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The Molecular Epidemiology and Clinical Phylogenetics of Rhinoviruses Among Paediatric Cases in Sydney, Australia

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ABSTRACT

Objectives: 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 increased disease severity. In order to better understand the potential associations between RV species and clinical features among paediatric cases, this study aimed to integrate genetic and epidemiological data using Bayesian phylogenetic methods.

Methods: 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 were uncovered using epidemiological and phylogenetic methods.

Results: It was found that RV-C was significantly more likely to be isolated from paediatric cases aged < 2 years compared with RV-A, although no significant differences in recorded symptoms were observed. Significant phylogenetic-trait associations between age and the VP4/VP2 capsid protein phylogeny suggest that age-specific variations in infectivity among subtypes may may be possible.

Conclusion: 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 understanding of the varied disease 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.

Introduction

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 (Mäkelä, Puhakka et al. 1998; Monto 2002). 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 aged underunder 5 years (Weiss and Sullivan 2001; Gaunt, Harvala et al. 2011). The direct and in-direct socioeconomic burden of non-influenza-related viral respiratory tract infections including RV has been estimated to cost approximately $40 billion/year in the United States of America (USA) alone (Fendrick, Monto et al. 2003). 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 (Lamson, Renwick et al. 2006) but had evaded serology-based detection until molecular methods became routine (Kistler, Avila et al. 2007). The transi-

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tion to sequence-based RV typing has also led to numerous taxonomic changes, as old serotypes were regrouped (such as RV-A98 into RV-A54 (Rathe, Liu et al. 2010)) and some species were reclassified entirely (such as RV-A87 to Enterovirus-D68 (EV-D68) (Blomqvist, Savolainen et al. 2002; Savolainen, Blomqvist et al. 2002)). Despite the sheer diversity of RV, they circulate freely around the world with no geographic structure of either species or subtype (McIntyre, Knowles et al. 2013).

Typical symptoms of upper respiratory infection with RV include rhinorrhoea, nasal congestion, cough, headache, and malaise (Fields, Knipe et al. 2013). However, asymptomatic infection is also prevalent, particularly within households (Peltola, Waris et al. 2008). It is now well established that RV can also compromise the lower respiratory tract, which is in contrast to previous evidence (Papadopoulos, Bates et al. 2000), and are sometimes associated with severe disease such as pneumonia and bronchiolitis (Hayden 2004). Exacerbations of asthma and other pre-existing diseases such as chronic obstructive pulmonary disease and cystic fibrosis in both adults and children have furthermore been associated with lower respiratory infection with RV (Varkey and Varkey 2008; Stelzer-Braid, Liu et al. 2017; Zheng, Wang et al. 2018; Cafferkey, Coultas et al. 2020; Michi, Love et al. 2020; Ortega, Nickle et al. 2020; Zhu, Mallia et al. 2020). There is also increasing evidence suggesting that RV-A and RV-C species may be associated with more severe disease compared with RV-B strains (Lee, Lemanske Jr et al. 2012; Megremis, Niespodziana et al. 2018; Ng, Oong et al. 2018; Baillie, Moore et al. 2020), and similar efforts have been made to examine the varied clinical manifestations between RV subtypes (Huang, Wang et al. 2009; Moreira, Kamikawa et al. 2011; Bruning, Thomas et al. 2015; Martin, Kuypers et al. 2015; Bergroth, Aakula et al. 2020). In order to better understand potential associations between RV species and clinical features among paediatric cases, this study aimed to integrate genetic and epidemiological data using Bayesian phylogenetic methods.

Methods

Compilation of Dataset and Sequencing

The sequence data of 91 RV isolates collected between 2006 and 2009 in Sydney, Australia, were extracted from GenBank. Thirty-eight sequences (42%) were originally collected as part of a household transmission study (MacIntyre, Cauchemez et al. 2009) and the other 53 (58%) were isolated from a hospital department (Ratnamohan, Zeng et al. 2016). Among these, 52 RV isolates (28 and 24 from each study, respectively) were sampled from paediatric cases defined as aged $<$ 18 years. Where available, these sequences were matched to corresponding clinical and demographic data collected during the same period: data on age waswas available for all 52 paediatric isolates, while clinical data (including symptoms) wasere available for 28 sequences. Sequences were first generated according to Ratnamohan, Zeng et al. 2016, with relevant detail included here. RNA was directly extracted using LabTurbo Viral DNA/RNA Extraction kits (TaiGen Biotechnology Inc., Taiwan), according to the manufacturer's specifications, and cDNAs synthesised 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 1. 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 (Ratnamohan, Zeng et al. 2016). This incongruence can be explained by inter- and intra-species recombination, particularly between the 5'UTR segment of RV-A and RV-C (Waman, Kolekar et al. 2014). Isolates were therefore recharacterised 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. Contingency tables were constructed by RV species and symptoms for all 28 paediatric cases with matching clinical data. All 52 paediatric isolates were considered for analysis by age groups and separated as $\geq$ 2 and $<$ 2 years of age. Fisher Exact tests were performed to measure statistical associations between age groups and clinical symptoms by RV species using R v3.4.4.

Phylogeny-Trait Association

This study confirmed the suitability of both 5'UTR and VP4/VP2 sequence regions for time-scaled phylogenetic analysis by producing preliminary Maximum Likelihood trees using RAxML v8.2 (Stamatakis 2014) via Geneious v11.1.5 (Kearse, Moir et al. 2012) and Tempest v1.5.1 (Rambaut, Lam et al. 2016) (Supplementary Figure 1). Seventeen sequences from the demographic phylogenetic analysis (N = 35) and seven from the clinical phylogenetic analysis (N = 21) were excluded since either the 5'UTR or VP4/VP2 sequences were not available. Sequences were aligned using MUSCLE v3.8 (Edgar 2004), and BEAST v1.10.4 (Suchard, Lemey et al. 2018) was used to produce posterior phylogeny distributions of both 5'UTR and VP4/VP2 independently and as a multi-locus partition model. A relaxed molecular clock (uncorrelated lognormal prior), prior constant demographic tree, and GTR+I+Γ_4 nucleotide substitution model were specified as determined in previous studies (Waman, Kolekar et al. 2014). Each model was individually run for 50 million Markov Chain Monte Carlo (MCMC) generations, sampling every 5,000 steps. Parameter convergence and sufficient mixing of the posterior (effective sample size $>$ 200) were checked using Tracer 1.7 (Rambaut, Suchard et al. 2014). For each model, phylogenetic parsimony score (PS) (Fitch 1971) association index (AI) (Wang, Donaldson et al. 2001) statistics were calculated as a measure of phylogeny-trait correlation for the matched demographic and clinical symptoms. Bayesian Tip-association Significance testing (BaTS) v0.9.0 (Parker, Rambaut et al. 2008) was used to account for statistical uncertainty in each phylogeny across a posterior distribution of 9,000 trees (after removing 10% burn-in) for each model. The PS and AI statistic between serotypes was also calculated as a positive control. This study was approved by the UNSW Human Research Ethics Committee (HC17284).

Results

Sequence Characterisation

Table 1 shows the complete distribution of RV species and subtype characterisation with additional metadata in Supplementary Table 2. Both 5'UTR and VP4/VP2 sequences were available for 35 (67%; N = 35/52) isolates, while 5'UTR was available for the remaining 17 (33%; N = 17/52) isolates. The majority (92%; N = 48/52) of isolated sequences were characterised as RV-A (48%; N = 25/52) and RV-C (44%; N = 23/52) species. The complete subtype recharacterisation for 43 isolates (83%; N = 43/52) was determined. The majority (78%; N = 7/9) of untyped isolates were among the 17 that were only sequenced for 5'UTR. Overall, RV-C11 was the most frequently detected subtype among characterised

Table 1
Distribution of species and genotypes detected by species among 52 paediatric rhinovirus cases in Sydney, Australia, between 2006 and 2009.

Species	Count	Genotypes
A	25	A00, A01, A12, A15, A20, A21, A22, A24, A28, A31, A33, A43, A49, A56, A57, A61, A67, A71, A89
B	4	B06, B17, B48, B91
C	23	C00, C02, C06, C11, C16, C26, C28, C32, C37, C40

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 characterised isolates).

Clinical and Demographic Epidemiology

Among the 28 RV isolates with matching clinical data, the majority were characterised as RV-A and RV-C. A single RV-B isolate had matching clinical data. Cough (96%; N = 27/28), nasal congestion (96%; N = 27/28), and sneezing (79%; N = 22/28) were reported in the majority of cases with matching clinical data (Supplementary Table 3). All other reported symptoms were less frequent (≤ 46%).

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. Therefore, only differences between RV-A and RV-C species were considered; however, there was no statistically significant difference between clinical symptoms by RV species (Supplementary Table 3). Among the demographic dataset of 52 RV isolates, 34 positive cases (65%; N = 34/52) were aged < 2 years. Among those aged < 2 years, 13 were positive for RV-A (38%, N = 13/34), one for RV-B (3%, N = 1/34), and 20 for RV-C (59%, N = 20/34). Of note, the majority of RV-C (89%, N = 20/23) and RV-A (52%, N = 13/25) positive cases were < 2 years, while most RV-B positive cases were ≥ 2 years (75%, N = 3/4). However, 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 < 2 years, and for consistency with the previous clinical analysis. In this case, RV-C was also significantly more likely to be isolated from cases < 2 years compared with RV-A ($p = 0.01$).

Phylogenetic Analysis and Trait Association

In each case, the positive control performed as expected, demonstrating significant phylogeny-trait associations by serotype for both PS and AI statistics (Supplementary Tables 4 and 5). 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.

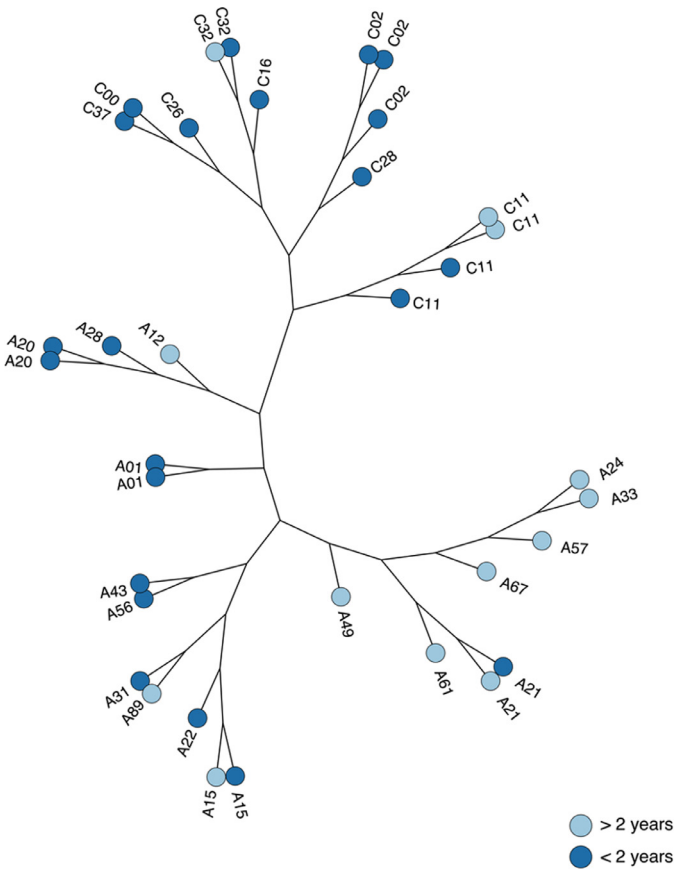

Figure 1. Unrooted radial phylogenetic tree of RV-A and RV-C species and subtype coloured by age group with branch lengths equally constrained.

Discussion

This study reported and compared the molecular epidemiology and clinical features of a small sample of paediatric RV cases in Sydney, Australia. Almost all RV species that were 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 have demonstrated the predominant circulation of RV-A and RV-C species compared with RV-B (Wildenbeest, van der Schee et al. 2016; Saraya, Kimura et al. 2017). This study found that RV-C was significantly more likely to be isolated from paediatric cases aged < 2 years compared with 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 (Supplementary Tables 4 and 5). More recent studies have also shown differences in the rates of RV infection by species among early and late childhood (Bochkov, Evans et al. 2018; Luka, Kamau et al. 2020). Together with the current results this reinforces the potential for age-specific infectivity by RV species, particularly RV-C in younger age groups. The clustering of clinical features within RV subtypes in the current 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 aged < 2 years) (Figure 1). Future studies could look at identifying 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.

This study 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 sneezing (Supplementary Table 3). 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 (Huang, Wang et al. 2009; Moreira, Kamikawa et al. 2011; Lee, Lemanske Jr et al. 2012; Bruning, Thomas et al. 2015; Chen, Arnold et al. 2015; Martin, Kuypers et al. 2015). For example, in a sample of hospitalised patients in Taiwan, upper respiratory tract infection and nasal congestion were significantly higher in paediatric cases than adults (Hung, Yang et al. 2019). In other studies, exacerbations of asthma were often observed among cases of RV-A and RV-C, but not RV-B (Zhao, Zhu et al. 2016). Furthermore, between species, lower respiratory compromise and pneumonia have commonly been reported among cases of RV-C but less so for RV-A (Linder, Kraft et al. 2013). In other molecular epidemiology and phylogenetic studies like the current one (Tapparel, Cordey et al. 2011), no significant differences in clinical features were observed between species (Supplementary Table 3). These contrasting results mean that it is difficult to conclusively determine the presence of epidemiological differences in the clinical presentation of RV species. The current approach to explore phylogeny-trait associations aimed to identify potential clustering by clinical traits within RV subtypes; however, these results were also not significant (Supplementary Tables 4 and 5). This suggests that potential variations in symptom presentation might be mostly dependant of individual host-pathogen responses rather than exclusively viral (Makris and Johnston 2018). The current study had a small sample size and further research with larger samples is required to understand the role these factors play in clinical RV presentation.

This study had a few limitations. First, the small sample size and limited availability of matched clinical data meant that any significant associations between RV species and clinical features were potentially concealed. Ninety-one RV isolates were found, 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 with 31% in RV-C) (Supplementary Table 3) they did not reach significance ($p = 0.13$). This 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. Second, the clinical features recorded in this 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 (Mäkelä, Puhakka et al. 1998; Monto 2002), coinfection with other viruses such as respiratory syncytial virus (RSV) is common, particularly among children (Franz, Adams et al. 2010). While this 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 the analysis. Translation of the approximately 7 kb RV genome produces a single polyprotein, which is cleaved to form mature capsid (VP1-4) and replication (2A-C, 3A-D) peptides (Palmenberg, Rathe et al. 2010). Studies have shown that the 5'UTR, which are common among species within the *Enterovirus* genus, have significant impacts on viral pathogenesis (Kawamura, Kohara et al. 1989; Guest, Pilipenko et al. 2004); however, differences in clinical manifestation are also attributed to other translatable peptides and genome regions, including the 3'UTR (Merkle, Van Ooij et al. 2002). Additionally, mutations within the *VP1* gene of EV-A71 are important determinants of pathogenesis in mice (Zaini, Phuektes et al. 2012), while *VP1* and *VP3* are critical for viral attachment of RV to nasal and bronchial epithelia (Blaas and Fuchs, 2016). 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

The results show that RV-C is significantly more likely to be isolated from paediatric cases aged < 2 years compared with 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 that 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 molecular epidemiological evidence concerning RV. Improving surveillance and testing for RV, including routine whole genome sequencing, may improve future understanding of the varied disease outcomes of RV species and subtypes.

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Ethics approval and consent to participate

The human sequence data used in this study were publicly available from GenBank. This study was approved by the UNSW Human Research Ethics Committee (HC17284).

Conflict of Interest

CRM has received funding for investigator-driven research from Merck, GSK and Seqirus, and support for laboratory testing unrelated to this study from Pfizer. CRM has also been on advisory boards for the same companies. DCA, XC, MS, DD and JK have no competing interests to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.06.046.

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