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ORIGINAL PAPER

Year-Long Rhinovirus Infection is Influenced by Atmospheric Conditions, Outdoor Air Virus Presence, and Immune System-Related Genetic Polymorphisms

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Abstract

Rhinovirus is a common picornavirus with over 150 serotypes and three species, which is responsible for half of the human common cold cases. In people with chronic respiratory conditions and elders, it may also cause life-threatening diseases. Transmission routes are not definitively established but may involve direct human-to-human and indirect transmission (surfaces and aerosols based). In the present study, year-long presence of virus was tested by qPCR in the nostrils of young healthy volunteers and indoor and outdoor air samples. Results were correlated to atmospheric conditions (meteorological and air quality parameters) and voluntaries immune system-related genetic polymorphisms (TOLLIP rs5743899, IL6 rs1800795, IL1B rs16944, TNFA rs1800629) typed by PCR–RFLP. Nasal samples showed increased frequency and viral titers of Rhinovirus in spring and autumn. No indoor air samples tested positive for Rhinovirus, whereas outdoor air samples tested positive in late autumn. Sun radiation, atmospheric SO_2, and benzene levels correlated with nostrils Rhinovirus detection. Both IL6 and TOLLIP polymorphisms but not TNFA or IL1B influenced Rhinovirus detection in the nostrils of voluntaries. Taken together, the results indicate that Rhinovirus circulation is determined by environmental conditions (weather, air-borne virus, and air pollution) and genetically encoded individual variation in immunity.

Keywords Rhinovirus · Environment · Polymorphism · Air-borne virus · Viral susceptibility

Abbreviation

RHV	Rhinovirus

Introduction

Human Rhinovirus (RHV) is a very common human virus with over 150 different serotypes and three species (Basnet et al. 2019; Palmenberg et al. 2009), causing more than half of the common cold cases (Blaas and Fuchs 2016). In addition to its involvement in this mild, naturally resolving infection, development of molecular-based multiplex detection has allowed the identification of its role among more severe acute respiratory infections (To et al. 2017). Indeed, it is now recognized as a frequent finding in acute respiratory infections (Jeon et al. 2019) leading to a significant risk (OR = 7.1) for this pathology in older adults (Shi et al. 2019). It is also involved in life-threatening disease in elders and individuals with chronic respiratory diseases (asthma, pulmonary obstructive disease, and cystic fibrosis) (Blaas and Fuchs 2016; Linden et al. 2019), and has even been found in association with at least one case of encephalopathy (Hazama et al. 2019). Rhinovirus has also been a frequent finding among hospitalized patients, in particular among young babies (Leotte et al. 2017; Wildenbeest et al. 2016) and patients undergoing chemotherapy (Aydin-Köker et al. 2019) or hematopoietic stem-cell transplantation (Pochon and Voigt 2019). Additionally, even if only common cold cases are taken into account, the abundance of these RHV-induced cases presents a social and economic burden including school and work absenteeism that should not be

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disregarded (Bertino 2002; Fendrick et al. 2003; Nichol et al. 2005; Roelen et al. 2011).

RHV is among the smallest RNA viruses (picornavirus = Pico RNA virus) and infects the upper and sometimes lower respiratory apparatus (Berrajah et al. 2018; Gern 2002; Jacobs et al. 2013; McErlean et al. 2008) via its cellular receptors (ICAM-1, LDLR, or CDHR3) (Basnet et al. 2019; Blaas and Fuchs 2016; Bochkov and Gern 2016). The large frequency of infection may be related to the large serological diversity and the low immune response cross-reactivity (Blaas and Fuchs 2016). Additionally, interindividual variation in the susceptibility to RHV infection may be related to genetically encoded immunological variation (Drysdale et al. 2016), but this has not been clearly demonstrated.

Rhinovirus inoculation usually occurs via the nose, mouth, or eyes (Blaas and Fuchs 2016). An important susceptibility factor may be environmental temperature as it impacts on the viral activity, which could favor its infection during the cold season and in the upper respiratory tract (Blaas and Fuchs 2016). Rhinovirus transmission has been the subject of some controversy but there is direct evidence of person-to-person and indirect transmission (via surfaces or aerosols). RHV is clearly stable for hours in objects (Ikonen et al. 2018) but aerosol transmission is certainly also a transmission route (La-Rosa et al. 2013; Myatt et al. 2003, 2004). RHV infection seasonality may be even related with viral stability in objects and aerosols which may be affected by meteorological conditions (temperature, humidity, UV index) (Du-Prel et al. 2009) or the chemical stability/reactivity of the transmission route (air pollution) (Myatt et al. 2003). However, a recent study has failed to detect significant association between RHV positivity and temperature, humidity, or dew point (Price et al. 2019) clearly indicating that more studies are needed to clarify this point. Pollutants such as ozone and nitrogen oxides and even air-borne particles may also impact on the respiratory tract immunology (Kaan and Hegele 2003; Miller and Peden 2014; Peden and Bush 2015; Romero et al. 2011; Spannhake et al. 2002; Yang and Marr 2011), making air parameters a double sword affecting not only viral stability and activity but also potentially modulating immune system responses.

In the last decades, polymorphisms in several cytokine genes (soluble modulators of immune system activity) have been described, indicating the genetically encoded immunological variation is not just MHC encoded. Thus, interindividual viral susceptibility may also be affected by these gene variations. Indeed, several studies have indicated polymorphisms in TOLLIP, lambda interferon, IL1B, IL8, IL6, and TNFA among others to influence immunological response to viral infections (Doyle et al. 2010; Huang et al. 2016; Noah et al. 1995; Russell et al. 2014; Wine and Alper 2012). Since the immune response to viral agents is significantly different before and after viral cell entry, different polymorphisms impacting on different components of the immune system could have a different effect on viral infectivity. In particular, TLR and TOLLIP polymorphisms can be expected to impact on the detection of free viral particles by TLR2 (Huang et al. 2016; Shah et al. 2012). Immune System response after infection may be more dependent on viral RNA recognition by TLR7 and TLR8, which is dependent on stress signals induced via IL6, making polymorphisms in these genes more relevant for viral clearance after infection (Doyle et al. 2007; O'Neill and Bowie 2007).

In the present work, we have searched for environment and genetic factors influencing the presence of RHV in the nostrils of young healthy volunteers. For that purpose, we tested by qPCR the year-long presence of RHV in the nostrils of a group of young volunteers. Concomitantly indoor and outdoor air samples were tested for the presence of RHV by the same method, and meteorological and air quality parameters have been recorded. Also, voluntaries were genotyped by PCR–RFLP for polymorphisms in 4 immune system-related genes (TOLLIP rs5743899, IL6 rs1800795, IL1B rs16944, TNFA rs1800629).

Materials and Methods

Population Studied

In this study, a total of 89 healthy young volunteers were enrolled, recruited from among the student population of the Faculty of Health Sciences of the Fernando Pessoa University in Porto, Portugal. The volunteers were aged between 20 and 41 years with females accounting for 2/3 of the volunteers. Each student provided a monthly swab sample collected for the characterization of the year-long presence of rhinovirus in the nostrils, and once during the observation period a blood sample or mouth endothelial cell cotton swab (if unwilling to donate blood) for DNA extraction and purification. At first appointment, voluntaries were asked to fill in a questionnaire characterizing themselves on socio-demographic aspects, health living style, and known disease/allergy preconditions.

Nasal Sample Collection

Voluntaries came monthly for nasal swab collection. Samples were collected from each nostril using sterile cotton swabs. Swabs from left and right nostrils were stored together in 200 µL of RNALatter (Invitrogen, USA) solution for 24 h at 4 °C as recommended by the manufacturer to allow the RNA stabilizer to embed the sample and prevent RNA decay, and then transferred to a deep freezer (– 70 °C) until RNA extraction procedures. At each appointment, voluntaries were asked to fill in a questionnaire about symptoms experienced in the last 30 days.

Viral RNA Extraction

No viral elution from swabs or filters was performed as viral lysis was performed in the collection material. Viral RNA extraction was performed using QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Final elution volume was 60 µL. RNA samples were stored at −70 °C.

Rhinovirus RNA Detection and Quantification

Rhinovirus RNA amplification was performed by qPCR on a Lightcycler 1.1 (Roche, Switzerland) as previously described (Myatt et al. 2004). Briefly, the primers RAF (5′-GACAGTGTTCYAGCCTGCG-3′), RBF (5′-RACHGTGTCYYAGCCTGCG-3′), and LR (5′-GAAACACGGACACCCAAAGTA-3′) were used at a final concentration of 800 nM together with the Taqman Probe (6-FAM-5′-TCCTCCGGCCCCTGAATGYGGC-3′-BHQ1) at 160 nM in a Quantitec Probe RT-PCR Master Mix (Qiagen, Germany). The reaction then took place on a Lightcycler 1.1 thermocycler (Roche, Switzerland). First a reverse transcription step was performed at 50 °C for 20 min followed by the RT enzyme inactivation and polymerase activation at 95 °C for 15 min. Real-time PCR was then performed using 45 cycles consisting of a denaturation step at 95 °C for zero seconds, and an annealing/polymerization step at 60 °C for 1 min. Fluorescence reading was done at the end of the amplification step. Semi-quantification was obtained relative to a dilution series of a positive sample.

Air Sample Collection

Indoor and outdoor air samples were collected as previously described (Myatt et al. 2004). Briefly, air was filtered for 1 h at a 4 L/min rate using 37 mm 2.0 µm Teflon filters (SKC, UK) in closed cassettes with the help of an air pump (Leland legacy single pump, SKC, UK). Filters were then cut in small pieces under sterile conditions using fresh sterile disposable scalpels and treated as described for the nasal samples. Air samples were always collected at the same spots. Outdoor air samples were collected outside a third-floor lab window at facilities not used for any other manipulation of the project samples. Indoor air samples were collected at a university cafeteria, a common meeting point for students and staff, on top of a balcony not usually manipulated by the cafeteria staff or clients.

Meteorological and Air Quality Data

Daily meteorological parameters were collected from the FP-ENAS meteorological station and the public air quality monitoring infrastructure. Data were used from the station closest to the university facilities where voluntaries spend most of their time. Meteorological parameters analyzed were wind speed, temperature, radiation, humidity, and air pressure. Air quality parameters analyzed were ozone, nitrogen oxides, carbon monoxide, sulfur dioxide, and benzene levels.

DNA Preparation

Genomic DNA was extracted from blood or epithelial mouth cells using Qiamp mini Blood DNA kit (Qiagen, Germany) according to the manufacturer's instruction. Genomic DNA was eluted with 200 µL of nuclease-free water and stored at −20 °C.

Genotyping was performed by PCR–RFLP using previously described primers and enzymes as described in Table 1. Briefly 5 µL of DNA were mixed with iProof High-Fidelity Master Mix (BioRad, USA), and 1 µM of each primer in a total volume of 20 µL and subjected to the thermal cycling conditions in Table 1. After PCR, 10 µL of the amplicon was mixed with the manufacturer-provided enzyme buffer and respective restriction endonuclease (New England Biolabs, UK; Table 1) for 2 h at 37 °C. PCR

Table 1 Experimental conditions used for genotyping

Gene/SNP	Primers	PCR thermal cycling	RE enzyme
TOLLIP (de Araujo et al. 2015) rs5743899	5′-GGCAATGGCAGTGGCCACCAGTGA-3′ 5′-CCGATGCCCGCACACCTGTGTGAT-3′	3 min—98 °C 35× [30 s—98 °C; 30 s—65 °C; 30 s—72 °C]	HhaI
IL6 (Honsawek et al. 2011) rs1800795	5′-TGACTTCAGCTTTACTCTTTGT-3′ 5′-CTGATTGGAAACCTTATTAAG-3′	3 min—98 °C 35× [30 s—98 °C; 30 s—55 °C; 30 s—72 °C]	SFaNI
IL1B (Ishii et al. 2000) rs16944	5′-TGGCATTGATCTGGTTCATC-3′ 5′-GTTTAGGAATCTTCCCACTT-3′	3 min—98 °C 35× [30 s—98 °C; 30 s—60 °C; 30 s—72 °C]	AvaI
TNFA (Sandhya et al. 2013) rs1800629	5′AGGCAATAGGTTTTGAGGGCCAT-3′ 5′TCCTCCCTGCTCCGATTC-3′	3 min—98 °C 35× [30 s—98 °C; 15 s—63 °C; 30 s—72 °C]	NcoI

RE restriction endonuclease

restriction fragments were size separated by electrophoresis in 4% NuSieve 3:1 TAE agarose gel (FMC, USA).

Statistical Analysis

Data were statistically analyzed using Statistical Package for Social Sciences (SPSS) version 22. Comparisons between RHV-positive and RHV-negative samples and between individuals with or without a specific allele were done by the Student's *t* test. Correlation between variables was analyzed using Pearson Correlation. All statistical analyses were performed at a significance level of 95%.

Ethical and Legal Aspects

The study was approved by the Fernando Pessoa University Ethical Committee. All voluntaries were explained the study, signed an informed consent, and were given the possibility to drop the study at any moment. The database with the study results was authorized and registered by the Portuguese National Committee for Personal Data Protection.

Results

RHV Detection in Nostrils of Voluntaries

As can be seen in Fig. 1, RHV was detected in the nostrils of voluntaries during the entire observation period with the exception of January 2018. The monthly frequency of positive samples showed a minimum of 0% in January and peaked to over 40% in November. A similar trend was observed for the RHV titers with a maximum RHV concentration in November but with a smaller peak in March/April.

Next, we tested the relation between detection of RHV in voluntaries and the self-reporting of common cold-associated symptoms. Results showed that RHV was more frequently found among individuals self-reporting the presence of common cold symptom (16%) then among individuals without them (6%). Interestingly, individuals with RHV in the nostrils but not reporting symptoms were very rare and only occurred from August to December, the period of increased RHV incidence, likely reflecting early RHV detection before symptoms arise which one would expect to be more likely in periods of high RHV circulation.

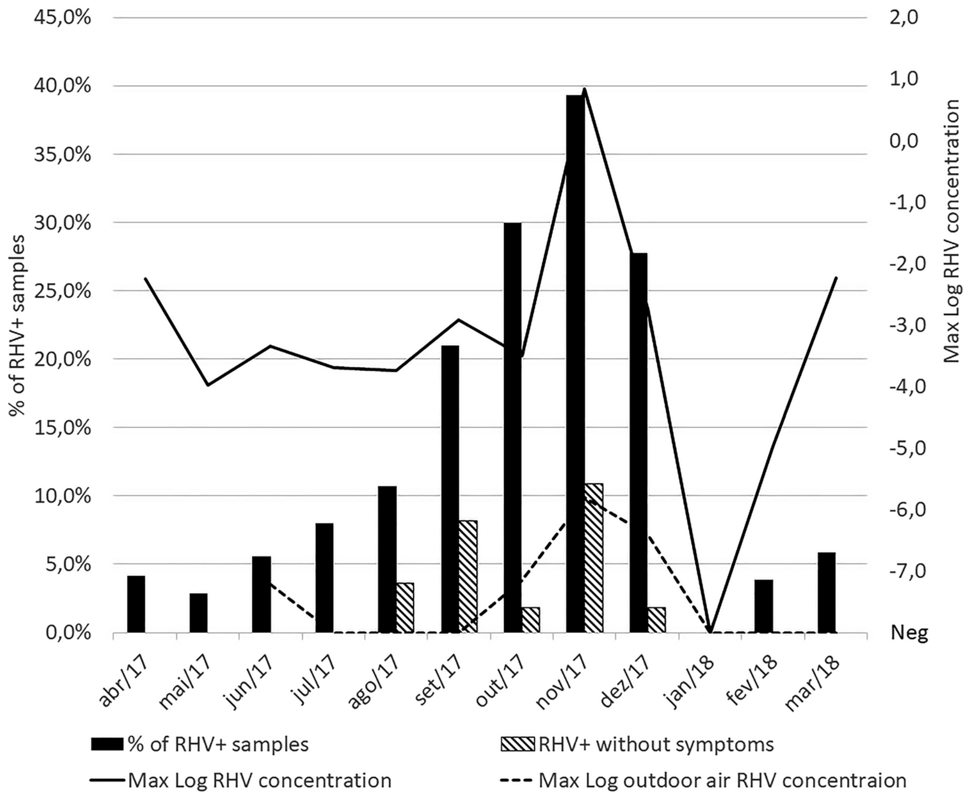

Fig. 1 Frequency of positive nasal swab samples (filled bars) versus nasal RHV RNA titer (continuous line) and outdoor air RHV RNA titer (dotted line). Concentration was measured as relative to one positive control sample from which a tenfold series dilution was performed and tested. Slashed bars indicate the frequency of RHV-positive nasal samples in individuals not reporting common cold symptoms

RHV Detection in Air Samples

Contrary to our expectations, no indoor air samples were found positive for RHV. However, during the period of higher nostril RHV incidence, positive outdoor air samples were found from October to December, thus coincident to the months of higher frequency of RHV in the nostrils of voluntaries (Fig. 1). Furthermore, the observed RHV concentrations in nostrils and in Air samples showed a similar pattern, although with clearly different scales (Fig. 1). In fact, a statistically significant correlation was found between nostril RHV concentration and outdoor air RHV concentration ($R=0.985$; $p<0.0001$).

RHV Nostril Detection and Meteorological and Air Quality Parameters

Next, we investigated whether meteorological and air quality parameters were somehow related to the frequency of RHV-positive samples. As can be seen in Fig. 2, sun radiation was significantly lower when positive RHV samples were found. This is also reflected on a tendency for a negative correlation between radiation and the percentage of RHV-positive samples, but it does not reach statistical significance. Similarly, levels of sulfur dioxide were significantly lower when positive samples were detected ($p<0.0001$) allowing a negative correlation between maximum SO_2 levels and the percentage of RHV+ nasal swabs (Fig. 3; $R=-0.843$; $p<0.009$). Interestingly, the inverse association was found between nasal swab RHV positivity and benzene atmospheric levels (Fig. 4). Higher benzene levels were associated with positive RHV samples, and a significant positive correlation was found (Fig. 4; $R=0.809$; $p<0.001$).

Influence of Genetic Polymorphisms on RHV Nostril Detection

No difference was found between individuals carrying the various alleles of IL1B (rs16944) and TNFA (rs1800629). However, the presence of the G allele of the TOLLIP rs5743899 SNP significantly increased ($p<0.048$) the likelihood of testing negative for RHV during the entire observation period (Fig. 5). Additionally, if this effect of the TOLLIP SNP is excluded by considering only individuals without the TOLLIC G allele, a statistically significant ($p<0.028$) higher viral titer is found among individuals with the IL6 rs1800795 C allele (Fig. 6).

Discussion

The present results indicate a year-long circulation of RHV, with an increase in incidence during late Autumn–early winter period. Additionally, an extra peak of RHV activity was found in spring when viral concentrations were taken into consideration which suggests a seasonal variation not only in viral infectivity but also in infection severity indicating that

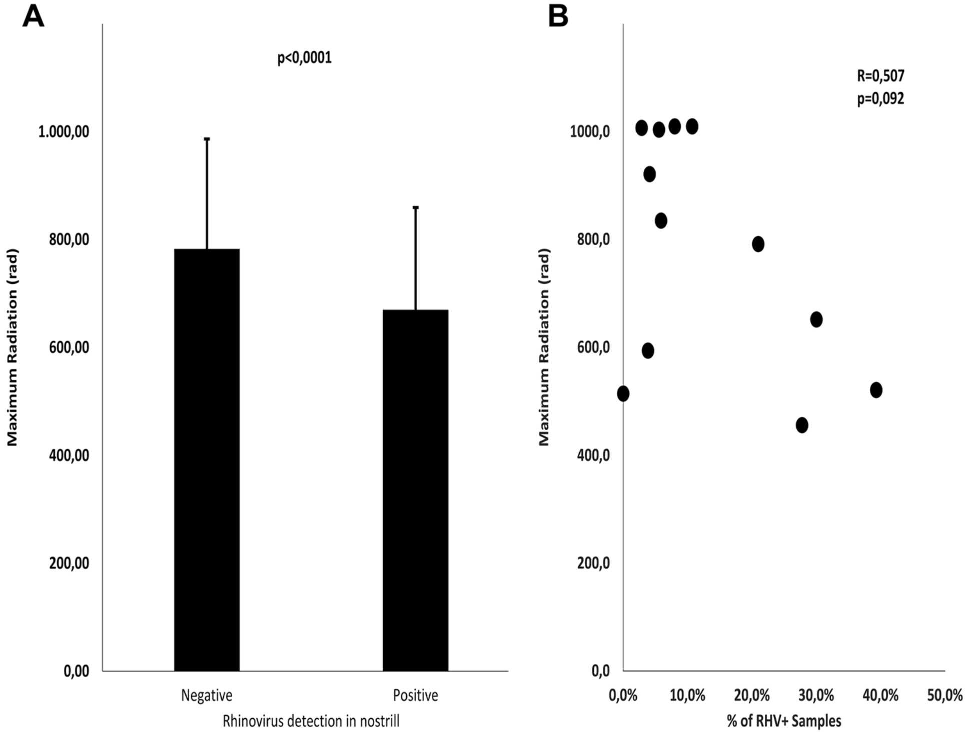

Fig. 2 Maximum monthly radiation versus RHV detection in the nostril of voluntaries. **a** Negative samples are associated with higher levels of sun maximum radiation, and positive samples with lower levels. **b** This produces a negative correlation between maximum monthly radiation and the percentage of monthly positive samples, but it does not reach statistical significance (lack of significance can be attributed to two outliers with both low percentage of RHV+ samples and low maximum sun radiation corresponding to the observations of January and February 2018)

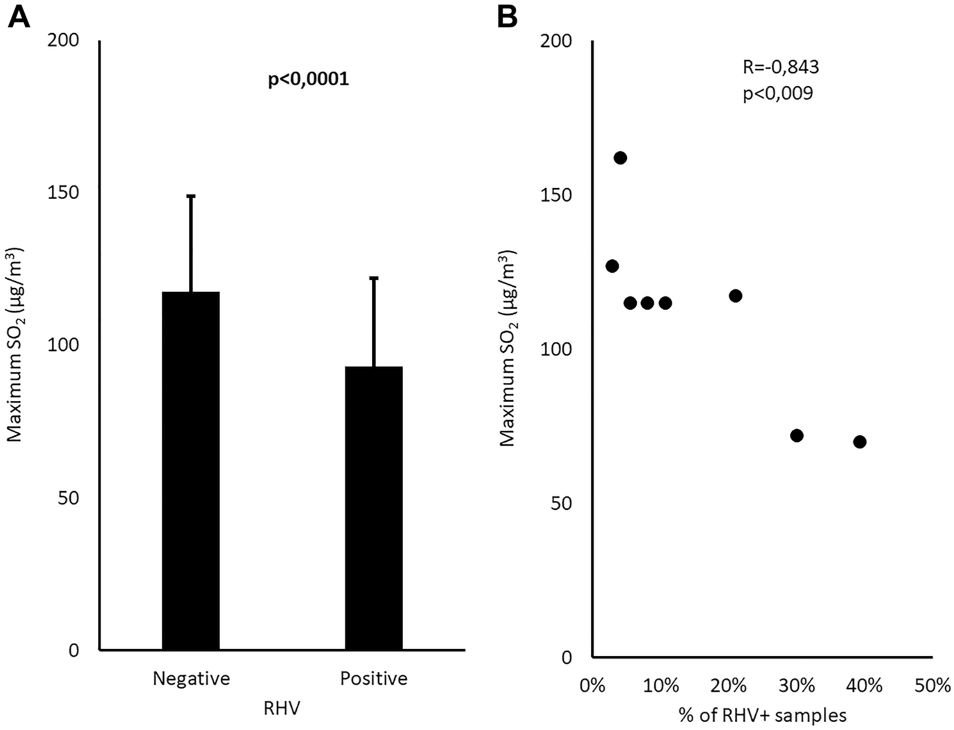

Fig. 3 Levels of atmospheric Sulfur dioxide versus nasal swab RHV positivity. **a** Atmospheric SO_2 levels were found to be lower when nasal swabs test positive for RHV. **b** This produces a negative correlation between maximum levels of SO_2 in the atmosphere and the percentage of nasal swabs positive for RHV ($R = -0.843$; $p < 0.009$)

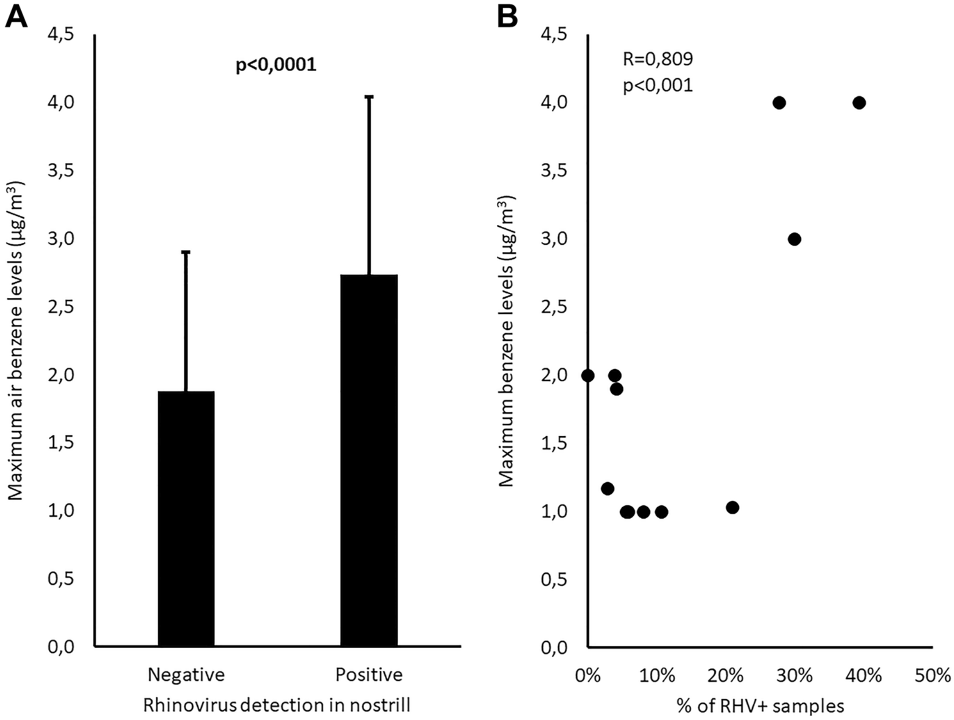

Fig. 4 Levels of atmospheric benzene versus nasal swab RHV positivity. **a** Atmospheric benzene levels were found to be higher when nasal swabs test positive for RHV. **b** This produces a positive correlation between benzene atmospheric levels and the percentage of nasal swabs positive for RHV ($R = -0.809$; $p < 0.001$)

viral concentration should also be assayed when studying the epidemiology of RHV infection.

RHV aerosol transmission is surely an active transmission RHV route (La-Rosa et al. 2013; Myatt et al. 2003, 2004), but is usually interpreted as only impacting on direct person–person transmissions. The present results support an outdoor air RHV transmission route as outdoor air RHV closely correlated to nasal RHV presence and concentration even if they do not allow to establish a definite causal effect. Confirmation of this would require the genotyping of

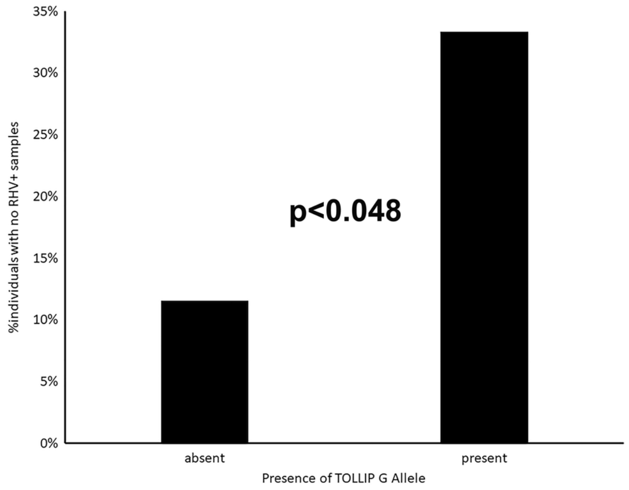

Fig. 5 The percentage of individuals that test negative for nasal RHV during the entire observation period is significantly higher ($p < 0.048$) among individuals with the TOLLIP rs5743899 G allele

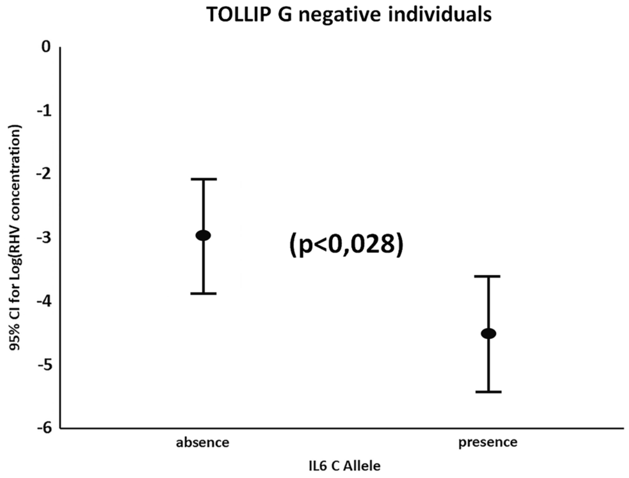

Fig. 6 When considering only individuals without the TOLLIP rs5743899 G allele, the nasal RHV concentration was found to be dependent on the presence of the IL6 rs1800795 C allele, with a significant ($p < 0.028$) lower RHV concentration being found among individuals with the IL6 C allele

the viruses detected, something that should be addressed in follow-up studies. Furthermore, the availability of sequencing data would also help clarify whether voluntaries with consecutive positive samples result from persistent infections or from reinfections.

Another interesting evidence supporting the air RHV transmission was the finding that atmospheric parameters that may affect atmospheric viral stability such as sun radiation (Myatt et al. 2003) and SO_2 levels were negatively correlated to RHV nostril detection. Interestingly, these are exactly the parameters long ago described to show virucidal effect in an experimental aerosol exposure of Venezuelan Equine Encephalomyelitis Virus (Berendt et al. 1972) suggesting a direct environmental exposure effect on the virus particles. However, experimental exposure of animals to viruses also showed a collaborative effect of SO_2 and influenza on short-term exposure (Lebowitz and Fairchild 1973), suggesting a dual role for SO_2 (decreased viral stability but increased immune system susceptibility) that could complicate analysis. This is also suggested by Asian results indicating a positive correlation between SO_2 and lower respiratory infections, but only during the dry season (Le et al. 2012), a period with also high radiation levels, thus making the SO_2 virucidal effect redundant and potentiating the effect of SO_2 on the immune system. Interestingly, benzene, an air pollutant previously described to potentially condition immune system status by decreasing immune system cells in blood and spleen and decrease immune system progenitor cells leaving the bone marrow (Wen et al. 2016; Zhang et al. 2010) showed a positive correlation with RHV detection in nostrils, further suggesting that air quality may affect not only virus stability and infectivity but also modulate immune system responses to virus. A similar effect has also been demonstrated by others for syncytial respiratory virus (Nenna et al. 2017) suggesting this to be an effect that may be relevant for several viral-associated pathologies.

The lack of detection of RHV in indoor air samples came as a surprising result. Previous work by others has shown the detection of RHV in closed environments reflecting virus shedding (Myatt et al. 2004). However, our results may reflect the unintended proximity of an air conditioning exhaust to the site of air collection, possibly indicating the efficiency of the air conditioning at clearing the air from viral particles. This result suggests that air conditioning filters should be examined in future experiments as it could indicate an easy to implement purification system during winter, especially in retirement houses where RHV infection severity and morbidity may be especially important.

TOLLIP is a negative regulator of TLR2 (Shah et al. 2012; Zhang and Ghosh 2002). In turn, TLR2 is an important immune system surveillance receptor needed for RHV capsid recognition before the virus enters the cells (Huang et al. 2016; Triantafilou et al. 2011). The TOLLIP rs5743899 G allele is known to decrease TOLLIP expression (Huang et al. 2016), therefore increasing TLR2 expression, which in turn contributes to better RHV detection and clearance by the immune system before viral cell entry (Bentley et al. 2019), effectively preventing infection. Additionally, a recent paper has shown that Tollip inhibits ST2 signaling in RHV-exposed airway epithelial cells, making the lower expression of TOLLIP a way of counteracting this inhibitory pathway, and achieving better RHV particle detection and clearance, even if it may lead to increased neutrophilic inflammation (Dakhama et al. 2019). After RHV enters cells, its recognition shifts from TLR2 to TLR7 and TLR8 which recognize viral single-stranded RNA (O'Neill and Bowie 2007). These receptors are known to send an emergency signal via the induction of IL6 (Doyle et al. 2007) which is a critical anti-inflammatory regulator of viral-induced immunopathology in the respiratory tract (Pyle et al. 2017). This IL6 pathway is therefore likely only relevant after viral cell entry and is thus independent of the TLR2-related TOLLIP effect we propose above. IL6 rs1800795 has been associated with lower gene expression which may counteract the increased expression stimulated by some viral components (Tanaka et al. 2014). However, since TOLLIP has been shown to sustain production of IL6 in several inflammatory conditions (Humbert-Claude et al. 2016; Kowalski and Li 2017), the effect of the IL6 gene polymorphism may only be seen when TOLLIP-induced sustained IL6 production is not taking place, which may explain why, in our results, the presence of the TOLLIP G allele masks the IL6 C allele effect. In the absence of the TOLLIP G allele, the absence of the IL6 C allele decreases IL6 levels, thus decreasing the emergency response to RHV after infection is established leading to higher viral titers found.

Conclusions

The present results confirm an autumn/winter increase in the frequency of RHV infections as reported by others (Jeon et al. 2019; Price et al. 2019; Reis and Shaman 2018; To et al. 2017), while also revealing an increase in the viral titers in positive cases in early spring, which is also in agreement with the previous reports (Leotte et al. 2017; Reis and Shaman 2018; To et al. 2017). A close association between the presence of RNA virus in the nostrils of voluntaries and the self-reporting of common cold symptoms was also found revealing that infection is usually associated with symptoms. The finding of air-borne RHV virus with a frequency and concentration closely resembling the nasal viral detection strongly suggests atmospheric air-carrying of virus as a potentially relevant transmission route. A similar conclusion may be drawn from the finding of environmental factors such as sun radiation and air pollution correlating with virus nostril detection. Interestingly, specific pollutants such as SO_2 or benzene showed differential effects, suggesting a dual-sword effect on virus stability in air and on immune system modulation effects. Both of these aspects deserve further clarification in controlled experimental conditions. Finally, two different polymorphisms have been found to differentially modulate the risk for RHV infection by two complementary mechanisms: TOLLIP rs5743899 G allele may increase resistance to infection, whereas IL6 rs1800795 C allele may decrease RHV titers when infection occurs.

Thus, the present results show that RHV infection is influenced by both environmental factors (meteorological factors, RHV air-borne particles, and air quality determinants) and by genetic-based differential susceptibility to RHV infection.

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Author Contributions Conceptualization: JMC and MEB; Methodology: JMC, MEB, AFR, AMS, AMF, and RM; Software development, Validation, Formal analysis, Resources, Data curation, Supervision, Project administration, Funding acquisition, and Writing—original draft preparation: JMC; Investigation: JMC, MEB, AFR, AMS, AMF, and RM; Writing—review and editing: JMC, MEB, AFR, AMS, AMF, and RM.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest.

Research Involving Human Participants and/or Animals The work was approved by the University Fernando Pessoa Ethics committee and done in accordance with the ethical standards as laid down by the 14964 Declaration of Helsinki and later amendments. All data acquisitions were done using a Individual ID code known only to the voluntary subject. Database was authorized and registered with the National Individual Data Protection committee and contains no data capable of identifying the subject under study.

Informed Consent Informed consent was obtained from all individual participants included in the study, prior to any sample or data acquisition. Informed consent forms were approved by the University ethics committee. Voluntaries were allowed to drop the study at any time during the observation period.

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