Viral load and sequence analysis reveal the symptom severity, diversity and transmission clusters of rhinovirus infections

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RV-C-infected subjects had higher viral load and was associated with more severe respiratory symptoms. Sustained RV transmission was attributed to multiple transmission clusters in the population. The relative humidity was the strongest predictor of RV seasonality.

Abstract

Background.

Rhinovirus (RV) is one of the main viral etiologic agents of acute respiratory illnesses. Despite the heightened disease burden caused by RV, the viral factors that increase the severity of RV infection, the transmission pattern and seasonality of RV infections remain unclear.

Methods.

An observational study was conducted among 3,935 patients presenting with acute upper respiratory illnesses in the ambulatory settings between 2012 and 2014.

Results.

The *VP4/VP2* gene was genotyped from all 976 RV-positive specimens, where the predominance of RV-A (49%) was observed, followed by RV-C (38%) and RV-B (13%). A significant regression in median nasopharyngeal viral load ($p<0.001$) was observed; from 883 viral copies/µl at 1-2 days to 312 viral copies/µl at 3-4 days and 158 viral copies/µl at 5-7 days, before declining to 35 viral copies/µl at ≥ 8 days. In comparison with RV-A (median viral load: 217 copies/µl) and -B (275 copies/µl), RV-C-infected subjects produced higher viral load (505 copies/µl; $p<0.001$). Importantly, higher RV viral load (median: 348 copies/µl) was associated with more severe respiratory symptoms (TSSS ≥ 17) ($p=0.017$). A total of 83 phylogenetic-based transmission clusters were identified in the population. Based on the partial ($r=0.520$, $p=0.011$) and bivariate ($r=0.491$, $p=0.009$) correlations and regression analyses (standard regression coefficient, beta=1.329, t=2.79, $p=0.011$), the relative humidity

was determined as the strongest environmental predictor (p=0.011) of RV seasonality.

Conclusions.

Our findings underlined the role of viral load in increasing disease severity attributed to RV-C infection, and unraveled the factors that fuel the population transmission dynamics of RV.

INTRODUCTION

Rhinovirus (RV) is a predominant and ubiquitous airborne viral pathogen. With the improvement in viral detection methods, the involvement of RV in lower respiratory compartment leading to severe and potentially fatal respiratory conditions are increasingly evident [1-3]. Furthermore, individuals with predisposing respiratory conditions such as asthma, chronic obstructive pulmonary disease and cystic fibrosis may experience increased risk of severe RV-associated complications [4, 5].

There are three confirmed RV species denoted as RV-A, -B and -C circulating worldwide. Despite the clinical burden of RV infections, large-scale molecular epidemiological data of RV are not extensively reported. Although the recent discovery of RV-C has incited renewed interest in investigating the epidemiology of RV infections [6], the effects of RV species on severity of respiratory illness remain insufficiently addressed. In the attempt to identify factors associated with RV morbidity and severity, studies have shown a possible correlation between high viral load and increased severity of RV infections [7]. However, different observations had also been reported elsewhere [8], potentially due to the variation in sample sizes and the inclusion of study subjects with predisposing conditions (asthma and pneumonia). Furthermore, the use of different viral load quantification methods that inherit certain technical limitations (limited RV type coverage) may also affect the quantification efficiency [9].

Spatiotemporal analyses based on viral sequence data and evolutionary history of human immunodeficiency virus type 1 have shown that the emergence of transmission clusters is responsible for the spread of infections, highlighting the role of transmission clusters in escalating viral transmission and disease expansion [10]. The importance of such phylogenetically-inferred transmission clusters in fueling the

onward disease transmission has also been observed in other viral infections, such as in the recent Ebola virus outbreaks [11]. Despite the high disease burden caused by RV, the evolutionary history and the dynamic of RV infections remains largely unexplored.

Climatological factors have also been implicated in the incidence of respiratory infections. For instance, findings from studies conducted in the temperate region have shown an association between high relative humidity and increased RV incidence [12]. However, studies of the meteorological factors and air pollutant on RV seasonality remain insufficiently explored in the regions with tropical climate [13].

In the present large-scale population-based RV molecular epidemiological study, we aimed to study the impact of RV species and the nasopharyngeal viral load on the symptom severity of acute upper respiratory tract infections. Next, we investigated the genetic diversity, evolutionary histories, and the spatiotemporal dynamics of RV transmission clusters that drive disease transmission. Finally, we analyzed the potential meteorological predictors that influence the seasonality of RV in the context of the tropical climate in Southeast Asia.

METHODS

The Study Subjects and Specimens

This study was approved by the University Malaya Medical Ethics Committee (MEC reference number: 890.1). Consenting outpatients who were presenting with symptoms of acute upper respiratory tract infections were recruited at the primary care clinics, University Malaya Medical Centre in Kuala Lumpur, Malaysia between February 2012 and May 2014. Nasopharyngeal swabs were collected in universal transport medium using standardized technique. The presence of symptoms associated with acute respiratory tract infection was determined based on previously published criteria [14]. At the point of patients recruitment, the number of days after the onset of symptoms (symptomatic phase) was recorded. To assess the severity of acute respiratory tract infection associated with RV species, previously described approach based on the total symptom severity score (TSSS) system was adopted [15], whereby higher score indicates greater severity of respiratory symptoms [2, 3].

Sequencing and Quantification of RV

Total viral RNA was extracted from 3,935 nasopharyngeal specimens and were screened for viral pathogens using the xTAG Respiratory Viral Panel (RVP) FAST Assay (Luminex Molecular, Toronto, Canada). Specific enteroviruses in HEV-positive specimens were further confirmed through nested PCR amplification and direct sequencing of the *VP4/VP2* gene [16]. The RV viral load was quantified using a newly developed one-step Taqman assay, and viral load was expressed in RV viral copies/µl of extracted RNA [17] (see Supplementary Material).

The categorical variables were compared using Chi-square test, while the differences and association between RV viral load and disease severity (based on

145 total symptom severity score, TSSS) were investigated through the non-parametric
146 Mann-Whitney U test, Kruskal-Wallis test, linear regression and multivariate analysis
147 using SPSS. To improve clarity, the recorded number of days after the onset of
148 symptoms (symptomatic phase) was grouped into sub-categories, namely day 1-2,
149 3-4, 5-7 and more than 8, based on previously described method [3].
150
151 **Phylogenetic and Phylodynamic Analysis of RV**
152 To determine the genetic types and to identify the possible transmission
153 clusters of RV in the present study, neighbour-joining and Bayesian maximum clade
154 credibility (MCC) trees were reconstructed based on an updated and comprehensive
155 list of global *VP4/VP2* sequence data (3,397 sequences). The time of most recent
156 common ancestor (tMRCA) of the respective transmission clusters observed in RV-
157 A, -B and -C were then estimated by the Bayesian coalescent-based relaxed
158 molecular clock model, performed in BEAST 1.7 (see Supplementary Material).
159
160 **Meteorological Parameters and Their Associations with RV cases**
161 To understand the seasonality of RV infections, meteorological data collected
162 from a weather station located within a 5 kilometers radius from the hospital were
163 obtained from the Malaysian Meteorological Department and were analyzed using
164 Statistical Package for Social Sciences version 22.0 (SPSS Inc., Chicago, USA) (see
165 Supplementary Material).
166

RESULTS

Distribution of RV types in patients with acute respiratory tract infection. A total of 3,935 consenting outpatients (median age: 38 years old, range: 7 – 95 years old) with symptoms of acute respiratory tract infection were recruited, of whom 51% (2,009/3,935) were positive for at least one viral pathogen in the multiplex respiratory virus panel screening assay. Among 2,009 subjects, 976 (49%) were tested positive for RV, highlighting its high prevalence in individuals with symptoms of acute upper respiratory tract infection (Supplementary Table 1). The species (and genetic types) of the infecting RV were determined through the neighbour-joining phylogenetic reconstruction (Figure 1a, 1b and 1c). Phylogenetic analysis of the *VP4/VP2* gene revealed the predominance of RV-A, infecting 49% (473/976) of the subjects, followed by RV-C (38%, 372/976), and RV-B (13%, 131/976). The prevalence of RV-A and -C infections were consistently higher than RV-B throughout the study period (Figure 1d). In total, 111 distinct RV types (RV-A: 54 types, RV-B: 16 types and RV-C: 41 types) were identified by phylogenetic analysis.

Clinical characteristics of RV infection, viral load dynamics and the association with symptom severity of acute respiratory infections. To investigate the variation in clinical manifestations during acute respiratory infection, the clinical characteristics among subjects positive for RV were compared to those infected with other respiratory viruses (Table 1). Of the 976 RV-infected subjects, 129 subjects were excluded from analysis due to co-infection with at least one other respiratory virus or incomplete data. It was observed that most of the RV-infected subjects experienced sneezing, nasal discharge, nasal congestion but fewer experienced muscle ache. A significant negative correlation between RV viral load

and the estimated number of days from onset of symptoms was observed, with a correlation coefficient (*r*) of -0.121 (*p*<0.001). A significant regression in median RV viral load (*p*<0.001) was observed; from 883 viral copies/μl at day 1-2 to 312 viral copies/μl at day 3-4 and 158 viral copies/μl at day 5-7, before declining further to 35 viral copies/μl at day ≥8 (Figure 2a and Supplementary Table 2). Of note, subjects with respiratory symptoms for ≥8 days had detectable RV RNA.

Taking other covariates (e.g. patient's demographic) into consideration, multiple linear regression analysis was performed to assess the difference in viral load between RV species at different symptomatic phases. At the species level, it was generally shown that subjects infected with RV-C had a significantly higher viral load as compared to those infected with RV-A and -B (Figure. 2b and Supplementary Table 3). Such difference in viral load was only evident at day 1-2 (*p*=0.006). The difference in viral load between RV-A and RV-B was not statistically significant. Interestingly, the multiple linear regression analysis revealed that patients with higher viral load generally had higher TSSS (*p*=0.017), indicating the increased severity of RV-associated acute respiratory tract infection (Figure 2c and Supplementary Table 4). Such association was profound at day 1-2 of the symptomatic phase (*p*=0.012), which coincided with the peak viral load. Taken together, analysis using multivariate analysis further indicated that patients infected with RV-C recorded higher viral load and higher TSSS (Supplementary Table 5), suggesting that the increased symptom severity among RV-C-infected individuals could be attributed to the high viral load.

The evolutionary histories of RV transmission clusters.

Phylogenetic reconstruction uncovered a total of 28 RV-A transmission clusters of varying sizes (2-9 subjects), predominantly involving RV-A32 (14%, 4/28).

Similarly, a total of 15 and 40 transmission clusters (involving 2-11 subjects) were observed among RV-B and RV-C, respectively, with the predominance of RV-B79 (27%, 4/15), B69 (27%, 4/15) and C22 (10%, 4/40) (Figure 1 and Supplementary Figure1). Based on Bayesian analyses, mean evolutionary rates of 3.21-3.24 (3.04-3.41) x 10^{-3} substitutions/site/year (across RV-A, -B and -C) were estimated and were used to elucidate the divergence times of RV transmission clusters (Supplementary Figure 2).

Seasonality of acute respiratory illness associated with RV infection in the tropical region. It was observed that the number of RV-infected cases peaked between October and December in 2012 and 2013, where the number of rain days, total amount of rain fall and relative humidity were high (Figure 3). During the peak detection periods, the ground temperature and air pollutant (PM_{10}) readings were low. Statistical analysis showed that relative humidity seemed likely to be the main predictor for the increased number of RV infections, based on both partial (r=0.520, p=0.011) and bivariate (r=0.491, p=0.009) correlations as well as regression analyses (standard regression coefficient, beta=1.329, t=2.79, p=0.011) (Supplementary Table 6). It is important to note that relative humidity showed a significant positive correlation with the number of rain days (r=0.886, p<0.001) and total amount of rain fall (r=0.833, p<0.001), and a significant negative correlation with mean temperature (r=-0.715, p<0.001) and PM_{10} (r=-0.700, p<0.001), underlining the indirect role of these meteorological factors and PM_{10} on the RV activities and seasonality.

DISCUSSION

Recent studies had investigated the potential role of viral load in molding the dynamics of viral-associated acute respiratory infection [20, 21]. However, limitations that may hamper accurate viral load quantification remains evident in many existing assays, such as the potential risk of viral load misestimation and the suboptimal sensitivity to detect broad array of RV types [22]. Here, a newly established Taqman assay with a broader coverage for improved detection and quantification of RV viral load was used [17]. From 847 RV-infected patients, RV viral load peak was observed in 1-2 days after symptoms onset, in line with previously reported observation [23]. A significant regression in RV viral load was observed thereafter, an indication of viral clearance by the immune system. Importantly, some patients had detectable viral load 1 week after the onset of symptoms. Such observation clearly suggests the prolonged RV shedding in the respiratory tract [24], which may facilitate viral transmission during the second week of infection.

Several studies have attempted to examine the influence of RV species on viral load during infection. For instance, study has demonstrated that in RV-C-infected patients with pneumonia, higher mean viral load was reported [3]. However, insignificant difference in median peak viral load between RV species was also reported in hospitalized patients elsewhere [26]. In the present analysis, which was established based on the large-scale RV molecular epidemiology and viral load data, a significant association between RV species and viral load was found, of whom RV-C-infected subjects exhibited higher nasopharyngeal viral load in comparison to subjects infected with RV-A and -B. Such notable difference in viral load between RV species could potentially due to, among others, the utilization of different cellular receptors for virus entry [27]. In comparison to RV-A and -B that utilize the

intercellular adhesion molecule 1, RV-C uses the highly-expressed cadherin-related family member 3 (CDHR3) as cellular receptor, in which a single nucleotide polymorphism (C529Y) in CDHR3 is associated with the upregulation of receptors on cell surface, promoting viral replication with a consequent increase in viral load [28].

Several other studies have investigated the impact of viral load on the virulence and severity of respiratory tract infection. For instance, it has been shown that higher viral load is a risk factor for the development of respiratory complications such as lower respiratory infection, bronchial hyperreactivity and respiratory failure, leading to prolonged hospitalization [20]. Here, statistical analysis revealed a significant correlation between higher viral load and increased symptoms severity, a manifestation that is associated with increased vascular permeability and stimulation of mucus hypersecretion during RV infection [18]. Several studies have also demonstrated the immunomechanism, whereby an increased production of interleukin-10 (IL-10) following heightened RV replication leads to an attenuated type 1 T helper (Th1) immune response, resulting in an increased symptom severity of acute respiratory infection [18]. However, such finding should be interpreted with caution as the present study focused primarily on nasopharyngeal viral load in outpatients (median age: 38 years old) with upper respiratory tract symptoms who sought medical care, and may not be reflected in those with lower respiratory illnesses that required hospitalization. Importantly, the inclusion of age and time matched asymptomatic controls are necessary to avoid potential biases.

Studies have shown the ability of viral sequence data in defining transmission clusters, highlighting the importance and advantage of viral genetic information in assessing the epidemic linkages [29, 30]. Here, multiple transmission clusters across RV-A, -B and -C species were observed (33%, 320/976 of RV *VP4/VP2* sequences),

suggesting that the observed RV disease burden was largely linked to multiple sub-epidemics. To the best of our knowledge, the transmission clusters of RV were mapped for the first time at the population level, providing significant insights in understanding the dynamics of RV transmission. However, it is important to acknowledge that the actual number of transmission clusters circulating in the population could have been underestimated due to sampling bias from a single study site. Genealogical analysis estimated that most of the RV transmission clusters originated around 2010, highlighting their recent ancestral origin, though RV-C seemed to have a more diverse and older origin. Such observation suggested that RV-C might have emerged earlier, but went undetected due to the lack of a reliable detection system [6].

In a recent molecular surveillance study of 29 months, it was reported that more than 100 RV types were found circulating during the study period [31], and that the circulating RV types could change over time [32]. In the present study that spanned a period of 27 months, a total of 111 distinct RV types were identified, in which up to fourteen distinct RV types were seen circulating concurrently in the study population within a given week (Supplementary Figure 3). However, it is important to note that the study subjects were recruited from a single study site, potentially leading to the underestimation of prevalence and distribution of circulating RV. In comparison to a study from Malaysia that detected 26 RV types in children presented with respiratory infections [33], a more diverse population of RV types were detected in adults in the present study. Although this may suggest that the adult population may play an important role in sustaining viral transmission and persistence in the general population, further investigation is necessary to test the hypothesis.

316 The impact of meteorological factors has been shown to correlate with
317 incidence and seasonality of respiratory viruses [12]. For instance, temperate winters
318 appeared to boost viral transmission by increasing the viral survivability in aerosols
319 and on surfaces. However, the effects of air pollutant such as PM_{10} remain
320 insufficiently reported. Malaysia has a tropical equatorial climate accompanied by the
321 Southwest Monsoon (spans between May and September) and Northeast Monsoon
322 (November to March) rainy seasons, of which, the Northeast Monsoon brings more
323 rainfall as compared to Southwest Monsoon. As anticipated, the peaks of total rain
324 fall, number of rain days and relative humidity coincided with the Malaysian
325 Northeast Monsoon. Statistical analysis revealed that relative humidity was the
326 strongest predictor for RV infections, in congruence with finding reported elsewhere
327 [12]. It has been reported that RV is more stable and viable in conditions with high
328 humidity, which extends the protective effect of droplets on viruses trapped on
329 fomites or aerosols [12, 34]. Although the direct effects of other meteorological
330 parameters and PM_{10} on RV prevalence were not observed, it has been shown that
331 relative humidity has a positive correlation with the number of rainy day and amount
332 of rainfall, while exhibiting negative correlation with mean ground temperature and
333 PM_{10}, suggesting a multifactorial contribution to the RV seasonality and incidence.
334 To have a thorough assessment, other factors such as fine particulate matter
335 ($PPM_{2.5}$) and oxidant pollutant levels (NO_2, and O_3), upon availability, should also be
336 taken into consideration. Likewise, the effects of these meteorological factors that
337 may alter human behavior, such as staying indoor during rainy seasons, which in
338 turn create a proxy for close contact RV transmission should also be investigated
339 further {Eggo, 2016 #11690}{Pica, 2012 #12864}.

Given the fact that RV is one of the most prevalent respiratory viruses, we believe that the burden of RV infections in Kuala Lumpur could be higher than documented. Also, since the proviso in analyzing evolutionary history more accurately relies on the depth of population-based sampling, a study of such nature should be continued and expanded to more recruitment centres in different countries to improve the resolution of RV genomic diversity and transmission dynamics.

Nevertheless, our data reveal that RV contributed to nearly half of acute viral respiratory tract infections in adult outpatients. Remarkably, RV-C and high viral load were shown to be the important determinants of the severity of acute respiratory illnesses. The phylogeny-based transmission clusters of RV were mapped for the first time, suggesting that the high RV disease burden in the population was largely linked to multiple sub-epidemics involving RV-A and -C. The detection of diverse RV types highlights the enormous genetic complexity and rapid evolution of circulating RV that warrant continuous molecular surveillance at the population level. Finally, the seasonality of RV in the tropical Southeast Asia region was largely influenced by the relative humidity in the environment.

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Author Contributions

Conceived and designed the experiments: K.T.N. and K.K.T. Performed the experiments: K.T.N., X.Y.O., J.B.C. and K.K.T. Analyzed the data: K.T.N., X.Y.O., S.H.L., J.B.C. and K.K.T. Contributed reagents/material: K.T.N., X.Y.O., J.B.C., Y.T., Y.F.C., K.G.C., N.S.H., Y.K.P., A.K. and K.K.T. Wrote the paper: K.T.N. and K.K.T. All authors reviewed the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

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Figure legends

Figure 1. Phylogenetic transmission clusters of RV-A, -B and -C *VP4/VP2* gene. Neighbour-joining trees based on global RV *VP4/VP2* sequence data (3,397 sequences) are shown. Phylogeny reconstructions indicated that the 976 Malaysian strains were classified as **(a)** RV-A (n=473), **(b)** RV-B (n=131), and **(c)** RV-C (n=372). A total of 111 distinct RV types (or serotypes) were identified and indicated at the tips of the tree (marked with parentheses). Eighty-three transmission clusters (filled circles) were observed across RV-A (28 clusters, size ranges between 2-9), RV-B (15 clusters, size ranges between 2-11) and RV-C (40 clusters, size ranges between 2-11). Newly sequenced RV that do not form transmission clusters were indicated (hollow circles). The statistical significance of the branching order was validated by bootstrap analysis of 1,000 replicates and the scale bar represents the nucleotide substitutions per site. **(d)** Bar chart illustrating the monthly distribution of RV-A, -B and -C infection in Kuala Lumpur, Malaysia, between March 2012 and May 2014.

Figure 2. The dynamics of population viral load in the upper respiratory tract during symptomatic phase of acute RV infection. (a) Boxplot illustrating the changes of RV viral load (copies/µl) during the course of acute respiratory tract infection. The regressive trendline highlighted a significant difference in median viral load as the estimated number of days from the onset of symptoms increased (p<0.001). **(b)** The difference in viral load between RV species during the symptomatic phase of acute RV infection. RV-C showed a significantly higher viral

493 load than RV-A and RV-B (p<0.001), particularly at day 1-2 (p=0.006). The
494 difference in viral load between RV-A and RV-B was not statistically significant. **(c)**
495 RV viral load and the symptom severity of acute respiratory tract infections during
496 the symptomatic phase of acute RV infection. The comparison of viral load between
497 TSSS groups (a = 1-8, b = 9-16 and c = 17-24) indicated that higher TSSS was
498 significantly associated with higher viral load (p=0.017), particularly at day 1-2
499 (p=0.012).
500
501 **Figure 3. Seasonality of RV infections and meteorological profiles in Kuala**
502 **Lumpur, Malaysia between March 2012 and May 2014.** Bar and line charts
503 illustrating the trends between RV incidence and meteorological factors were
504 depicted. Meteorological data from February 2012 were omitted due to the
505 incomplete sampling period. The number of RV-infected cases was higher during the
506 months where the number of rain days, total amount of rain fall and relative humidity
507 were high, while the ground temperature and air pollutant (PM_{10}) readings were low.
508 The increased or decreased (arrows) in meteorological readings that coincided with
509 the annual peak of RV detection between October and December were indicated.
510
511 **Table 1:** Clinical manifestations in patients presented with acute respiratory
512 infections.
513
514 **Supplementary Figure 1. Pie charts summarizing the distribution of RV types**
515 **that formed transmission clusters**. A total of 83 transmission clusters, inferred
516 from neigbour-joining tree, were observed across RV-A (28 clusters), RV-B (15
517 clusters) and RV-C (40 clusters), with the predominance of RV-A32 (14%, 4/28), RV-

B79 (27%, 4/15), B69 (27%, 4/15) and C22 (10%, 4/40). The number of clusters observed in each RV types are indicated in parentheses.

Supplementary Figure 2. Divergence times of RV-A, -B and -C transmission clusters among subjects presented with acute respiratory tract infections in Kuala Lumpur, Malaysia. The divergence times (in calendar years) of the RV transmission clusters and the 95% highest posterior distribution were estimated based on the *VP4/VP2* gene in BEAST software. The Bayesian coalescent relaxed clock-based analysis was performed using a general time-reversible nucleotide substitutions model with a gamma-distributed among-site rate variation. It was estimated that the divergence times of RV-A and -C transmission clusters were dated around early 2000s to mid-2010s, while the divergence times of RV-B types were dated more recently than that of RV-A and -C.

Supplementary Figure 3. Line chart illustrating the number of circulating RV types between February 2012 and May 2014. A total of 111 distinct RV types were identified, in which up to fourteen distinct RV types (indicated in red) were seen circulating concurrently in the study population within a given week.

Supplementary Table 1: Demographic Table

Supplementary Table 2: Rhinovirus (RV) viral load among RV-positive subjects at different symptomatic phase (in days).

Supplementary Table 3: Viral load of RV-A, -B and -C among RV-positive subjects at different symptomatic phase (in days).

Supplementary Table 4: Rhinovirus (RV) viral load among RV-positive subjects (with different TSSS) at different symptomatic phase (in days).

Supplementary Table 5: Viral load of RV-A, -B and -C among RV-positive subjects (with different TSSS) at different symptomatic phase (in days).

Supplementary Table 6: Linear correlation and regression between meteorological factors and number of RV cases.