Title: The Molecular Epidemiology and Clinical Phylogenetics of Rhinoviruses among Paediatric Cases in Sydney, Australia.

Abstract

Background

Rhinoviruses (RV) represent the most common aetiological agent of all acute respiratory tract infections across all age groups and a significant burden of disease among children. Recent studies have shown that RV-A and RV-C species are associated with varying degrees of disease severity and clinical symptoms.

Methods

In this study, we uncovered potential associations between RV species and subtypes, and clinical disease severity using a matched dataset of 52 RV isolates sampled from children (<18 years) in Sydney, Australia between 2006 and 2009 using epidemiological and phylogenetic methods.

Results

We found that RV-C was significantly more likely to be isolated from paediatric cases under two years of age compared to RV-A, although no significant differences in recorded symptoms were observed. Significant phylogenetic-trait associations between age and the VP4/VP2 capsid protein phylogeny suggests age-specific variations in infectivity among subtypes might also be possible.

Conclusions

This study adds to the growing body of epidemiological evidence concerning RV. Improving surveillance and testing for RV, including routine whole genome sequencing may improve our understanding of the varied diseashe outcomes of RV species and subtypes. Future studies could aim to identify specific genetic markers associated with age-specific

infectivity of RV which could inform treatment practices and public health surveillance of RV.

Keywords: rhinoviruses, epidemiology, phylogenetics, paediatric infections

List of abbreviations

RV	Rhinoviruses
DALY	Disability adjusted life years
USA	United States of America
EV-D68	Enterovirus-D68
5'UTR	5' untranslatable regions
RSV	Respiratory syncytia virus
MCMC	Markov Chain Monte Carlo
PS	Parsimony scores
AI	Association index

Background

Rhinoviruses (RV) are a highly prevalent and diverse respiratory pathogen of the *Enterovirus* genus and are a major cause of all acute respiratory illnesses throughout the year in both adults and children (1, 2). The attributable burden of RV in both developed and developing countries is significant, accounting for approximately 34.4 disability adjusted life years (DALY) per 1000 population among children under five (3, 4). The direct and in-direct socio-economic burden of RV has also been estimated to cost approximately $60B/year in the United States of America (USA) alone (5). There are three recognised species of RV denoted RV-A, RV-B, and RV-C, among which there are currently 179 classified subtypes based on sequence homology to former serology-based prototype strains. Since the shift from serology-based to sequence-based typing of RV, our understanding of the RV genome and diversity has increased. For example, RV-C was first recognised as a distinct RV species in 2006 (6) but had evaded serology-based detection until molecular methods became routine (7). The transition to sequence-based RV typing has also led to numerous taxonomic changes as old serotypes were regrouped (such as RV-A98 into RV-A54 (8)), and some species re-classified entirely (such as RV-A87 to Enterovirus-D68 (EV-D68) (9, 10)). Despite the sheer diversity of RV, they circulate freely around the world with no geographic structure of either species or subtype (11).

Typical symptoms of upper respiratory infection with RV include rhinorrhoea, nasal congestion, cough, headache, and malaise (12). However asymptomatic infection is also prevalent, particularly within households (13). It is now well established that RV can also compromise the lower respiratory tract, in contrast to previous evidence (14), and are sometimes associated with severe disease such as pneumonia and bronchiolitis (15). Exacerbations of asthma and other pre-existing diseases such as chronic obstructive pulmonary disease and cystic fibrosis in both adults and children has furthermore been associated with lower respiratory infection with RV (16). There is increasing (17), but yet inconclusive (18, 19) evidence to suggest RV-A and RV-C species may be associated with more severe disease compared to RV-B strains, and similar efforts have been made to

examine the varied clinical manifestations between RV subtypes, again with inconclusive or contrasting results (20, 21).

In order to better understand potential associations between RV species and clinical features among paediatric cases, we aimed to integrate genetic and epidemiological data using Bayesian phylogenetic methods.

Methods

Compilation of Dataset & Sequencing

We extracted the sequence data of 91 RV isolates collected between 2006 and 2009 in Sydney, Australia from GenBank. Thirty-eight sequences (42%) were originally collected as part of a household transmission study (22) and the other 53 (58%) isolated from a hospital department (23). Among these, 52 RV isolates (28 & 24 from each study respectively) were sampled from paediatric cases defined here as under the age of 18 years. Where available, we matched these sequences to corresponding clinical and demographic data collected during the same period: data on age was available for all 52 paediatric isolates, while clinical data (including symptoms) was only available for 28 sequences. Sequences were first generated according to (23) with relevant detail included here. RNA was extracted directly using LabTurbo Viral DNA/RNA Extraction kits (TaiGen Biotechnology Inc., Taiwan) according to the manufacturer's specifications, and cDNAs synthesized using SuperScript III reverse-transcriptase (Invitrogen, USA). Both 5' untranslatable regions (5'UTR) and VP4/VP2 capsid protein segments were sequenced in both directions using respective forward and reverse primers listed in Supplementary Table S1. Amplicons were purified from agarose gel using QIA PCR purification kits (Qiagen GmbH, Hilden, Germany) and sequenced bi-directionally to confirm specificity in an ABI-3730 XL DNA Analyzer (Applied Biosystem Inc., Foster City, CA).

Characterisation and Epidemiology

Previous phylogenetic analyses found serotype disagreements between the phylogenetic results of RV-A and RV-C species using the 5'UTR region (23). This incongruence can be explained by inter and intra-species recombination, particularly between the 5'UTR segment of RV-A and RV-C (24). We therefore recharacterized isolates by RV species and genotype if possible, primarily using assembled VP4/VP2 sequences (or 5'UTR sequences where VP4/VP2 amplification or assembly failed) with NCBI BLAST. We constructed contingency tables by RV species and symptoms for all 28 paediatric cases with matching clinical data. We considered all 52 paediatric isolates for analysis by age groups here separated as over and under two years of age. We performed Fisher Exact tests to measure statistical associations between age groups and clinical symptoms by RV species using R v3.4.4.

Phylogeny-Trait Association

We confirmed the suitability of both 5'UTR and VP4/VP2 sequence regions for temporal Bayesian phylogenetic analysis by producing preliminary Maximum Likelihood trees using RAxML v8.2 (25) via Geneious v11.1.5 (26) and Tempest v1.5.1 (27) (Supplementary Figure S1). We excluded 17 sequences from the demographic phylogenetic analysis (N = 35) and seven from the clinical phylogenetic analysis (N = 21) since either the 5'UTR and VP4/VP2 sequences were not available. We aligned sequences using MUSCLE v3.8 (28) and used BEAST v1.10.4 (29) to produce posterior phylogeny distributions of both 5'UTR and VP4/VP2 independently and as a multi-locus partition model. We specified a relaxed molecular clock (uncorrelated lognormal prior), constant demographic tree prior, and GTR+I+Γ_4 nucleotide substitution model as determined per previous studies (24). We ran each model individually for 50 million Markov Chain Monte Carlo (MCMC) generations sampling every 5,000 steps. We checked for parameter convergence and sufficient mixing of the posterior (effective sample size > 200) using Tracer 1.7 (30). For each model, we calculated phylogenetic parsimony scores (PS) (31) association index (AI) (32) statistics as a measure of phylogeny-trait correlation for the matched demographic and clinical symptoms. We used BaTS v0.9.0 (33) to account for statistical uncertainty in each phylogeny across a

posterior distribution of 9,000 trees (after removing 10% burn-in) for each model. As a positive control, we also calculated the PS and AI statistic between serotypes. This study was approved by the UNSW Human Research Ethics Committee (HC17284).

Results

Sequence Characterisation

In Table 1, we show the complete distribution of RV species and subtype characterisation with additional metadata in Supplementary Table S2. Both 5'UTR and VP4/VP2 sequences were available for 35 (67%; N=35/52) isolates while 5'UTR was only available for the remaining 17 (33%; N=17/52) isolates. The majority (92%; N=48/52) of sequences isolated were characterised as RV-A (48%; N=25/52) and RV-C (44%; N=23/52) species. We determined the complete subtype recharacterization for 43 isolates (83%; N=43/52). The majority (78%; N=7/9) of untyped isolates was among the 17 only sequenced for 5'UTR. Overall, RV-C11 was the most frequently detected subtype among characterised isolates (9%; N=4/43) followed by RV-A01 (7%; N=3/43). Just over half of all characterised isolates (51%; N=22/43) were unique i.e. had no common subtype among all isolates characterised.

Clinical and Demographic Epidemiology

Among the 28 RV isolates with matching clinical data, the majority were characterised as RV-A and RV-C. Only a single RV-B isolate had matching clinical data. Cough (96%; N=27/28), nasal congestion (96%; N=27/28) and sneezes (79%; N=22/28) were reported in the majority of cases with matching clinical data (Table 2). All other reported symptoms were less frequent (46% and below).

Cough was absent in a single case of subtype RV-C11 while nasal congestion was absent in a case of RV-C02, however, both subtypes were detected among other cases reporting these symptoms. Diarrhoea was the least reported symptom (14%; N=4/28). The

limited number of RV-B isolates in the dataset precluded inclusion in subsequent statistical comparisons. Differences therefore between RV-A and RV-C species were considered only, however there was no statistically significant difference between clinical symptoms by RV species (Table 2). Among the demographic dataset of 52 RV isolates, 34 cases (65%; N=34/52) were under the age of two years. Within species, RV-A was isolated from 13 cases under two years (52%; N=13/25), one from RV-B (25%; N=1/4), and 20 from RV-C (89%; N=20/23). Again, only RV-A and RV-C were tested for significant differences by demographic age group due to low numbers of RV-B isolated from cases under two years, and for consistency with the previous clinical analysis. In this case RV-C was significantly more likely to be isolated from cases under two compared to RV-A ($p = 0.01$).

Phylogenetic Analysis & Trait Association

In each case, our positive control performed as expected, demonstrating significant phylogeny-trait associations by serotype for both PS and AI statistics (Tables 3 and 4). Overall, no clinical symptom demonstrated significant phylogeny-trait associations between or within species. There was a significant association between the VP4/VP2 capsid protein and age group for the PS statistic ($p < 0.01$) but not AI statistic ($p = 0.12$). Figure 1 shows an unrooted maximum clade credibility tree generated from the Bayesian posterior tree distribution coloured by age group.

Discussion

In this study, we report and compare the molecular epidemiology and clinical features of a small sample of paediatric RV cases in Sydney, Australia. Almost all RV species isolated were characterised as RV-A (48%; N=25/52) or RV-C (44%; N=23/52) with very few RV-B (8%; N=4/52) cases observed. This is consistent with many molecular epidemiology studies on RV that demonstrate the predominant circulation of RV-A and RV-C species compared to RV-B (34, 35). We found that RV-C was significantly more likely to be isolated from paediatric cases under two years of age compared to RV-A ($p = 0.01$). This significant difference was also apparent ($p < 0.01$) in one of two Bayesian phylogeny-trait association test statistics (PS not AI) of the VP4/VP2 capsid gene (Tables 3 and 4). More recent studies

have also shown differences in the rates of RV infection by species among early and late childhood (36, 37). Together with our results, this reinforces the potential for age-specific infectivity by RV species, particularly RV-C at younger age groups. The clustering of clinical features within RV subtypes in our dataset may also indicate associations with age of infection beyond the current species classification system, e.g. RV-C02, RV-A01 and RV-A20 in children under two years of age (Figure 1). Future studies could look to identify genetic markers exclusively observed among these subtypes which could be associated with susceptibility at younger ages. For this purpose, larger phylogenetic-trait association studies should be implemented.

In our study we examined a wide range of clinical RV features. The most frequent symptoms reported among the 28 cases with matching clinical data were cough, nasal congestion and sneezes (Table 2). A variety of other symptoms such as loss of appetite, abdominal pain and vomiting were also reported in the minority. Previous studies have reported inconsistent results concerning the clinical presentation and severity between RV species (17-21, 38). For example, in a sample of hospitalised patients in Taiwan, upper respiratory tract infection and nasal congestion was significantly higher in paediatric cases compared to adults (39). In other studies, exacerbations of asthma were often observed among cases of RV-A and RV-C, but not RV-B (40). Furthermore, between species, lower respiratory compromise and pneumonia has been commonly reported among cases of RV-C but less so for RV-A (41). In other molecular epidemiology and phylogenetic studies like ours (42), no significant difference in clinical features were observed between species (Table 2). These contrasting results mean it is difficult to determine conclusively the presence of epidemiological differences in the clinical presentation of RV species. Our approach to explore phylogeny-trait associations aimed to identify potential clustering by clinical traits within RV subtypes, however these results were also not significant (Tables 3 and 4). This suggests that potential variations in symptom presentation might be mostly dependant of individual host-pathogen responses rather than exclusively viral (43). We had a small sample size, and further research with larger samples is required to understand the role these factors play on clinical RV presentation.

Overall this study has a few limitations. Firstly, the small sample size and limited availability of matched clinical data means any significant associations between RV species and clinical features are potentially concealed. We could only find 91 RV isolates, including 52 isolates from paediatric cases in Sydney collected between 2006 and 2009 available in GenBank, however no additional RV sequences were available in Sydney from more recent years. While some differences were observed, e.g. cough was reported among 64% of RV-A cases compared to 31% in RV-C (Table 2) they did not reach significance ($p = 0.13$). Our approach, particularly the unique application of clinical RV phylogeny-trait association testing within a Bayesian phylogenetic framework could be replicated across larger datasets containing match clinical data to overcome this in the future. Secondly, the clinical features recorded in our study could be attributable to coinfection with other viruses. Although RV continue to represent the most common aetiological agent of all acute respiratory tract infections across all age groups (1, 2), coinfection with other viruses such as respiratory syncytia virus (RSV) is common particularly among children (44). While our study did not determine any significant associations between clinical presentation and RV species or subtype, any future study must consider the potential for any confounding due to viral coinfection. Finally, this study only incorporated the commonly sequenced 5'UTR and VP4/VP2 gene regions into our analysis. Translation of the approximately 7kb RV genome produces a single polyprotein which is cleaved to form mature capsid (VP1-4) and replication (2A-C, 3A-D) peptides (45). Studies have shown that the 5'UTR common among species within the *Enterovirus* genus have significant impacts on viral pathogenesis (46, 47), however differences in clinical manifestation are also attributed to other translatable peptides and genome regions including the 3'UTR (48). Additionally, mutations within the VP1 gene of EV-A71 are important determinants of pathogenesis in mice (49), while VP1 and VP3 are critical for viral attachment of RV to nasal and bronchial epithelia (50). Studies incorporating whole genome sequencing could be used to uncover significant clinical associations between RV strains, with the aim of identifying genetic markers of severity in the future.

Conclusions

Our results show that RV-C is significantly more likely to be isolated from paediatric cases under two years of age compared to RV-A despite near equal predominance in circulation. These results were supported by Bayesian phylogenetic-trait association testing of the VP4/VP2 capsid protein suggesting genetic factors may influence the age-specific infectivity of RV species. No statistically significant difference in clinical symptom manifestation was detected between species or subtypes using phylogenetic methods, however the small sample size likely limited statistical power. These results add to the growing body of epidemiological evidence concerning RV. Improving surveillance and testing for RV, including routine whole genome sequencing may improve our understanding of the varied disease outcomes of RV species and subtypes.

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Figure Legend

Figure 1. Unrooted radial phylogenetic tree of RV-A and RV-C species and subtype coloured by age group with branch lengths equally constrained