. 1). The majority of the HRV sequences we found can be grouped into the HRV A strain (39/71; 55%). A smaller proportion of the HRV subtypes belonged to the HRV B strain (5/71; 7%) and the HRV C strain (27/71; 38%).

To visualize the dynamics of HRV infections in children during the observation period, we constructed individual timelines of the HRV positive samples in Fig. 2. During the observation period we observed re-infections with the same HRV subtype in two children (children 1 and 3). These children showed re-infection with an identical HRV subtype with periods of other HRV subtypes in between. In most instances HRV positivity concerned a new infection with a new HRV subtype. Continuity of identical HRV subtypes for 14 days or more was observed in 3 children (1, 9 and 10).

Finally, we analyzed the influence of age on the occurrence of HRV and the diversity of HRV subtypes. In the youngest age group (children <5 years, 1–10; Fig. 2) 44% of the samples taken were HRV positive, compared to 28% of the samples in the oldest age group (children ≥5 years, 11–18) (Chi-square test, $p = 0.01$). The number of different HRV subtypes found was higher in the youngest age group with a median of 4.0 (range 1–6) compared to the oldest group with a median of 2.5 (range 1–5) (Mann Whitney *U* test $p = 0.05$). All

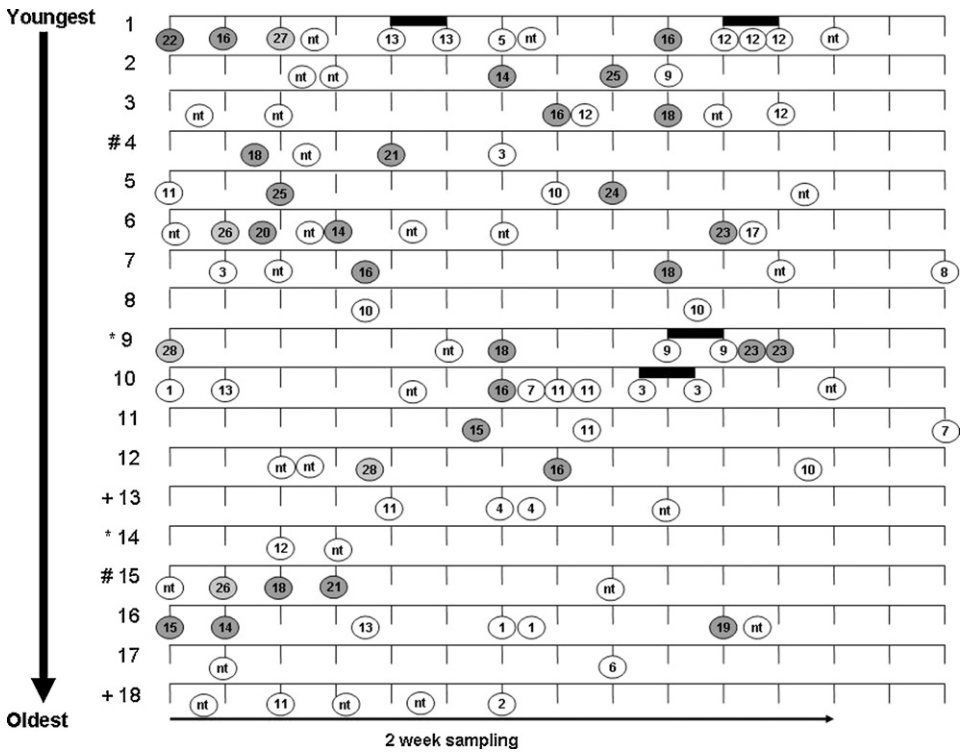

Fig. 2. Timelines of detected HRV subtypes for all children during the study period. Each line represents a child in order of increasing age; the time between two vertical lines accounts for approximately 2 weeks. The numbers are the 32 different HRV subtypes found. The black bars refer to a period of continuity (≥2 weeks) with the same HRV subtype. The white circles are HRV A, the light grey circles are HRV B and the dark grey circles are HRV C subtypes. nt, non-typable. The symbols #, * or + refer to children being siblings.

children with a re-infection and/or continuity with an identical HRV subtype belonged to the youngest age group (children <5 years). We could not find any relationship between specific HRV subtypes and age.

5. Discussion

This longitudinal study shows that HRV is highly prevalent in young children due to a high infection rate with a huge diversity of HRV subtypes. We also observed re-infections and continuity of identical HRV subtypes during this 6-month observation period. Younger age is associated with a higher infection rate with HRV.

This is the first study using longitudinal analysis and phylogenetic subtypes to describe the dynamics of HRV infections over time in healthy children. Recently, Olenec et al.[18] studied the effect of rhinovirus infections on asthmatic children. They also showed a high frequency of HRV infection with different strains. The high sampling frequency and sequencing of HRV subtypes of our study gives a detailed picture of HRV acquisition of young children and makes it possible to distinguish prolonged continuity with the same subtype from repeated infections with different HRV subtypes. In this study we sampled from November through April, the "respiratory season" but still this could limit the generalizability of our results, especially because HRV infections occur throughout the year with a peak incidence in August. Besides not all HRV-PCR positive samples were successfully sequenced, possibly due to low RNA concentrations in samples that were nevertheless positively identified by Southern blotting. Successful sequencing in 70% seems acceptable to draw conclusions and is even higher compared to results of other studies.[19]

In this study, HRV was found in 37% of all samples. This high detection rate is in line with a similar designed longitudinal study[20] where picornavirus was detected in 26% of the samples from periods of both illness and wellness in children. In addition, in cross-sectional studies HRVs are also frequently found in up to 50% of the children during respiratory tract illnesses.[21–23] The systematic surveillance for HRVs in this study revealed that the occurrence of HRV is even higher than thought before with an average of almost 4 HRVs in a six-month period.

Our data show that the prevalence of HRV is high due to a high infection rate with a huge diversity of HRV subtypes. A recent study[24] showed that HRVs are frequently transmitted from children to other family members and multiple HRV types circulated simultaneously within these families. In this study children had a median of 3 different HRV subtypes with a maximum of 6 during a 6-month period. The majority of the HRV sequences we found belonged to HRV A species; this is in line with other studies.[25–27]

We investigated the longitudinal course of infections with HRV subtypes. In our study re-infection with the same HRV subtype was seen in 2 children with a period of other HRV subtypes in between. Apparently, re-infections with the same HRV subtype occur during a winter season. It would be interesting to observe whether different HRV subtypes can be found in successive seasons or if HRV subtypes disappear and/or reappear in following years.[19,28] Continuity of the same HRV subtype was seen in 3 different children. The maximum period for a prolonged infection with an identical HRV subtype was 14 days. Continuity of HRV was also described in a few other studies. Our data shed new light on these studies because sequence data have been lacking in all studies until now. The Finnish study[13] longitudinally followed children for the persistence of HRV after hospitalisation for wheezing illness. Follow up was done after 2, 5 and 8 weeks; HRVs could be detected until 2–5 weeks after onset of symptoms. In another study[19] samples were taken weekly to identify picornavirus infections in children. Here, detection of the picornavirus was episodic lasting for a period of 1–3 weeks. In the third study[26], HRV-RNA was detected in 40%

of the asthmatic children till 6 weeks after an acute exacerbation. Considering the high number of different HRV subtypes we found in our study, only genetic analysis can prove persistence of HRV subtypes instead of the discovery of different subtypes.

Finally, we studied the influence of age on the dynamics of HRV infections. The frequency of symptomatic viral respiratory tract infections is higher in young children compared to adults.[2,24,29] Therefore, we hypothesised that young children might be more often infected with HRV than older children. In this study we found that children <5 years of age acquire significantly more HRV infections compared to children from ≥5 years of age (50% versus 28%, respectively). Moreover, they have significantly more different HRV subtypes, although we could not find a relationship between age and specific HRV subtypes. Perhaps the higher infection rate in younger children is due to differences in immune reaction between younger and older children. Several studies have shown that there are differences in cytokine profiles, T cell proliferation and Natural Killer Cell (NKC) activity between young children and adults.[30–33] This is supported by the fact that both re-infection and prolonged infection of HRV subtypes were only seen in the youngest children of the study population. Further studies are needed to unravel the association between age and the diversity of HRVs.

In conclusion, we found that there is a high HRV exposition with a considerable diverse population of HRV subtypes in young children. Future studies into the pathogenic role of HRV should differentiate for HRV subtypes to discriminate between continuity and re-infections of viruses.

Funding

None competing.

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgements

We thank all the parents and children who were willing to participate in our study. The authors thank T. Yimam and B. Zwan from the Laboratory for Infectious Diseases and Screening, National Institute of Public Health & Environment Bilthoven for their assistance in performing the PCR studies.

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