Catching the common cold
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Chapter 7

A molecular epidemiological perspective of rhinovirus types circulating in Amsterdam from 2007 to 2012

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ABSTRACT

Rhinoviruses (RVs) are frequently detected respiratory viruses that cause mild common cold symptoms, but may also lead to more severe respiratory tract infections. The large number of RV types, classified into species A, B and C, hampers clear insights in the epidemiology and clinical significance of each RV type. The aim of this study was to map the circulation of RV types in the Amsterdam area. RV-positive nasopharyngeal and oropharyngeal samples, collected from 2007 to 2012 in the Academic Medical Centre (Amsterdam, the Netherlands), were typed based on the sequence of the region coding for capsid proteins VP4 and VP2. RV-A, RV-B, and RV-C were found in a ratio of 52.4% (334/637), 11.3% (72/637), and 36.2% (231/637), respectively. We detected 129 of the 167 currently classified types. RVs circulated throughout the entire year with a peak in the autumn and a decline in the summer. Some RV types were observed throughout the entire sampling period and others more in a seasonal pattern. Nine RV-A and four RV-B novel provisionally assigned types were identified. This study provides an insight in the molecular epidemiology of RVs in the Amsterdam area. The RVs circulating are diverse and include several provisionally new types.
INTRODUCTION

Rhinoviruses (RVs) are causative agents of upper and lower respiratory tract infections (1). Symptoms range from a common cold to more serious infections such as bronchiolitis, and pneumonia. Also, RVs can cause acute exacerbations of asthma, chronic obstructive pulmonary disease, and cystic fibrosis and have been implicated in the pathogenesis of asthma (2).

RVs belong to the enterovirus (EV) genus of the picornavirus family, and currently 167 RV types have been classified into three species, named A, B, and C (3). Species RV-A and RV-B have been known since the 1950s (1), but species RV-C has only been discovered in 2006 upon the introduction of molecular techniques because these viruses cannot be cultured on standard immortalized cell lines (4–7).

Studies suggested that RV-C infections may elicit more severe disease compared with infections with RV-A or RV-B types (8). However, this has been refuted by several studies which did not observe a difference in outcome between RV-C and RV-A infections (8). Due to the large number of RV types, the severity of infections caused by individual RV types is even more elusive.

As a result of small sample sizes, short observation periods and the large number of RV types there is a lack of insight in the prevalence and circulation patterns of RV types. Compared to the other EVs, RVs co-circulate to a much larger extent, and seasonal patterns are less prominent (1,9,10). Increased insight into prevalence, circulation patterns and clinical significance is not only of importance for surveillance purposes, but also for the future development of antiviral therapy and vaccines. The aim of our study was therefore to investigate the prevalence of RV types in the hospital population of the Academic Medical Centre (Amsterdam, the Netherlands), by genotyping all RV-positive samples submitted for respiratory viral diagnostics from 2007 to 2012.

MATERIALS AND METHODS

Study design

This study was conducted at the Academic Medical Center (AMC) in Amsterdam, the Netherlands. From 2007 to 2012, a total of 6258 nasopharyngeal and oropharyngeal samples were submitted to the Laboratory of Clinical virology, department of Medical Microbiology of the Academic Medical Centre in Amsterdam for diagnostic evaluation. The Academic
Medical Centre receives mainly samples from the southeast area of Amsterdam. The samples were acquired from hospitalized patients and non-hospitalized patients visiting the outpatient clinics or emergence room. The reason for sampling was not systematically documented on the accompanying form for the laboratory and could therefore not be adequately monitored or analysed. Respiratory samples collected for research purposes and those that were not tested for RV were excluded from the analyses. A total of 1102 (17.6%) respiratory samples were positive for RV. There was no material available from five samples for additional gene sequencing. All available RV-positive samples were further characterised by sequencing the VP4/VP2 region. The sampling and virological testing were part of routine care and was executed according to hospital ethical guidelines and the Dutch code of conduct for responsible use of human tissue and medical research 2011.

**Virological assessments**

RNA was extracted from naso- and/or oropharyngeal samples with the MagnaPure LC instrument® using the total nucleic acid isolation kit (Roche Diagnostics). Samples were tested for the presence of RV, EV, human parechovirus (HPeV), influenza virus A and B (InfA, InfB), para-influenzavirus 1 to 4 (PIV1-4), human bocavirus (hBoV), human coronavirus (hCoV: HKU1, NL63, 229E and OC43), respiratory syncytial virus (RSV), adenovirus (AdV) and human metapneumovirus (hMPV), with a multiplex real-time PCR as described previously (11). Primers used for the detection of RV were reported in Jaramillo-Gutierrez, 2013 (12). Ct values of ≥ 40 were considered negative.

**RV genotyping**

RV-positive samples were genotyped based on a 540-base pair fragment of the VP4/VP2 region as described previously (13) using a two-step semi-nested protocol with primers in the 5′-untranslated region (UTR) and in VP2 (Table 1). The VP4/VP2 sequences were phylogenetically compared to published reference sequences as proposed and provided by McIntyre et al (14). Sequences were analyzed using CodonCode Aligner version 3.7.1, aligned using Clustal X version 3.0.11 and edited using Genedoc version 2.7 software (15,16). Phylogenetic trees were constructed using neighbor-joining trees under a p-distance model as implemented in MEGA version 5.10 (17). Trees were unrooted and bootstrap values were determined from 100 bootstrap resamplings of the original data.

Sequences for which the genetic distance, i.e., nucleotide divergence, was above the threshold of 10%, 9.5%, and 10% for RV-A, RV-B, and RV-C, respectively (14), were submitted to Genbank
(nr KP003842-KP003896 and KT272022-KT272030) and to the Picornavirus Studygroup to be designated a provisionally assigned type.

Table 1. Primers

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>HRV-VP4-1</td>
<td>GGG ACC AAC TAC TTT GGG TGT</td>
</tr>
<tr>
<td>Antisense</td>
<td>9565-reverse</td>
<td>GCA TCI GGY ARY TTC CAC CAN CC</td>
</tr>
<tr>
<td>Step 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>HRV-VP4-2-forward</td>
<td>GGG GAC CAA CTA CTT TGG GTG TCC GTG T</td>
</tr>
</tbody>
</table>

Definitions

If multiple samples were available from the same patient, infections were defined as a new infection when the sample was the first RV-positive sample from that patient, when the sample yielded a different type than the previous sample from the same patient, and/or when the interval period between the two samples was >3.0 months.

As a result of the close genetic relationship between RVs and non-RV EVs, diagnostic detection assays may be cross-reactive and result in a false-positive outcome. An RV false-positive sample was defined as a sample that was RV-positive by real-time PCR and resulted in a non-RV EV sequence after phylogenetic analysis. A sample was only classified as an EV co-infection if the RV infection was verified by RV typing and if either of two EV typing protocols (18) yielded a non-RV EV sequence. The EV-A/EV-B protocol was adapted to a semi-nested PCR and used the same forward outer primer for the first and the second PCR for both EV-A and EV-B.

Statistical analysis

For data analysis, all new infections were considered statistically independent. Data were analyzed with the IBM SPSS Statistics software (version 22.0, IBM Corporation) and GraphPad Prism (version 5.01, GraphPad Software). Categorical variables were compared by means of chi-square test. Differences between continuous variables were determined using Student’s t-test or ANOVA if normally distributed and non-parametric tests if not normally distributed. A two-sided p-value of <0.05 was considered statistically significant.
RESULTS

RV genotyping

We analyzed 1098 RV-positive naso- and oropharyngeal samples. VP4/VP2 sequences were obtained for 745 samples (67.9%), yielding 709 samples with an RV sequence (64.6%, 64/1098) and 36 samples with a non-RV EV sequence (3.3%, 36/1098) (see web-only Appendix, Table A1).

A total of 637 new RV infections were observed in 557 patients of whom 310 (55.7%) were male and 247 (44.3%) were female. Overall, the patients were young children, with a median age at time of infection of 1.6 years (interquartile range (IQR) 0.5-17.6).

Distribution of RV species

Typing of the available samples revealed that 334 of 637 infections (52.4%) typed were caused by RV species A, 72 (11.3%) by species RV-B, and 231 (36.2%) by RV-C. RV-B contains the smallest number of RV types (32 types, compared to 80 RV-A types and 55 RV-C types) and thus one would expect a lower prevalence of RV-B infections if all RV types were equally prevalent. After correction for the skewed proportions of the different species, RV-B types were less prevalent than RV-A and RV-C types (chi-square test, p<0.0001).

Patients infected with RV-B types were significantly older (median 10.4 years, IQR 0.7-35.3) than those infected with a RV-A types (median 1.8 years, IQR 0.5-26.5) or RV-C (median 1.2 years, IQR 0.5-4.3) (Kruskal-Wallis test, p=0.007) (Figure 1). An analysis including only the first infection of each patient resulted in a similar outcome (Kruskal-Wallis test, p=0.002). To investigate whether the low median age of our population could explain the underrepresentation of RV-B infections, we calculated the ratio of RV species in patients over 10 years of age. In this selected patient population the underrepresentation of RV-B in our study was retained (chi-square test, p=0.005), indicating that the lower proportion of RV-B infections was not (solely) due to the lower detection of RV-B in the young children.

The detection of co-infections

In 130 RV infections (20.4%, 130/637) there was a viral co-infection (Figure 2). In the majority of these viral co-infections, there was one co-infecting viral agent (n=104), but up to four co-infecting viruses were detected, of which hBoV (n=42) and AdV (n=33) were the most prevalent.

Co-infection rates were similar between the different infecting RV species (22.4% [74/334] RV-A, 20.8% [15/72] RV-B and 17.7% [41/231] RV-C infected patients, chi-square test, p=0.44).
Figure 1. Age distribution of patients infected with rhinovirus type A (RV-A), RV-B or RV-C. The median and interquartile range are depicted as well as p-values calculated with Dunn-Bonferroni post hoc test.

Figure 2. Observed rhinovirus (RV) co-infections. Patient samples were tested with a multiplex real-time PCR for other respiratory viruses. The viral co-infections detected in RV-positive samples are depicted. Enterovirus (EV) infections were only included if EV genotyping yielded a non-RV EV sequence.
Circulation of RV types

In our population we could detect the majority of the currently classified 167 RV types (77.2% [128/167] of all RV types, 83.8% [67/80] of all RV-A types, 62.5% [20/32] of all RV-B types, and 74.5% [41/55] of all RV-C types) (Figure 3), suggesting that most types circulate ubiquitously. In addition, we detected eight previously described provisionally assigned types (PATs). These are virus strains predicted to be new types based on the VP4/VP2 sequence, but for which the VP1 sequence is not yet available (14). VP1 is regarded as the gold standard for type identification because it shows more sequence variation than VP4/VP2. Furthermore, we detected 31 strains which were classified as 13 novel PATs (9 RV-A and 4 RV-B). Phylogenetic trees including these PATs are provided as supplementary data (see web-only Appendix, Figure S1).

Most types were detected at low frequency, but some types, such as RV-A12 (n=23), RV-A78 (n=24), and RV-C2 (n=27), were present at a higher rate.

Figure 3. Detection of rhinovirus (RV) types. The frequency of all currently known RV types and all detected provisionally assigned types are indicated.
**Time of circulating species and types**

RVs circulated throughout the year, with a slightly higher number of infections occurring in the autumn and a decline in the summer (Figure 4 and web-only Appendix, Figure S2). In the second half of 2009 there was an exceptional large increase in RV-positive samples compared with other years (see web-only Appendix, Figure S2).

Over the year, the species distribution fluctuated slightly (Figure 4). RV-C tended to be more dominant in the winter months, while the rest of the year RV-A infections were more prevalent. RV-B showed low-level circulation throughout the year with a moderate increase of infections in the second half of the year.

Specific RV types (e.g., RV-A29) were consistently detected throughout the study period, while other RV types were mainly detected in peak seasons (see web-only Appendix, Figure S3). For example, RV-A12 was detected in clusters in the winters of 2007/2008, 2009/2010, and 2011/2012. Furthermore, RV-A101 was only detected during a small outbreak in August/September 2008 (n=11) and a few times in September/October 2011 (n=4). Part of these RV-A101 infections may have been hospital-acquired as deduced from the reason for admission and the time between the admittance of the patient and the sampling, but other RV infections were clearly contracted outside of the hospital (sample taken within one day of admittance to the hospital for respiratory illness). RV-C2 and RV-A78, the most commonly detected RV types in our population, were detected every year and followed a similar pattern as RVs in general: a higher detection rate in the autumn/winter and less infections in the summer.

**Figure 4. Frequency of rhinovirus type A (RV-A), RV-B and RV-C infections per month.** The amount of infections with RV species A, B and C observed in each month, pooling the years 2007-2012. The year 2009 was excluded, as RV circulation in that year was atypical.
DISCUSSION

In our study we described the prevalence of RV types in Amsterdam by typing RV-positive samples submitted for diagnostics in 2007-2012 to the Academic Medical Center. In our population we found a large number of different RV types, of which most belonged to species RV-A, and the least belonged to species RV-B. Relatively many RV strains were classified as novel PATs.

Among the 637 typed RV infections, 52.4% (334/637) belonged to RV-A, 11.3% (72/637) belonged to RV-B, and 36.2% (231/637) of the typed RV samples belonged to RV-C. The detection rates in our study are in line with previous studies that reported ratios of 47-64%, 2-13%, and 21-45% for respectively RV-A, RV-B, and RV-C (19–23). RV-A and RV-C infections are consistently reported to be more frequent than RV-B infections.

We could detect most of the currently classified types even though our samples were obtained in a geographically small area. These results suggest that most RVs are widespread, which is in agreement with previous reports that also describe the simultaneous circulation of a high number of types (20,24,25). Nevertheless, some types have repeatedly been detected at higher frequencies than other types over the world (14,22,26,27). Most of these types were also present at intermediate-to-high frequency in our population, such as RV-A12, RV-A78, RV-A101, RV-B104, RV-C2, RV-C16, and RV-C43. A limitation of our study was that we used population sequencing, which in most cases only detects the major type present and misses most RV-RV co-infections. However, there were no indications for RV-RV co-infections, such as sequence ambiguities.

4.8 percent (36/745) of all sequences obtained from RV-positive samples were characterised as an EV sequence. The finding of EV sequences is reflective of the well-reported cross-reactivity of the RV detection PCR for non-RV EVs, due to their close relationship and the conservation of the 5′-UTR (12,28). Almost half of the EVs (15/36) were EV-D68, an EV which is associated with respiratory diseases. The same holds true for EV-C104, which was detected in five samples (from four infections). The other EVs detected are found only occasionally in respiratory samples (8,29).

Our study monitored circulation of RVs over a relatively long time period, which allowed us to examine the circulation of RV species and types over six years, thereby limiting the influence of single outbreaks. Our observation that RVs circulate during the whole year has repeatedly been found (1,8). Interestingly, Linder et al. also reported a dominance of RV-C in the winter months.
We found that some types circulate persistently, but for other types we saw a different circulation pattern where the type was detected mainly in the winter months and occasionally early spring but not throughout the rest of the year. Further studies are required to determine if this circulation pattern is type-specific or due to the small sample size of specific RV types in our study.

We were able to detect no less than 13 putative new RV types. The finding of this many possible new types was unexpected compared to the 20 new RV types / PATs reported since 2010 (complete genome, VP1 or VP4/VP2 sequence published in 2010 or later, or sequence made available to the Genbank in 2010 or later if unpublished, www.picornastudygroup.com), though VP1 sequencing is in progress for our strains to confirm that these are new types. Theoretically, the large amount of new PATs could be due to local circulation of specific RV types, but as widespread occurrence has been reported for most RV types (14), we hypothesise that there may be many more RV types to be discovered and classified.

In July and August 2009 an atypical high number of RV infections was detected. This may be due to increased sampling as a result of the start of the influenza H1N1 epidemic (31). This emphasizes that one should be aware that the number of incoming samples can confound the estimated incidence when studying epidemiology by typing samples submitted for diagnostics. This is especially the case for a virus like RV, of which infections occur at high frequency and often asymptotically.

Our study has some limitations. We were not able to perform associations between the clinical symptoms of the patients and the different RV types due to the retrospective character of the study and the limited information provided by the clinicians on the accompanying form to the laboratory. Because of the limited clinical information and heterogeneous composition of our study group, we were not able to evaluate the prevalence of various RV types in specific populations. A selection bias is that our study is composed of individuals attending the hospital and therefore might not completely reflect the prevalence of RV types in the general population. Nevertheless, the large variety in RV types in our population points towards continuous variations in RV types co-circulating in the community.

In summary, in this study we present an overview of the RV types circulating in the Amsterdam area in the Netherlands in the years 2007 to 2012. RV epidemiology is complex and many RV types are co-circulating simultaneously. RV-targeted vaccination and antiviral strategies should therefore aim for broad-spectrum activity and can probably not afford to focus on a selection of dominant RV types.
Conflict of interest and funding

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REFERENCES


**Supplementary table A1.** Enterovirus types detected

<table>
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<td>E6*</td>
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<tr>
<td>EV-D68</td>
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</table>

*Typed by Blast analysis. * Including one repeat sample.
Supplemental Figure S1.
Supplementary Figure 2.

![Bar graph showing rhinovirus circulation in Amsterdam over different years and types (RV-A, RV-B, RV-C).](image)
Supplementary Figure 3.