Advances in diagnostics of respiratory viruses and insight in clinical implications of rhinovirus infections

Anneloes Liesbeth van Rijn-Klink

Advances in diagnostics of respiratory viruses and insight in clinical implications of rhinovirus infections

Anneloes Liesbeth van Rijn-Klink

Colophon

Cover design by Lodewijk van Rijn Consultancy

Advances in diagnostics of respiratory viruses and insight in clinical implications of rhinovirus infections.

PhD thesis, University of Leiden, The Netherlands

ISBN: 978-94-6380-795-1

Print by ProefschriftMaken, www.proefschriftmaken.nl

Advances in diagnostics of respiratory viruses and insight in clinical implications of rhinovirus infections

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,

volgens besluit van het College voor Promoties

te verdedigen op dinsdag 9 juni 2020

klokke 16:15 uur

door

Anneloes Liesbeth van Rijn-Klink

Geboren 7 maart 1984

te Lelystad

Promotor

Prof. dr. A.C.M. Kroes

Co-promotoren

Dr. J.J.C. de Vries

Dr. E.C.J. Claas

Leden Promotiecommissie

Prof. dr. N. Fischer (University Medical Center Hamburg-Eppendorf)

Prof. dr. P.S. Hiemstra

Prof. dr. H.G.M. Niesters (Universitair Medisch Centrum Groningen)

Dr. P.L.A. Fraaij (Erasmus Medisch Centrum)

Table of contents

PART II

CLINICAL IMPLICATIONS OF RHINOVIRUS

CHAPTER 1

General introduction

RESPIRATORY INFECTIONS

Respiratory infections are among the most common infections in humans and are a major health issue[1,2]. Overall, they are the cause of high morbidity and mortality, and as such associated with high costs due to absence of work and hospitalization, sometimes even in isolation[3-5].

Respiratory infections can be categorized into upper respiratory tract infections and infections of the lower airways. Although upper respiratory tract infections are usually relatively mild, more serious infections occur in specific high-risk groups. The most prevalent and well known upper respiratory tract infection is the common cold. Infections of the lower airways, mainly bronchiolitis and pneumonias, are in general more serious and the leading infectious cause of death world-wide[1,6].

Respiratory infections are caused by a variety of pathogens, but primarily by viruses, with overlapping clinical symptoms. The common cold is a syndrome of upper respiratory tract infections caused by viral pathogens, with rhinovirus being the most prevalent, detected in 40-50% of the cases of the common cold[6-8]. Other common respiratory viruses are respiratory syncytial virus, adenovirus, para-influenza viruses, bocavirus and metapneumovirus[6].

Viruses are the most prevalent pathogens detected in hospitalized adults with community acquired pneumonia (Figure 1). As with upper respiratory tract infections, rhinovirus is the most frequent cause, alone or as co-infection with other viruses or bacteria. Strikingly, in up to 62% of the community-acquired pneumonias no causative agent is found with current diagnostic procedures (Figure 1)[7,8].

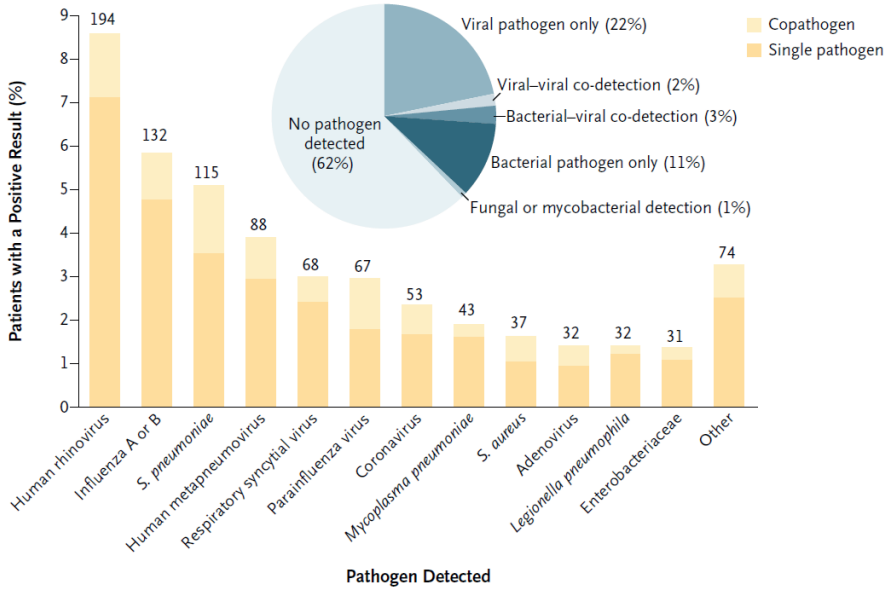


Figure 1. Pathogens detected in adults with community acquired pneumonia requiring hospitalization. (Adapted from Jain et al.[7])

DIAGNOSTICS OF RESPIRATORY INFECTIONS

Respiratory viral causes of infections are hard to distinguish by their clinical presentation, even so from other pathogens such as bacteria. Therefore, a precise diagnosis is important for adequate therapy and isolation measures. Over the years the diagnostic possibilities have expanded and the conventional routine assays, based on viral culture, antigen detection and serology, have been replaced by molecular methods. A routine diagnostic respiratory assay has to be fast, sensitive, specific, with limited hands-on time, cost-effective and has to be able to detect a wide variety of respiratory pathogens.

History of diagnostics of respiratory viruses

In the 1950's, diagnosing viruses was performed by in vitro culture on mono-layer cell lines[9]. Virus culture has been the gold standard for a long time. However, the time to results is considerable and generally too slow for clinical actions. Not only different cell lines and skilled staff are required, but also special safety requirements regarding viable virus[10]. Additionally, some viruses as for example rhinovirus species C, are hard or even impossible to propagate in cell-culture.

Later, new diagnostic options were introduced with antigen detection assays and serology. Antigen detection assays, such as rapid lateral-flow immunoassays and direct fluorescent antibody assays, have been designed for several viruses and are rapid but in general less sensitive and specific[10]. Serology is often limited by the need to detect seroconversion, IgG antibody titer rise or IgM detection, which is less sensitive in case of repeated exposure to for example rhinoviruses. As the induction of a measurable antibody response may take at least a week, the value of serology in the acute phase is limited[10].

In 1985, an *in-vitro* nucleic acid amplification method was designed. The polymerase chain reaction (PCR) relied on exponential amplification and subsequent detection of specific parts of nucleic acid sequences, with much improved sensitivity and specificity. Initially, after the amplification protocol was completed, the amplified PCR products were visualized by gel electrophoreses, southern-blotting or ELISA-like detection systems. In 1992, the real-time PCR concept was launched, which enabled detection of PCR products while they were generated using fluorescent probes. This greatly enhanced the possibilities as it enabled quantitative detection of targets. Real-time PCR is fast, sensitive, specific and has relative low hands-on time. Initially one virus per run was tested, but, using multiple fluorophores that could be differentiated, also duplex and multiplex PCR assays could be designed. However, the amount of targets that could be detected is limited to a maximum of five (in most platforms) by the number of fluorescent probes that could be differentiated and by applying an efficient workflow with batch-wise testing the time to results could be a day[11-13].

Advances in molecular respiratory viral diagnostics

Recently introduced molecular platforms offer the possibility of syndromic testing, as extended multiplex PCRs detect a wide panel of respiratory pathogens implicated in the clinical syndrome. Another benefit is the random and continuous access resulting in very fast and reliable results throughout the day. Whether these super-fast time to results diagnostic tests have clinical implications needs to be determined in more detail[14,15].

Molecular amplification based methods are still limited by the need to pre-define the targets of interest in a diagnostic panel. This is complicated by the high number of possible respiratory pathogens, the genetic diversity of the viruses implicated and the occurrence of new respiratory pathogens. Rare causes are easily overlooked with sometimes great consequences, as encountered for example in the Netherlands during Legionella and Q-fever outbreaks, and MERS-coronavirus in South-Korea[16-18]. The emergence of new clinically relevant respiratory viruses is a real threat, which will be clear to anyone after the devastating appearance of SARS-CoV-2 early in 2020 and indeed, NGS played a crucial role in its initial diagnosis and phylogenetic analysis[19-22]. But in addition, such new viruses may also be present already, without causing a pandemic, like the pigeon paramyxovirus type 1 that was coincidentally found to cause a fatal infection in a stem cell recipient[23]. We can never be informed about the role of such viruses without applying NGS as a broad 'catch all' approach.

While for a large proportion of the pneumonias the causative agent is still unknown, it is suspected that the percentage attributed to viral pathogens is higher than currently is assumed. Therefore there is a need for an unbiased catch-all method (see figure 2).

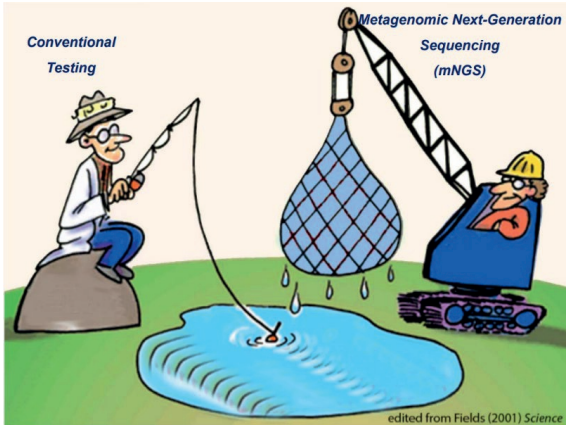


Figure 2. Cartoon representation of selective conventional testing compared to catch-all metagenomic next-generation sequencing.
(adapted from Chiu et al.[24]).

The newest diagnostic development, metagenomic next-generation sequencing (mNGS) is such an unbiased catch-all method, detecting all the genetic material in a sample. Standardized protocols for application of mNGS in the diagnostic field are still lacking and also the time to results and the cost of this approach are reason for concern, although the latter is rapidly declining (figure 3). To use mNGS as routine diagnostic tool, there is a need for fast, automated, combined DNA and RNA pre-treatment and analysis protocols with high sensitivity and specificity.

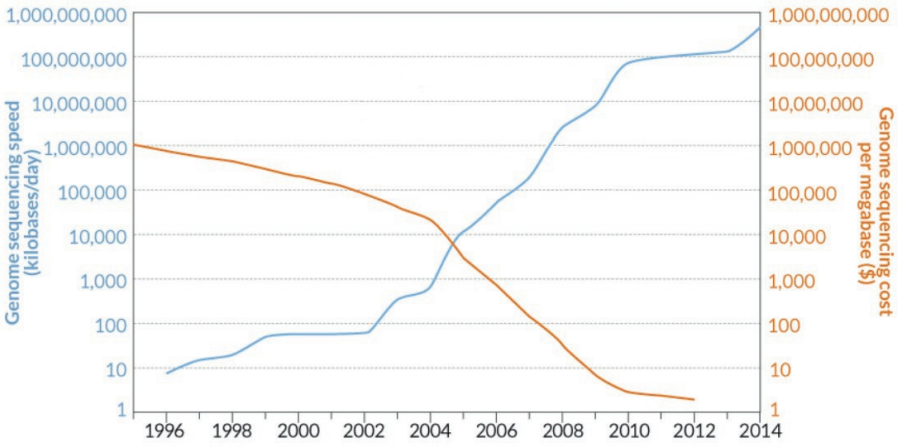


Figure 3. Improvements in costs and speed of genome sequencing.
(adapted from Otwell[25]).

Another advantage of this catch-all technique is that additional information is provided on strain characteristics, genotyping, susceptibility and virulence markers, pathogen evolution and the virome in health and disease. The respiratory virome and its correlation to clinical data is not yet studied extensively.

RHINOVIRUSES

Rhinoviruses are single stranded positive-sense RNA viruses in the family Picornaviridae and the genus *Enterovirus*. Since their discovery in the 1950s over 160 types, subdivided in species A, B and C, have been discovered[26,27] (Figure 4).

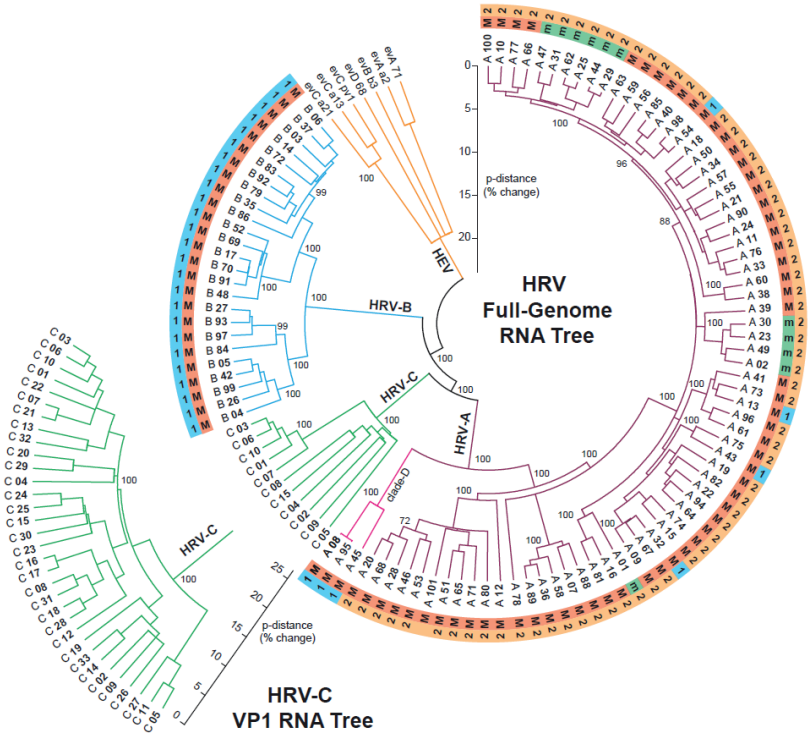


Figure 4. Phylogenetic tree of rhinovirus.
(adapted from Fields et al.[28])

The genome of rhinoviruses is approximately 7,200 bp long and consists of a single open reading-frame that encodes a large polyprotein of nearly 2200 amino acids, which is cleaved to produce 11 viral proteins (VP). VP1, VP2, VP3 and VP4 compose the viral capsid that embeds the RNA, while the non-structural proteins are involved in replication and assembly. At the 5' end of the genome the rhinoviruses have a long untranslated region (UTR), with internal ribosome entry sites (IRES), for initiation of translation. The 3' end untranslated region is much shorter and enables efficient replication[26,29], figure 5.

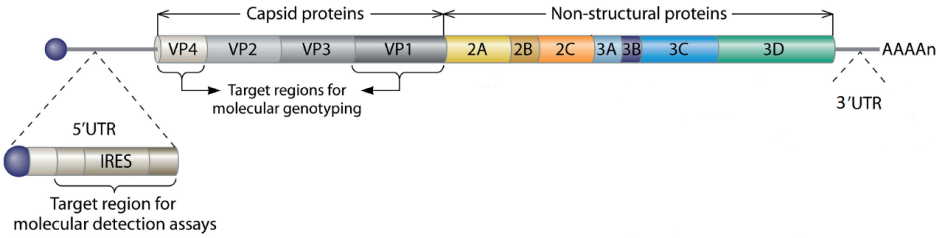


Figure 5. The genome structure of rhinoviruses.
UTR, untranslated region. IRES, internal ribosome entry site (Adapted from Jacobs et al.[26])

Epidemiology of rhinoviruses

Rhinoviruses have a worldwide distribution with a high prevalence, especially in young children. Although rhinovirus infections occur all year round, they have a peak incidence in early fall and spring[30-33].

Rhinoviruses are transmitted by direct contact, droplets or aerosols, but most important experimental evidence supports efficient transmission occurring through (in)direct contact mainly through hands, after which the virus attaches and replicates in the nasal mucosa, with an additional role for aerosol transmission[34-39].

Clinical manifestations of rhinoviruses

Rhinoviruses can cause a wide variety of respiratory symptoms, but asymptomatic shedding does occur as well. Asymptomatic infection tends to be more prevalent in younger patients and was even found in 12-32% of children under the age of four[40-45].

The most frequently encountered symptomatic presentation of rhinovirus infection is the, self-limiting, common cold. This usually starts with a sore throat followed by nasal obstruction and rhinorrhea, but a variety of symptoms has been observed. Rhinoviruses can also cause (rhino)sinusitis and otitis media, but in those cases co-infection with bacteria is common. Although rhinoviruses have long been considered relatively mild viruses, causing benign upper respiratory tract infections, they are now implicated in more serious lower respiratory tract infections in high-risk groups. They have been associated with the development of asthma, asthma and COPD exacerbations, bronchiolitis and life-threatening pneumonia, mainly in children and the elderly[7,26,46-48].

The development of more serious infections by rhinovirus is probably multifactorial and dependent on both host and environmental factors (Figure 6). Though several factors associated with more severe disease are known, much remains unknown.

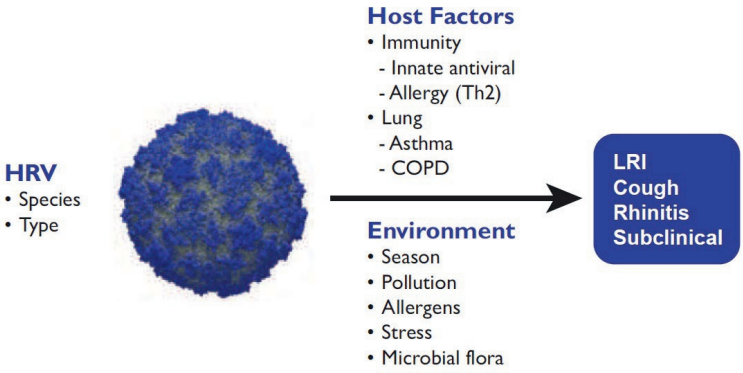


Figure 6. Spectrum of human rhinovirus infections and factors involved.
(adapted from Fields' Virology [28])

More severe rhinovirus infections have been shown in elderly patients, immunocompromised patients and patients with underlying chronic lung disease[49-53]. In children, risk factors for more serious infections are prematurity, congenital heart disease, respiratory syncytial virus co-infections, and non-infectious underlying respiratory disease. Finally, rhinoviruses have been associated with a complicated post-operative course after cardiac surgery[54-56]. In children, higher viral loads and viremia have been found in association to higher disease severity and more extensive clinical symptoms[57-65]. Whether rhinovirus viremia does occur in adults and is associated with more severe disease is still unknown.

Another gap in knowledge exists on whether rhinovirus infection can lead to serious complications in case of extreme stress and special circumstances, for example in children undergoing cardiac surgery.

In addition, the association of specific rhinovirus types to more serious disease is still open for debate. Some studies showed rhinovirus type C to cause more severe illness, while others failed to demonstrate a difference between the rhinovirus types[48,66-70]. A longitudinal study with a large number of patients with a variety of rhinovirus types is needed to determine the influence of rhinovirus species on disease severity.

Although rhinovirus infections have been associated with mortality, the major disease burden is caused by the high frequency of infections, the duration of illness (a median of 7 days) and the associated economic burden[29,46,71].

Treatment and prevention

There is currently no licensed treatment available for rhinovirus infections, although several antiviral treatments have been described or are under development[26,72]. Therefore, at the moment, the best way to prevent rhinovirus infections is adequate hand hygiene measures[73].

SCOPE OF THIS THESIS

The research presented in this thesis aims to determine the implications and performance of new viral respiratory diagnostic methods and the aspects of the disease severity of the most common respiratory virus, rhinovirus.

Part I: Application and added value of advanced respiratory viral diagnostic methods

In this part, advanced diagnostic methods for respiratory infections were studied to determine their clinical implications and their performance, when applied to routine diagnostics.

An in-house developed diagnostic mNGS protocol, with simultaneous RNA and DNA detection, was developed and compared with real-time PCR (chapter two). This protocol was used to study respiratory infections and the respiratory virome in patients with COPD exacerbations and to correlate these results with clinical data and real-time PCR (chapter three).

Rapid molecular syndromic testing by an innovative automated amplification platform was compared to lab-developed multiplex real-time PCR assays, focusing on the difference in time to results and its implications for clinical decision making, regarding isolation, and antimicrobial therapy (chapter four).

Part II: Clinical implications of rhinovirus

In this part, rhinovirus is studied in different patient populations to determine the clinical impact and factors influencing the course of the disease. To determine whether rhinovirus infections, symptomatic or asymptomatic, have a negative effect on the post-operative course of children undergoing cardiac surgery, a large prospective study screening all children at the time of their operation was performed (chapter five).

A retrospective study to detect rhinovirus viremia and its association with disease severity was performed in adult patients with high rhinovirus loads in bronchoalveolar lavage (chapter six). The difference in disease severity between different rhinovirus species and types was studied in a prospective study in an adult population in general practices throughout Europe (chapter seven).

REFERENCES

1. WHO -The top 10 causes of death. https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death. Last access 17-10-2019. who top 10 death (accessed 31-05-2019.
2. WHO- The global impact of respiratory disease, second edition. https://www.who.int/gard/publications/The_Global_Impact_of_Respiratory_Disease.pdf?ua=1. Last access 17-10-2019. (accessed 19-09-2019.
3. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory tract infections in 195 countries: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet Infectious diseases* 2017; **17**(11): 1133-61.
4. Trucchi C, Paganino C, Orsi A, et al. Hospital and economic burden of influenza-like illness and lower respiratory tract infection in adults >/=50 years-old. *BMC health services research* 2019; **19**(1): 585.
5. HCUP- Costs for hospital stays in the United States, 2011.https://www.hcup-us.ahrq.gov/reports/statbriefs/sb168-Hospital-Costs-United-States-2011.jsp. Last access 17-10-2019.
6. Mandell GLB, J.E; Dolin, R. The common cold. Priciples and practice of infectious diseases: 809-14.
7. Jain S, Self WH, Wunderink RG, et al. Community-Acquired Pneumonia Requiring Hospitalization among U.S. Adults. *N Engl J Med* 2015; **373**(5): 415-27.
8. Ieven M, Coenen S, Loens K, et al. Aetiology of lower respiratory tract infection in adults in primary care: a prospective study in 11 European countries. *Clinical microbiology and infection* 2018; **24**(11): 1158-63.
9. Zuckerman B, Schoub, Griffiths, Mortimer. Principles and practices of clinical virology: wiley.
10. Loeffelholz M, Chonmaitree T. Advances in diagnosis of respiratory virus infections. *Int J Microbiol* 2010; **2010**: 126049-.
11. Mackay IM. Real-time PCR in the microbiology laboratory. *Clinical Microbiology and infection* 2004; **10**(3): 190-212.
12. Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science (New York, NY)* 1985; **230**(4732): 1350-4.
13. Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. *Biotechnology* 1992; **10**(4): 413-7.
14. Nijhuis RH, Guerendiain D, Claas EC, Templeton KE. Comparison of the ePlex(R) Respiratory Pathogen Panel with Laboratory Developed Real-Time PCR for the Detection of Respiratory Pathogens. *Journal of clinical microbiology* 2017; **55**(6): 1938-45.
15. Huang HS, Tsai CL, Chang J, Hsu TC, Lin S, Lee CC. Multiplex PCR system for the rapid diagnosis of respiratory virus infection: systematic review and meta-analysis. *Clinical Microbiology and Infection* 2018; **24**(10): 1055-63.
16. W Den Boer J, Yzerman E, Schellekens J, et al. A Large Outbreak of Legionnaires' Disease at a Flower Show, the Netherlands, 1999. *Emerging infectious diseases* 2002; **8**(1): 37-43.
17. van der Hoek W, Morroy G, Renders NH, et al. Epidemic Q fever in humans in the Netherlands. *Advances in experimental medicine and biology* 2012; **984**: 329-64.
18. Kim KH, Tandi TE, Choi JW, Moon JM, Kim MS. Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak in South Korea, 2015: epidemiology, characteristics and public health implications. *The Journal of hospital infection* 2017; **95**(2): 207-13.
19. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. *Nature* 2020.
20. Ji W, Wang W, Zhao X, Zai J, Li X. Cross-species transmission of the newly identified coronavirus 2019-nCoV. *Journal of medical virology* 2020; **92**(4): 433-40.
21. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020.
22. Zhu N, Zhang D, Wang W, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med* 2020; **382**(8): 727-33.
23. Kuiken T, Breitbart M, Beer M, et al. Zoonotic Infection With Pigeon Paramyxovirus Type 1 Linked to Fatal Pneumonia. *The Journal of infectious diseases* 2018; **218**(7): 1037-44.
24. Chiu C. UCSF-Metagenomic approaches to diagnosis of infectious diseases. https://impaactnetwork.org/DocFiles/LabCenter/LabRetreat/2017/Chiu_IMPAACT_Lab_Retreat_25JAN17.pdf. Last access 17-10-2019.
25. Science news-The gene sequencing future is here. https://www.sciencenews.org/article/gene-sequencing-future-here. Last access 17-10-2019.
26. Jacobs SE, Lamson DM, St George K, Walsh TJ. Human rhinoviruses. *Clinical microbiology reviews* 2013; **26**(1): 135-62.

27. ICTV. Genus: Enterovirus. https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/picornavirales/w/picornaviridae/681/genus-enterovirus Date last update: 02-2019 . Last access 25-07-2019.
28. J G, A P. Rhinoviruses. Fields virology, 6th edition. Philadelphia, PA.: Wolters Kluwer Health/Lippincott Williams & Wilkins.
29. Mandell GLB, J,E,; Dolin, R. Rhinovirus. Principles and practice of infectious diseases: 2389-98.
30. Galanti M, Birger R, Ud-Dean M, et al. Longitudinal active sampling for respiratory viral infections across age groups. *Influenza and other respiratory viruses* 2019; **13**(3): 226-32.
31. Aydin Koker S, Demirag B, Tahta N, et al. A 3-Year Retrospective Study of the Epidemiology of Acute Respiratory Viral Infections in Pediatric Patients With Cancer Undergoing Chemotherapy. *Journal of pediatric hematology/oncology* 2019; **41**(4): e242-e6.
32. Winther B, Hayden FG, Hendley JO. Picornavirus infections in children diagnosed by RT-PCR during longitudinal surveillance with weekly sampling: Association with symptomatic illness and effect of season. *Journal of medical virology* 2006; **78**(5): 644-50.
33. Miller EK, Lu X, Erdman DD, et al. Rhinovirus-associated hospitalizations in young children. *The Journal of infectious diseases* 2007; **195**(6): 773-81.
34. Dick EC, Jennings LC, Mink KA, Wartgow CD, Inhorn SL. Aerosol transmission of rhinovirus colds. *The Journal of infectious diseases* 1987; **156**(3): 442-8.
35. Hendley JO, Gwaltney JM, Jr. Mechanisms of transmission of rhinovirus infections. *Epidemiologic reviews* 1988; **10**: 243-58.
36. Gwaltney JM, Jr., Moskalski PB, Hendley JO. Hand-to-hand transmission of rhinovirus colds. *Annals of internal medicine* 1978; **88**(4): 463-7.
37. Ikonen N, Savolainen-Kopra C, Enstone JE, et al. Deposition of respiratory virus pathogens on frequently touched surfaces at airports. *BMC infectious diseases* 2018; **18**(1): 437.
38. Rodrigues AF, Santos AM, Ferreira AM, Marino R, Barreira ME, Cabeda JM. Year-Long Rhinovirus Infection is Influenced by Atmospheric Conditions, Outdoor Air Virus Presence, and Immune System-Related Genetic Polymorphisms. *Food and environmental virology* 2019.
39. Kutter JS, Spronken MI, Fraaij PL, Fouchier RA, Herfst S. Transmission routes of respiratory viruses among humans. *Current opinion in virology* 2018; **28**: 142-51.
40. Singleton RJ, Bulkow LR, Miernyk K, et al. Viral respiratory infections in hospitalized and community control children in Alaska. *Journal of medical virology* 2010; **82**(7): 1282-90.
41. Nokso-Koivisto J, Kinnari TJ, Lindahl P, Hovi T, Pitkaranta A. Human picornavirus and coronavirus RNA in nasopharynx of children without concurrent respiratory symptoms. *Journal of medical virology* 2002; **66**(3): 417-20.
42. van Benten I, Koopman L, Niesters B, et al. Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants. *Pediatric allergy and immunology* 2003; **14**(5): 363-70.
43. Iwane MK, Prill MM, Lu X, et al. Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. *The Journal of infectious diseases* 2011; **204**(11): 1702-10.
44. Fry AM, Lu X, Olsen SJ, et al. Human rhinovirus infections in rural Thailand: epidemiological evidence for rhinovirus as both pathogen and bystander. *PloS one* 2011; **6**(3): e17780.
45. Birger R, Morita H, Comito D, et al. Asymptomatic Shedding of Respiratory Virus among an Ambulatory Population across Seasons. *mSphere* 2018; **3**(4).
46. Choi SH, Huh JW, Hong SB, et al. Clinical characteristics and outcomes of severe rhinovirus-associated pneumonia identified by bronchoscopic bronchoalveolar lavage in adults: comparison with severe influenza virus-associated pneumonia. *Journal of clinical Virology* 2015; **62**: 41-7.
47. Bergroth E, Aakula M, Elenius V, et al. Rhinovirus Type in Severe Bronchiolitis and the Development of Asthma. *The journal of allergy and clinical immunology In practice* 2019.
48. Lambert KA, Prendergast LA, Dharmage SC, et al. The role of human rhinovirus (HRV) species on asthma exacerbation severity in children and adolescents. *The journal of asthma* 2018; **55**(6): 596-602.
49. Bruning AHL, Thomas XV, van der Linden L, et al. Clinical, virological and epidemiological characteristics of rhinovirus infections in early childhood: A comparison between non-hospitalised and hospitalised children. *Journal of Clinical Virology* 2015; **73**: 120-6.
50. Louie JK, Yagi S, Nelson FA, et al. Rhinovirus outbreak in a long term care facility for elderly persons associated with unusually high mortality. *Clinical infectious diseases* 2005; **41**(2): 262-5.
51. Kraft CS, Jacob JT, Sears MH, Burd EM, Caliendo AM, Lyon GM. Severity of human rhinovirus infection in immunocompromised adults is similar to that of 2009 H1N1 influenza. *Journal of clinical microbiology* 2012; **50**(3): 1061-3.

52. Gunawardana N, Finney L, Johnston SL, Mallia P. Experimental rhinovirus infection in COPD: implications for antiviral therapies. *Antiviral research* 2014; **102**: 95-105.
53. Piralla A, Zecca M, Comoli P, Girello A, Maccario R, Baldanti F. Persistent rhinovirus infection in pediatric hematopoietic stem cell transplant recipients with impaired cellular immunity. *Journal of Clinical Virology* 2015; **67**: 38-42.
54. Costa LF, Queiroz DA, Lopes da Silveira H, et al. Human rhinovirus and disease severity in children. *Pediatrics* 2014; **133**(2): e312-21.
55. Simsic J, Phelps C, Yates A, Galantowicz M. Management strategies after cardiac surgery in an infant with human rhinovirus. *Pediatric cardiology* 2013; **34**(8): 1922-4.
56. Delgado-Corcoran C, Witte MK, Ampofo K, Castillo R, Bodily S, Bratton SL. The impact of human rhinovirus infection in pediatric patients undergoing heart surgery. *Pediatric cardiology* 2014; **35**(8): 1387-94.
57. Esposito S, Daleno C, Scala A, et al. Impact of rhinovirus nasopharyngeal viral load and viremia on severity of respiratory infections in children. *European journal of clinical microbiology & infectious diseases* 2014; **33**(1): 41-8.
58. Urquhart GE, Grist NR. Virological studies of sudden, unexplained infant deaths in Glasgow 1967-70. *Journal of clinical pathology* 1972; **25**(5): 443-6.
59. Urquhart GE, Stott EJ. Rhinoviraemia. *British medical journal* 1970; **4**(5726): 28-30.
60. Fuji N, Suzuki A, Lupisan S, et al. Detection of human rhinovirus C viral genome in blood among children with severe respiratory infections in the Philippines. *PloS one* 2011; **6**(11): e27247.
61. Tapparel C, L'Huillier AG, Rougemont AL, Beghetti M, Barazzone-Argiroffo C, Kaiser L. Pneumonia and pericarditis in a child with HRV-C infection: a case report. *Journal of Clinical Virology* 2009; **45**(2): 157-60.
62. Xatzipsalti M, Kyrana S, Tsolia M, et al. Rhinovirus viremia in children with respiratory infections. *American journal of respiratory and critical care medicine* 2005; **172**(8): 1037-40.
63. Lupo J, Schuffenecker I, Morel-Baccard C, et al. Disseminated rhinovirus C8 infection with infectious virus in blood and fatal outcome in a child with repeated episodes of bronchiolitis. *Journal of clinical microbiology* 2015; **53**(5): 1775-7.
64. Las Heras J, Swanson VL. Sudden death of an infant with rhinovirus infection complicating bronchial asthma: case report. *Pediatric pathology* 1983; **1**(3): 319-23.
65. Granados A, Peci A, McGeer A, Gubbay JB. Influenza and rhinovirus viral load and disease severity in upper respiratory tract infections. *Journal of Clinical Virology* 2017; **86**: 14-9.
66. Lauinger IL, Bible JM, Halligan EP, et al. Patient characteristics and severity of human rhinovirus infections in children. *Journal of Clinical Virology* 2013; **58**(1): 216-20.
67. Xiang Z, Gonzalez R, Xie Z, et al. Human rhinovirus C infections mirror those of human rhinovirus A in children with community-acquired pneumonia. *Journal of Clinical Virology* 2010; **49**(2): 94-9.
68. Jacobs SE, Lamson DM, Soave R, et al. Clinical and molecular epidemiology of human rhinovirus infections in patients with hematologic malignancy. *Journal of Clinical Virology* 2015; **71**: 51-8.
69. Choi SH, Hong SB, Kim T, et al. Clinical and molecular characterization of rhinoviruses A, B, and C in adult patients with pneumonia. *Journal of Clinical Virology* 2015; **63**: 70-5.
70. Hasegawa K, Jartti T, Bochkov YA, et al. Rhinovirus Species in Children With Severe Bronchiolitis: Multicenter Cohort Studies in the United States and Finland. *The Pediatric infectious disease journal* 2019; **38**(3): e59-e62.
71. Fendrick AM, Monto AS, Nightengale B, Sarnes M. The economic burden of non-influenza-related viral respiratory tract infection in the United States. *Archives of internal medicine* 2003; **163**(4): 487-94.
72. Casanova V, Sousa FH, Stevens C, Barlow PG. Antiviral therapeutic approaches for human rhinovirus infections. *Future Virol* 2018; **13**(7): 505-18.
73. Jefferson T, Del Mar CB, Dooley L, et al. Physical interventions to interrupt or reduce the spread of respiratory viruses. *The Cochrane database of systematic reviews* 2011; (7): Cd006207.

APPLICATION AND ADDED VALUE OF ADVANCED RESPIRATORY VIRAL DIAGNOSTIC METHODS

PART I

CHAPTER 2

Retrospective validation of a metagenomic sequencing protocol for combined detection of RNA and DNA viruses using respiratory samples from paediatric patients

S. van Boheemen[a#], A. L. van Rijn[a#], N. Pappas[b], E.C. Carbo[a], R.H.P. Vorderman[b], I. Sidorov[a], P.J. van`t Hof[b], H. Mei[b], E.C.J. Claas[a], A.C.M. Kroes[a*], J.J.C. de Vries[*$]

[a] Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands
[b] Sequencing Analysis Support Core, Department of Biomedical Data Sciences, Leiden University Medical Center, Leiden, The Netherlands
[#] These authors contributed equally to this work
[*] These authors contributed equally to this work

Journal of molecular diagnostics 2020 22 (2): 196-207

ABSTRACT

Viruses are the main cause of respiratory tract infections. Metagenomic next-generation sequencing (mNGS) enables unbiased detection of all potential pathogens. To apply mNGS in viral diagnostics, sensitive and simultaneous detection of RNA and DNA viruses is needed. Herein, were studied the performance of an in-house mNGS protocol for routine diagnostics of viral respiratory infections with potential for automated pan-pathogen detection. The sequencing protocol and bioinformatics analysis were designed and optimized, including exogenous internal controls. Subsequently, the protocol was retrospectively validated using 25 clinical respiratory samples. The developed protocol using Illumina NextSeq 500 sequencing showed high repeatability. Use of the National Center for Biotechnology Information's RefSeq database as opposed to the National Center for Biotechnology Information's nucleotide database led to enhanced specificity of classification of viral pathogens. A correlation was established between read counts and PCR cycle threshold value. Sensitivity of mNGS, compared with PCR, varied up to 83%, with specificity of 94%, dependent on the cutoff for defining positive mNGS results. Viral pathogens only detected by mNGS, not present in the routine diagnostic workflow, were influenza C, KI polyomavirus, cytomegalovirus, and enterovirus. Sensitivity and analytical specificity of this mNGS protocol were comparable to PCR and higher when considering off-PCR target viral pathogens. One single test detected all potential viral pathogens and simultaneously obtained detailed information on detected viruses.

INTRODUCTION

Respiratory tract infections pose a great burden on public health, causing extensive morbidity and mortality among patients worldwide[1-3]. The majority of acute respiratory infections is caused by viruses, such as rhinovirus (RV), influenza (INF) A and B viruses, metapneumovirus (MPV), and respiratory syncytial virus (RSV)[4]. However, in 20-62% of the patients, no pathogen is detected[4-6]. This might be the result of diagnostic failures or even infection by unknown pathogens, such as the Middle East respiratory syndrome coronavirus (MERS-CoV), in 2012[7].

Rapid identification of the respiratory pathogen is critical to determine downstream decision-making such as isolation measures or treatment, including cessation of antibiotic therapy. Current diagnostic amplification methods as real-time polymerase chain reaction (qPCR) are very sensitive and specific, but are only targeting predefined virus species or types. Genetic diversity within the virus genome and the sheer number of potential pathogens in many clinical conditions pose limitations to predefined primer and probe based approaches, leading to false negative results[8]. These limitations, combined with the potential emergence of new or unusual pathogens highlight the need for less restricted approaches that could improve the diagnosis and subsequent outbreak management of infectious diseases.

Metagenomics relates to the study of the complete genomic content in a complex mixture of (micro)organisms[9]. Unlike bacteria, viruses do not display a common gene in all virus families, and therefore pan-virus detection relies on catch-all analytic methods. Metagenomics or untargeted next-generation sequencing (mNGS) offers a culture and nucleotide-sequence-independent method that eliminates the need to define the targets for diagnosis beforehand. Besides primary detection, mNGS immediately offers additional information, on virulence markers, epidemiology, genotyping, and evolution of pathogens[7,10-12]. Furthermore, quantitative assessment of the presence of virus copies in the sample is enabled by the number reads[8].

While original mNGS studies typically aim at analysis of (shifts in) population diversity of abundant DNA microbes, detection of viral pathogens in patient samples requires a different technical approach because of 1) the usually very low abundance of viral pathogens (<1%) in clinical samples and 2) the requisite of detecting both DNA and RNA viruses. Hence, a low limit of detection for RNA and DNA in one single assay is essential for implementation of mNGS for routine pathogen detection in clinical diagnostic laboratories. Current viral mNGS protocols are optimized for either RNA or DNA detection[11,13-15]. Consequently, detection of both RNA and DNA viruses requires parallel work-up of both RNA and DNA pre-treatment methods. Additionally, to increase the relative concentration of viral sequences, viral particle enrichment techniques are often applied[8,12]. These techniques are laborious and not easily automated for routine clinical diagnostic use. Moreover, during enrichment directed at viral particles, intracellular viral nucleic acids as genomes and mRNAs are being discarded. Following sequencing, the bioinformatic classification and interpretation of the results remain a major challenge. Bioinformatic classifiers are often developed for usage in either microbiome studies or classification of high abundant reads whereas extensive validation for clinical diagnostic usage in settings of very low abundance is very limited. After bioinformatics classification, the challenge remains to discriminate between viruses that play a role in disease aetiology and non-pathogenic

viruses[16]. Before considering mNGS in routine diagnostics, there is a need for critical evaluation and validation of every step in the procedure.

In this study, we evaluated a metagenomic protocol for NGS-based pathogen detection with sample pre-treatment for DNA and RNA in a single tube. The method was validated using a selection of 25 respiratory paediatric samples with in total 29 positive and 346 negative viral PCR results. The main study objective was to define a sensitive and specific method for mNGS to be used as a broad diagnostic tool for viral respiratory diseases with the potential for automated pan-pathogen detection.

MATERIAL AND METHODS

Sample selection

Twenty-five stored clinical respiratory samples (-80 ºC) from paediatric patients, sent to the microbiological laboratory for routine viral diagnostics in 2016, were selected from the laboratory database (general laboratory information management system, MIPS, Ghent, Belgium) at the Leiden University Medical Center (LUMC). Based on previous PCR test results, a variety of 21 positive and four negative respiratory virus samples with a wide range of quantification cycle (Cq) values were included. The sample types represented routine diagnostic samples from paediatric patients that had been sent to our laboratory: 19 nasopharyngeal washings , two sputa, two broncho-alveolar lavages (BAL), one bronchial washing and one throat swab (in viral transport medium). The patient selection (age range 1.2 months – 15 years) represented the paediatric population with respiratory diagnostics in our university hospital in terms of (underlying) illness.

Sample pre-treatment

Total nucleid acids (NA) were extracted directly from 200 µL of clinical material using the MagNAPure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics, Almere, the Netherlands) with 100 µL output eluate.

Internal controls

Clinical material was spiked with equine arteritis virus (EAV) and phocine herpesvirus 1 (PhHV1, kindly provided by prof. dr. H.G.M. Niesters, UMC Groningen, the Netherlands), as internal controls for RNA detection[17] and DNA detection respectively[18]. To determine the optimal concentration of the internal controls a ten-fold dilution series of PhHV1/EAV was added to a mix of two pooled influenza A positive throat swabs (Cq value 25) and read count and Cq values were compared. Concentration was based on the number of mNGS reads.

Quality control

Before sequencing the DNA input concentration was measured with the Qubit (ThermoFisher Scientific, Waltham, USA), to determine whether there was sufficient DNA in the sample to obtain sequencing results. The range of DNA input for library preparation was 0.5 ng/µl for throat swabs (see reproducibility experiment) up to 300 ng/µl for bronchoalveolar lavages and sputa.

Fragmentation

To compare the effect of different DNA fragmentation techniques, six PCR positive (containing one to three viruses) and three PCR negative samples were 1) chemically fragmented using zinc (10 min.) as part of the NEB (New England Biolabs) Library Prep Kit protocol as described below (see library preparation) and 2) physically fragmented using sonication with the Bioruptor® pico (Diagenode, Seraing, Belgium, on/off time: 18/30s, 5 cycli)[19]. Three samples were also tested with the 3) high intensity settings of the Bioruptor® pico (on/off time: 30/40s, 14 cycli).

Library preparation

Libraries were constructed with 7µL extracted nucleic acids using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England Biolabs, IpsWich, USA) using single, unique adaptors. This kit has been developed for transcriptome analyses. We made several adaptations to the manufacturers protocol in order to enable simultaneous detection of both DNA and RNA viruses: the following steps were omitted: Poly A mRNA capture isolation (Instruction manual NEB #E7420S/L, version 8.0, Chapter 1), rRNA depletion and DNase step (Chapter 2.1-2.4, 2.5B, 2.11A).

The size of fragments in the library was 300-700 bp. Adaptors were diluted 30 fold given the low RNA/DNA input and 21 PCR cycli were run post-adaptor ligation.

Nucleotide Sequence Analysis

Sequencing was performed on Illumina HiSeq 4000 and NextSeq 500 sequencing systems (Illumina, San Diego, CA, USA), obtaining 10 million 150 bp paired-end reads per sample.

Detection limit

To determine the detection limit of mNGS, serial dilutions (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of an influenza A positive sample was tested with both mNGS and lab developed real-time PCR . Based on run-off transcript experiments the typical limit of detection of our real-time RNA PCRs was estimated to be 10-50 copies/reaction (data not shown).

Repeatability (within run precision)

To estimate the reproducibility of metagenomic sequencing an influenza A positive clinical sample (throat swab) was divided into four aliquots, nucleic acids were extracted, library preparation and subsequent sequence analysis on the Illumina HiSeq 4000 was performed in one run.

Bioinformatics: taxonomic classification

All FASTQ files were processed using the BIOPET Gears pipeline version 0.9.0 developed at the LUMC (*http://biopet-docs.readthedocs.io/en/stable/* accessed 9-12-2018). This pipeline performs FASTQ pre-processing (including quality control, quality trimming and adapter clipping) and taxonomic classification of sequencing reads. In this project, FastQC version 0.11.2 (*https://www.bioinformatics.babraham.ac.uk/projects/fastqc/* accessed 9-12-2018*)* was used for checking the quality of the raw reads. Low quality read trimming was done using Sickle[20] version 1.33 with default settings. Adapter clipping was performed using Cutadapt[21] version 1.10 with default settings. Taxonomic classification of reads was performed with Centrifuge[22] version 1.0.1-beta. The pre-built NT index, which contains all sequences from NCBI's nucleotide database, provided by the Centrifuge developers was used (*ftp://ftp.ccb.jhu.edu/pub/infphilo/centrifuge/data/old-indices* accessed 16-11-2017) as the reference database. An overview of the bioinformatic process is shown in Figure 1.

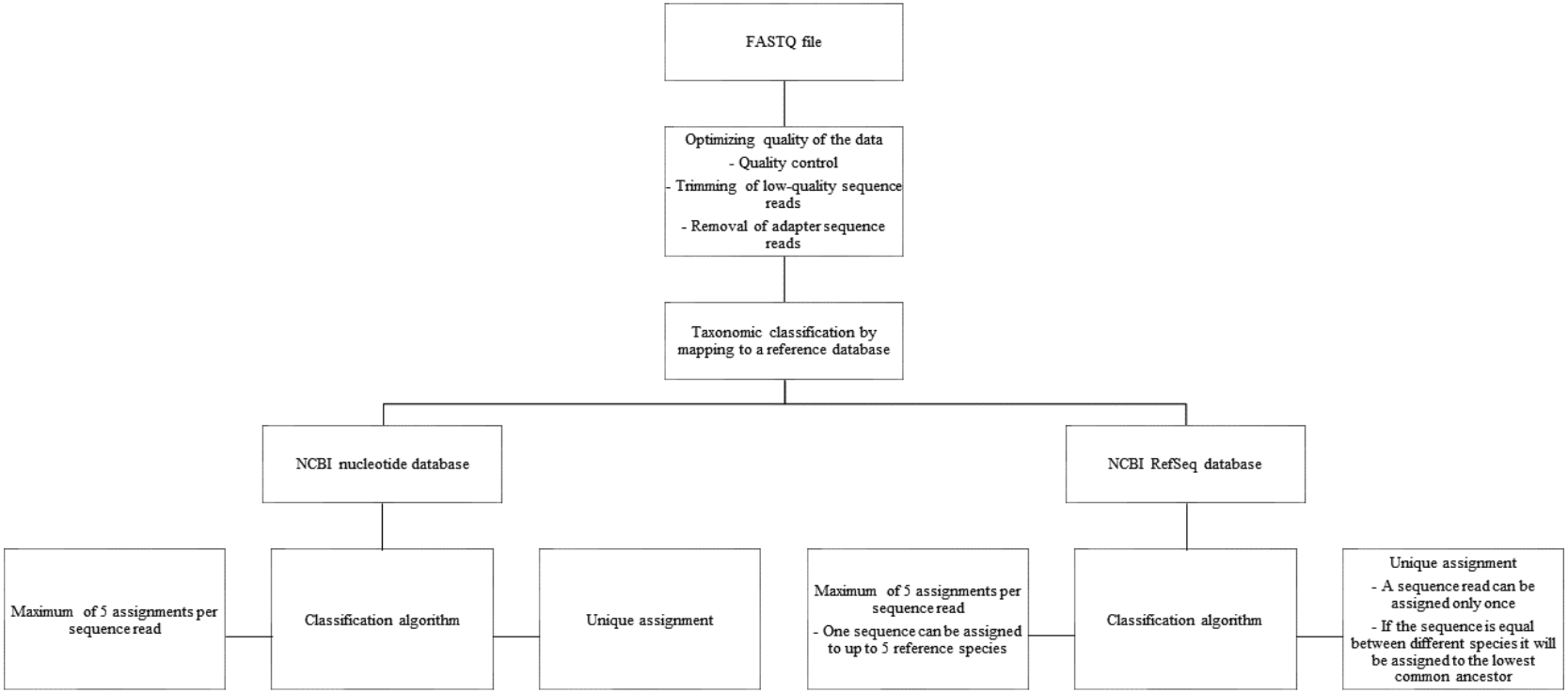


Figure 1. **The bioinformatic workflow of the mNGS protocol studied.**

In addition, a customized reference centrifuge index with sequence information obtained from the NCBI's RefSeq[23] (accessed February 2019) database was built. RefSeq genomic sequences for the domains of bacteria, viruses, archaea, fungi, protozoa, as well as the human reference, along with the taxonomy identifiers, were downloaded with the Centrifuge-download utility and were used as input for Centrifuge-build.

Centrifuge settings were evaluated to increase the sensitivity and specificity. The default setting, with which a read can be assigned to up to five different taxonomic categories, was compared to one unique assignment per read[22] where a read is assigned to a single taxonomic category, corresponding to the lowest common ancestor of all matching species.

Kraken-style reports with taxonomical information were produced by the Centrifuge-kreport utility for all (default) options. Both unique and non-unique assignments can be reported, and these settings were compared. The resulting tree-like structured, Kraken-style reports were visualized with Krona[24] version 2.0.

Horizontal coverage (%) was determined using GenomeDetective website[25] version 1.111 (*https://www.genomedetective.com/*, accessed 5-4- 2019).

In silico simulated EAV reads were analysed in different databases (NCBI's nucleotide vs RefSeq), classification algorithms (max 5 labels per sequence, vs unique, lowest(common ancestor) and reporting (non-unique vs unique) to determine the most sensitive an specific bioinformatic analyses using Centrifuge.

To determine the amount of reads needed, results of one and 10 million reads were compared. A total of one million reads were randomly selected of the 10 million reads of one FASTQ file and analysed. The random selection was performed with the FastqSplitter (*https://github.com/biopet/biopet/blob/v0.9.0/docs/tools/FastqSplitter.md* accessed 9-12-2018), which cuts a FASTQ file of 10 million reads is into 10 pieces, of which one was selected. Read counts were normalized by the total read count and target virus genome size.

Bioinformatics: assembly of PhHV1 sequences

Since NCBI's databases were lacking a complete PhHV1 genome sequence, PhHV1 was sequenced and based on the gained sequence reads the genome was build using SPAdes[26]. Assembly of PhHV1 was done using the biowdl virus-assembly pipeline 0.1 *(https://github.com/biowdl/virus-assembly* accessed 9-12-2018*)*. The QC part of the biowdl pipeline determines which adapters need to be clipped by using FastQC version 0.11.7 (*https://www.bioinformatics.babraham.ac.uk/projects/fastqc/* accessed 9-12-2018*)* and cutadapt version 1.16[21], with minimum length setting "1''. The resulting reads were downsampled within bowdl to 250 000 reads using seqtk 1.2 *(https://github.com/lh3/seqtk* accessed 9-12-2018*)* after which SPADES version 3.11.1[26] was run to get the first proposed genome contigs.

To retrieve longer assembly contigs a reiterative assembly approach was used by processing the proposed contigs by the biowdl reAssembly pipeline 0.1. This preassembly pipeline aligns reads to

contigs of a previous assembly, then selects the aligned reads, downsamples them and runs a new assembly using SPADES. Subtools used for this consisted of BWA 0.7.17[27] for indexing and mapping, SAMtools 1.6[28] for creating bam files, SAMtools view (version 1.7) for filtering out unmapped reads using the setting "-G 12", Picard SamToFastq (version 2.18.4) and seqtk for creating FASTQ files with 250 000 reads. The contigs from the reAssembly pipeline were then processed for a second using SPADES, with setting the 'cov-cutoff' to 5. The resulting contigs were then processed with the reAssembly pipeline for the third and last time setting the 'cov-cutoff' in SPADES to 20.

The contigs from the last reAssembly step were then run against the blast NT database using blastn 2.7.1[29] Out of 23 contigs only 5 contigs, that showed the lowest % in identity matches with any other possible non herpes virus species, were selected. The final 5 contigs contained sequence lengths of 97893, 8170 3710, 3294 and1279 nucleotides, the average coverage was 206, 131, 211, 285 and 154, respectively. The proposed almost complete genome of PhHV1 was added to NCBI's GenBank database (https://www.ncbi.nlm.nih.gov/genbank/, accession number GenBank MH509440, release date 4 Dec 2018).

Retrospective validation

Clinical sensitivity was analysed using the optimized procedure, which in short consisted of total NA extraction including internal controls (1:100 dilution),the adapted NEB Next library preparation protocol including fragmentation with zinc, for combined RNA and DNA detection (see library preparation), and sequencing of 10 million reads(Illumina NextSeq 500). Bioinformatic analyses was performed using Centrifuge with NCBI's RefSeq database and unique assignment of the sequence reads.

Sensitivity and specificity of the metagenomic NGS procedure was compared to a published updated version of our lab developed multiplex qPCR[30]. The routine multiplex PCR panel consisted of 15 respiratory target pathogens: influenza virus A/ B, respiratory syncytial virus (RSV), metapneumovirus (MPV), adenovirus (ADV), human bocavirus (HBoV), parainfluenza viruses (PIV) 1/ 2/ 3/ 4, rhinovirus (RV), and the coronaviruses HKU1, NL63, 227E and OC43. Thus, in total 375 PCR results were available (15 targets x 25 samples) of which 29 PCR positive and 346 PCR negative for comparison with mNGS.

Ethical approval of patient studies

The study design was approved by the medical ethics review committee of the Leiden University Medical Center (reference B16.004).

RESULTS

Internal controls

Serial dilutions of EAV and PhHV1 were added to an influenza A PCR positive sample. Serial dilution 1:10,000 detected EAV with a substantial read count in the presence of a viral infection and without a significant decline in target virus family reads (Table 1). Based on these results we determined the concentration of internal controls for further experiments.

The EAV Cq value of the dilutions correlated with the number of EAV reads from the Centrifuge analysis.

Fragmentation

The comparison of fragmentation methods was done using a selection of samples with relevant target reads and performed on the Illumina Nextseq 500 As shown in Figure 2, the total reads were comparable among the three protocols. The protocol with Zinc fragmentation had higher yield in target virus reads for all RNA viruses tested and adenovirus.

Table 1. Internal controls EAV/PhHV-1: serial dilutions against a clinical sample background and within-run precision (INFA)

Sample EAV/PhHV-1 dilution	INFA Cq	EAV Cq	PhHV-1 Cq	INFA reads (log) Centrifuge	EAV reads (log) Centrifuge	PhHV-1 reads (log) Centrifuge
1:100	24.52	21.59	23.52	4438 (3.6)	12925 (4.1)	347 (2.5)
1:1,000	24.67	24.91	26.83	3742 (3.6)	1202 (3.1)	49 (1.7)
1:10,000	24.76	28.45	30.33	4628 (3.7)	95 (2.0)	14 (1.1)
1:100,000	24.79	30.85	32.55	4093 (3.6)	18 (1.3)	14 (1.1)

Abbreviations:, Cq: quantification cycle value, INFA: influenza A, EAV: equine arteritis virus, PhHV-1 phocine herpesvirus 1.

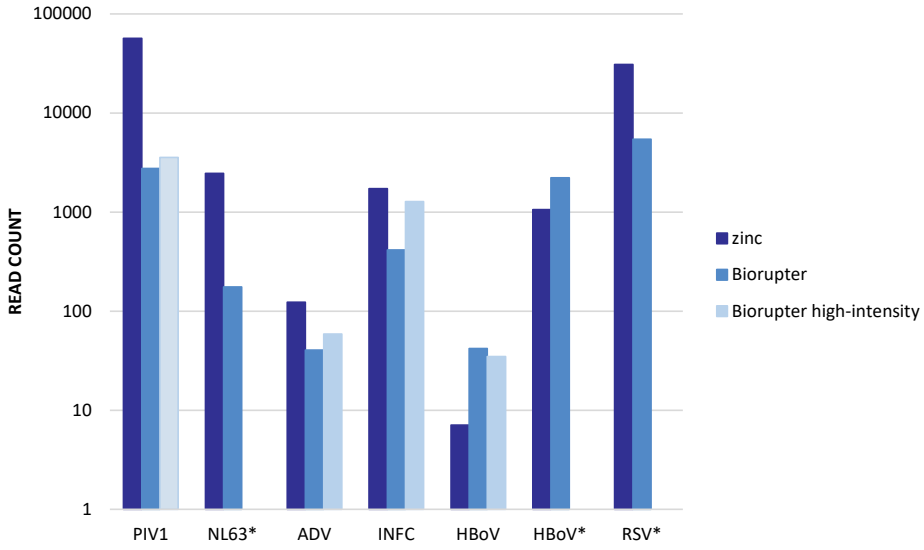


Figure 2. Comparison of fragmentation methods on target reads (species level, log scale).
*Not tested with Bioruptor setting high intensity.
PIV parainfluenza, NL63: coronavirus NL63, ADV: adenovirus, INFC: influenza C , hBoV: human bocavirus, RSV: respiratory syncytial virus

Detection limit

The detection threshold of our NGS limit, deduced from serial dilutions of influenza A (Figure 3) and EAV (table 1) was comparable with a real time PCR Cq value of >35, corresponding to, approximately <50-250 copies/reaction.

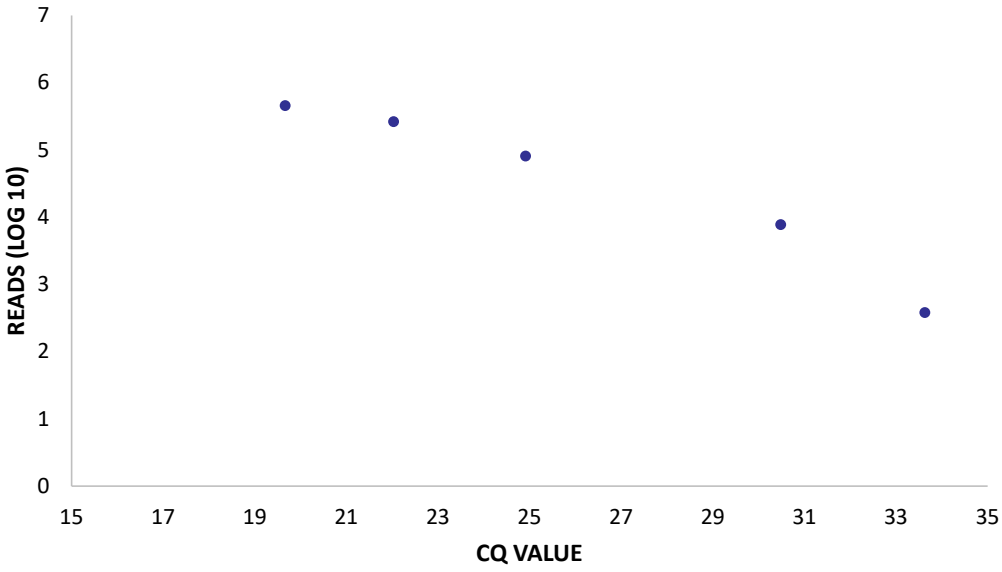


Figure 3. Serial dilutions of an influenza A positive clinical sample.

Repeatability: within run precision

The mNGS results of an influenza A positive sample tested in quadruple could be reproduced with only minor differences (table 1): coefficient of variation of 1.1%: 0.04 log SD/ 3.6 log average.

Bioinformatics: taxonomic classification

The Centrifuge default settings, with NCBI's nucleotide database and assignment of sequence reads to a maximum 5 labels per sequence, resulted in various spurious classifications (Figure 4), for example Lassa virus (Figure 5), evidently highly unlikely to be present in patient samples from the Netherlands with respiratory complaints. The specificity could be increased by using NCBI's RefSeq database instead of NCBI's nucleotide database. The classification was further improved by changing the Centrifuge tool settings to limit the assignment of homologous reads to the lowest common ancestor (maximum 1 label per sequence).

The Centrifuge reporting of shared sequences between different organisms/ subtypes differs dependent of the classification and reporting algorithm. The default classification will assign a shared read to a maximum of 5 organisms (one read will be assigned 5 times) and with the lowest common ancestor classification setting this read will only be assigned once, namely to the lowest ancestor these organisms/ subtypes have in common. Classification with maximum 5 labels per read resulted in two different outcomes using the report with all mappings and the report with unique mappings, with the latter not reporting the reads assigned to multiple organisms.

Comparison of classification using these different settings shows the highest sensitivity and specificity using NCBI's RefSeq database with one label (lowest common ancestor) assignment, both with *in silico* prepared datasets containing solely EAV sequence fragments (Figure 4) and with clinical datasets (with highly abundant background) (Figure 5).

To determine the effect of the total number of sequencing reads obtained per sample on sensitivity, one million and 10 million total reads were compared by *in silico* analysis (Table 2). One million total reads resulted in an approximate tenfold decrease in target virus read count as compared to 10 million total reads, implicating a reduction of sensitivity.

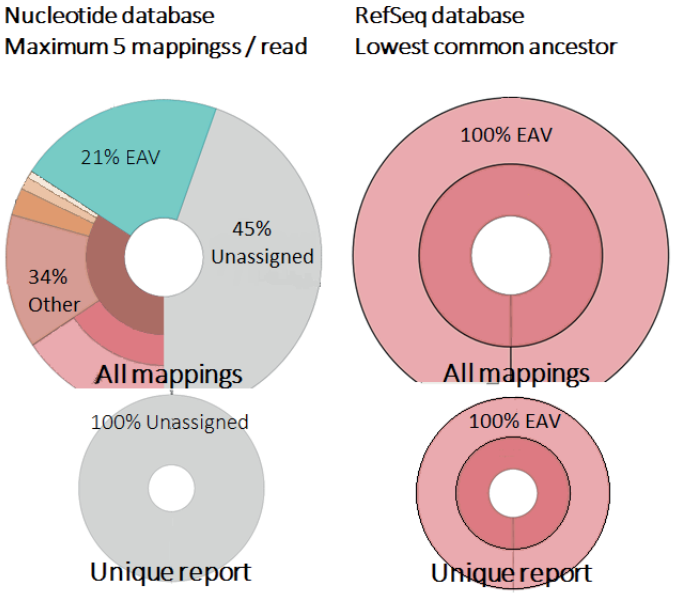


Figure 4. Analysis of *in silico* simulated EAV reads with the different bioinformatic settings of the Centrifuge pipeline.

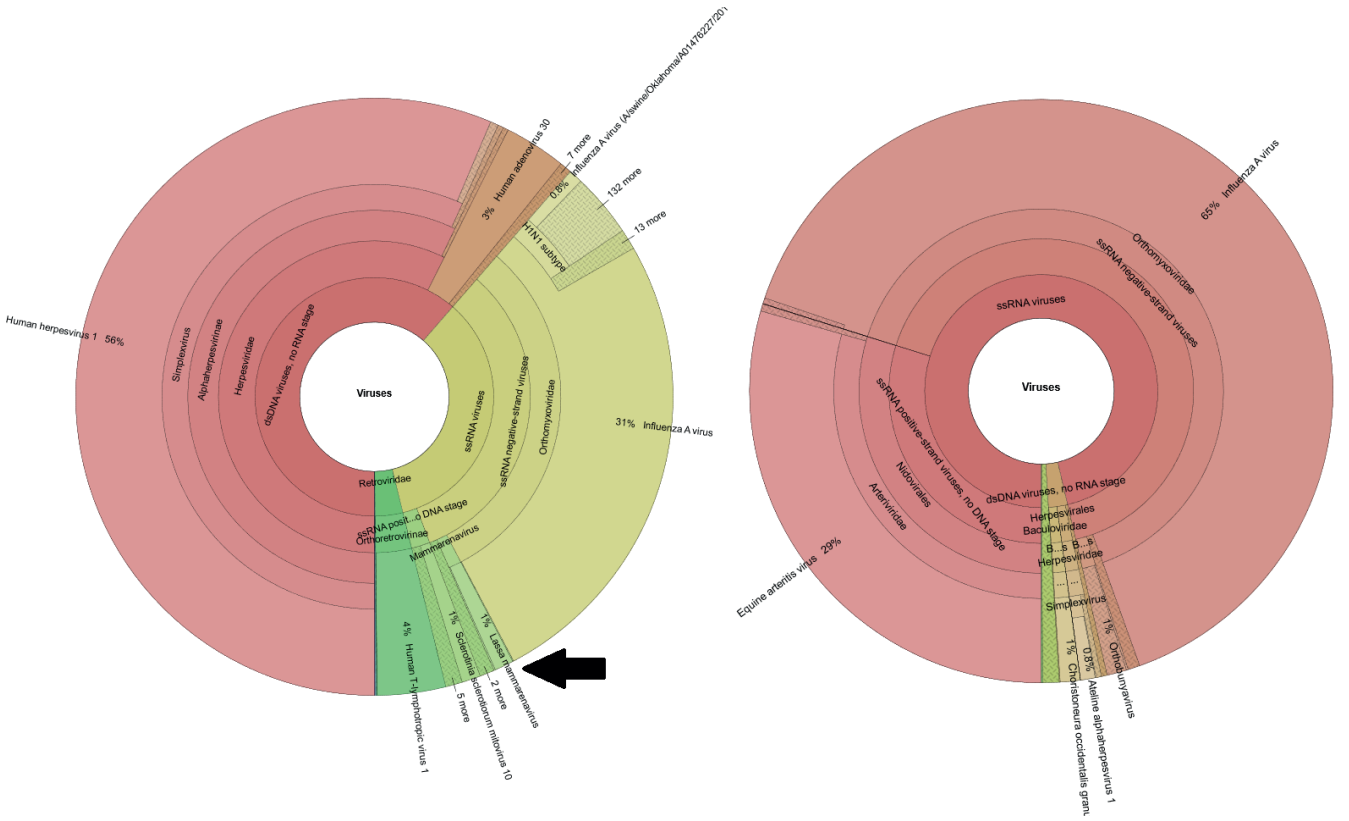


Figure 5. Spurious Lassa virus reads detected using NCBI's Nucleotide (NT) database, versus NCBI's RefSeq database.
Black arrow points to the spurious Lassa virus reads.

Table 2. **Comparison of analysis of 1 million vs 10 million reads.**

virus	virus family	Cq value	10 million reads Total reads	virus family reads	% of total	% of viral	1 million reads Total reads	virus family reads	% of total	% of viral
RV	*Picornaviridae*	37.7	8203894	8941	0.06	84.37	822218	889	0.07	86.11
PIV4	*Paramyxoviridae*	24.9	10886798	2136	0.04	41.90	1088067	199	0.08	40.73
CMV	*Herpesviridae*	34.5	15889428	22	00.01	10.88	1588922	2	0.04	11.87
ADV	*Adenoviridae*	30.2	11146488	0	0	0	1115135	0	0	0
RSV	*Pneumoviridae*	27.3	10191995	1477	0.02	53.29	1019415	163	0.04	59.25
INFB	*Orthomyxoviridae*	30	8535672	652	0.01	48.67	853149	61	0.02	46.58
NL63	*Coronaviridae*	36.2	10386928	0	0	0	1038469	0	0	0
INFA	*Orthomyxoviridae*	27.5	10981601	8403	0.11	70.28	1097872	855	0.17	69.84
MPV	*Pneumoviridae*	34.1	12972626	2	0	0.10	1297151	0	0	0
HBOV	*Parvoviridae*	32.2	11819805	0	0	0	1181738	0	0	0
RV	*Picornaviridae*	23.1	11819805	58695	0.42	84.27	1183738	5754	0.49	84.25

Abbreviations: Cq: quantification cycle value, % of total: percentage of total reads, % of viral: percentage of all viral reads , RV: rhinovirus, PIV4: parainfluenza 4, CMV: cytomegalovirus, ADV: adenovirus, RSV: respiratory syncytial virus, INF: influenza, NL63: coronavirus NL63, MPV: metapneumovirus, hBoV: human bocavirus

Retrospective validation

Clinical sensitivity based on PCR target pathogens

Clinical sensitivity was analysed using the optimized mNGS procedure. The sample collection consisted of 21 clinical specimens positive for at least one of the following PCR target viruses: rhinovirus, influenza A&B, parainfluenza 1 &4 (PIV), metapneumovirus, respiratory syncytial virus, coronaviruses NL63 and HKU1 (CoV), human bocavirus (hBoV), and adenovirus (ADV). Fourteen samples were positive for one virus, six samples for two and one sample for three viruses with the lab-developed respiratory multiplex qPCR. Cq values ranged from Cq 17 to Cq 35, with a median of 23.

With mNGS 24 of the 29 viruses demonstrated in routine diagnostics were detected (Table 3), resulting in a sensitivity of 83% for PCR targets. If a cut-off of 15 reads was handled, sensitivity declined to 66% (19/29) (Table 4). A Receiver-operating Characteristic (ROC) curve for mNGS detection of PCR target viruses, depending on the cut-off level of the number of mapped sequence reads for defining a positive result, is shown in Figure 6.

mNGS target read count (log value) showed a correlation (Pearson correlation coefficient -0.582, p=0.003), with the Cq values of the qPCR (Figure 7).

Table 3. **Detection of qPCR viruses positive respiratory samples with mNGS**

	Routine diagnostics		Metagenomic NGS			
Material	**PCR positive**	**Cq values**	**Virus genus**	**Genus reads***	**Virus species**	**Species reads***
NP wash	RV	30.7	*Enterovirus*	0	*Rhinovirus*	0
	PIV1	17.1	*Respirovirus*	58619	*Human respirovirus 1*	56407
	ADV	33.6	*Mastadenovirus*	0	*Human mastadenovirus C*	0
NP wash	MPV	24	*Metapneumovirus*	127	*Human metapneumovirus*	123
BAL	NL63	24.4	*Alphacoronavirus*	1999	*Human coronavirus NL63*	2176
	HKU1	28.2	*Betacoronavirus*	1	*Human coronavirus HKU1*	1
Sputum	RV	32	*Enterovirus*	2326	*Rhinovirus C*	2204
NP wash	INFA	22.2	*Alphainfluenzavirus*	1490	*Influenza A virus* (A/California/07/2009 (H1N1))	1490
NP wash	MPV	33.4	*Metapneumovirus*	1	*Human metapneumovirus*	3
	ADV	19.3	*Mastadenovirus*	125	*Human mastadenovirus C*	123
Sputum	PIV4	21	*Orthorubulavirus*	7729	*Human rubulaviurs 4* (subtype a)	6798
NP wash	HBoV	22.3	*Bocaparvovirus*	7	Human bocavirus	7
NP wash	MPV	22.2	*Metapneumovirus*	139	*Human metapneumovirus*	312
NP wash	INFB	16.5	*Betainfluenzavirus*	4971	*Influenza B virus* (B/Lee/1940)	4971
NP wash	RV	25.4	*Enterovirus*	8	*Rhinovirus A*	6
	RSV	30.7	*Orthopneumovirus*	32	*Human orthopneumovirus*	32
NP wash	INFB	21.4	*Betainfluenzavirus*	2686	*Influenza B virus* (B/Lee/1940)	2686
NP wash	RSV	17.8	*Orthopneumovirus*	29900	*Human orthopneumovirus*	22483
NP wash	RV	34.4	*Enterovirus*	0	*Rhinovirus*	0
	INFB	22.6	*Betainfluenzavirus*	68972	*Influenza B virus* (B/Lee/1940)	68972
BAL	INFB	34.8	*Betainfluenzavirus*	0	*Influenza B virus*	0
	HBoV	34.1	*Bocaparvovirus*	0	Human bocavirus	0

NP wash	HKU1	24.3	*Betacoronavirus*	534	*Human coronavirus HKU1*	535
NP wash	RV	16.8	*Enterovirus*	3877	*Rhinovirus A*	1721
NP wash	RV	27.4	*Enterovirus*	1	*Rhinovirus B*	2
	HBoV	19	*Bocaparvovirus*	1014	Human bocavirus	1064
NP wash	INFA	22.1	*Alphainfluenzavirus*	657	*Influenza A virus* (A/California/07/2009 (H1N1))	657
NP wash	RSV	17.2	*Orthopneumovirus*	31179	*Human orthopneumovirus*	72
NP wash	RV	17.7	*Enterovirus*	50642	*Rhinovirus A*	29293

Abbreviations: NGS: next-generation sequencing, nr: number, Cq: quantification cycle value, NP wash: nasopharyngeal wash, BAL: bronchoalveolar lavagae, RV: rhinovirus, PIV parainfluenza, ADV: adenovirus, MPV: metapneumovirus, NL63: coronavirus NL63, HKU1: coronavirus HKU1, INF: influenza , hBoV: human bocavirus, RSV: respiratory syncytial virus

*number of reads assigned to the genus or species of the target virus

Table 4. Sensitivity and specificity of the mNGS protocol tested, based on PCR target viruses, with different sequence read cut-off levels for defining a positive result.

	All reads	≥15 sequence reads	≥50 sequence reads
Sensitivity	83 (24/29)	66 (19/29)	62 (18/29)
Specificity	94 (325/346)	100 (345/346)	100 (346/346)

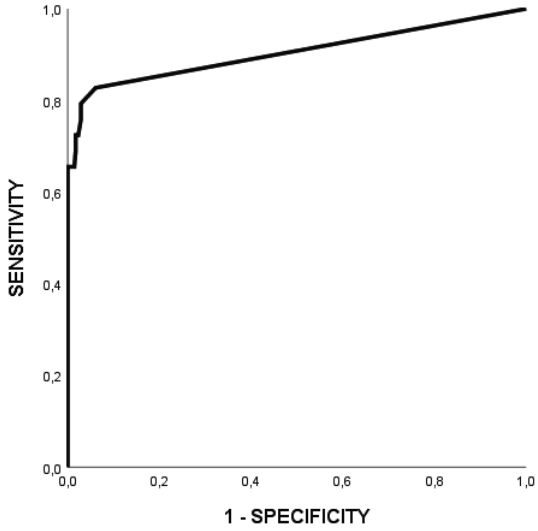


Figure 6. Receiver-operating characteristic (ROC) curve for mNGS detection of PCR target viruses depending on the cut-off level of the number of mapped sequence reads for defining a positive result

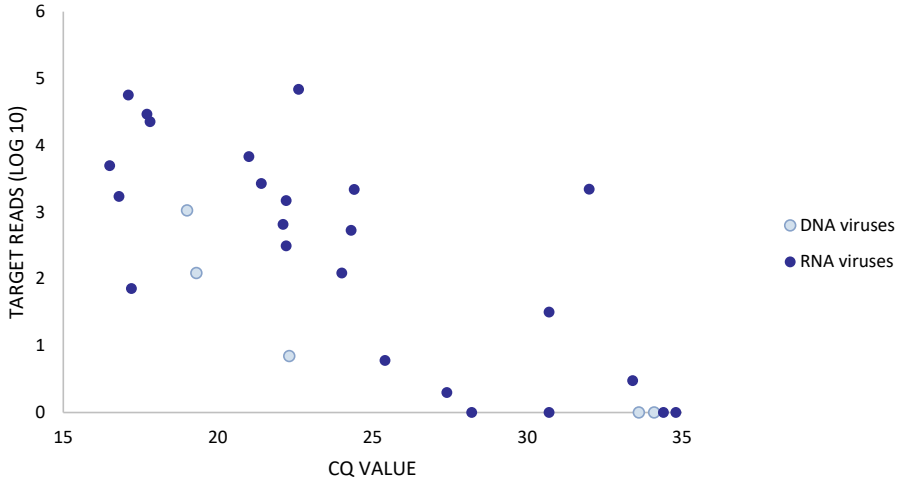


Figure 7. Semi-quantification of the mNGS assay for target virus detection in clinical samples with qPCR confirmed human respiratory viruses.

Detection of additional viral pathogens by mNGS: off-PCR target viruses

Next to the viral pathogens tested by PCR, mNGS also detected other pathogenic viruses, indicating additional viral sequences uncovered by mNGS but not included in the routine diagnostics, with influenza C virus being the most prominent. A high amount,2221 reads (99% horizontal coverage), of influenza virus C reads (58% of all viral reads and 0.02 of the total reads) was found in one sample, confirmatory PCR was not routinely available. Other potential respiratory pathogens detected by mNGS and not included in PCR analysis were KI polyomavirus (2 samples: 262 and 46 reads respectively, retrospective in-house PCR Cq 25 (1:10 dilution) and 26 respectively), cytomegalovirus (human betaherpesvirus 5) (55 and 3 reads, retrospective in-house PCR Cq 22 and 27 respectively) and enterovirus (10073 reads, retrospective in-house PCR rhinovirus/ enterovirus Cq 18). All of these viruses are not included routinely in the diagnostic multiplex qPCRs.

Internal controls

The spiked-in internal controls were detected by mNGS in all samples. EAV sequence reads ranged from 14 - 19894 (median 362) and PhHV1 ranged from 41 - 1206 (median 121).

Analytical specificity based on PCR target viruses

In total 25 paediatric respiratory samples were available to evaluate the analytical specificity of mNGS: 4 samples were negative for all 15 viral pathogens in the multiplex PCR panel (influenza A/B,

RSV, HMPV, ADV, HBoV, PIV1/2/3/4, RV, HKU1, NL63, 227E, OC43) and 21 samples were negative for 12-14 of these PCR target pathogens.

Out of in total 346 negative target PCR results of these 25 samples, 325 results corresponded with the finding of 0 target specific reads by mNGS. If a cut-off of 15 reads was used 345 of the 346 negative PCR targets were negative with mNGS. The sample positive by mNGS and negative by PCR was human parainfluenzavirus 3 (18 reads). Though no conclusive proof for neither true or false positive mNGS results could be found, specificity of mNGS was 94% (325/346) when encountering all reads and ≥99% (345/346) with a 15 reads cut-off (Table 4, ROC curve in Figure 6).

Antiviral susceptibility

Additional to subtyping (Table 3), using the metagenomic sequence data we analysed the nucleotide positions that conferred resistance to either oseltamivir or zanamivir. Sequence data of amino acids I117, E119, D198, I222, H274, R292, N294 and I314 showed susceptibility to oseltamivir and V116, R118, E119, Q136, D151, R152, R224, E276, R292 and R371 revealed susceptibility to zanamivir[31,32].

Data access

The raw sequence data of the samples, after removal of human reads have been deposited to Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/sra; accession number SRX6715205-SRX6715229).

DISCUSSION

Metagenomic sequencing has not yet been implemented as routine tool in clinical diagnostics of viral infections. Such application would require the careful definition and validation of several parameters to enable the accurate assessment of a clinical sample with regard to the presence or absence of a pathogen, in order to fulfil current accreditation guidelines. For this purpose, this study has initiated the optimization of several steps throughout the pre- and post-sequencing workflow, which are considered essential for sensitive and specific mNGS based virus detection. Many virus discovery or virus diagnostic protocols have focussed on the enrichment of viral particles[33] with the intention to increase the relative amount of virus reads. However, these methods are laborious and intrinsically exclude viral nucleic acid located in host cells. Here, a sample pre-treatment protocol was designed with potential for: 1) automation, 2) pan-pathogen detection and 3) detection of intracellular viral nucleic acids. Consequently, any type of viral enrichment was excluded (filtration, centrifugation, nucleases, rRNA removal). The current protocol enabled high throughput sample pre-treatment by means of automated NA extraction and without depletion of bacterial nor human genome, with potential for pan-pathogen detection. Several adaptations in the bioinformatic script resulted in more accurate reporting of the classification output.

Addition of an internal control to a PCR reaction is commonly used for quality control in qPCR[34]. While the addition of internal controls in mNGS is not yet an accepted standard procedure, we employed EAV and PhHV1 as an RNA and DNA control, respectively, to monitor the workflow in this diagnostic application. The amount of internal control reads and target virus reads have been reported to be dependent of the amount of background reads (negative correlation)[35]. In our protocol, the internal controls were used as qualitative controls but may be used as indicator of the amount of background. PhHV1 showed less linearity in the dilution series, as compared to EAV, which may be indicative for a potential relative difference in efficiency of amplification of PhHV1 viral sequences. Since NCBI's databases were lacking a complete PhHV1 genome, the Centrifuge index building and classification was limited to classification on a higher taxonomic rank. In order to achieve classification of PhHV1 at species level, the whole genome of PhHV1 was sequenced, and based on the gained sequence reads the genome was built[26]. The proposed nearly complete genome of PhHV1 was submitted to NCBI's GenBank database.

Sensitivity of the mNGS protocol was maximum 83% based on PCR target viruses and depended on the cut-off level of reads for defining a positive result. Five viruses, that were not recovered by mNGS had high Cq values, over 30, i.e. a relatively low viral load. This may be a drawback of the retrospective nature of this clinical evaluation as RNA viruses may be degraded due to storage and freeze-thaw steps, resulting in lower sensitivity of mNGS. A correlation was found between read counts and PCR Cq value, demonstrating the quantitative nature of viral detection by mNGS. Discrepancies between the Cq values and the number of mNGS reads may be explained by 1) unrepresentative Cq values, e.g. by primer mismatch for highly divergent viruses like rhino/enteroviruses and 2) differences in sensitivity of mNGS for several groups of viruses, as has been reported by others[36]. Additionally, viral pathogens were detected that were not targeted by the routine PCR assays, including influenza C virus, which is typical of the unbiased nature of the method. In addition, though not within the scope of this study, bacterial pathogens, including *Bordetella pertussis* (qPCR confirmed), were also detected. In the current study only viruses were targeted since these could be well compared to qPCR results, bacterial targets remain to be studied in clinical sample types as sputum or broncheo-alveolar lavages that are more suitable for bacterial detection. The analytical specificity of mNGS appeared to be high, especially with a cut-off of 15 reads. However, the clinical specificity, the relevance of the lower read numbers, still needs further investigation in clinical studies.

Sequencing using Illumina HiSeq 4000 with single, unique indexes resulted in rhinovirus-C sequences (55-909 reads) in all samples run on one lane, which appeared to be identical sequences. Retesting of the samples with Illumina Nextseq 500 resulted in disappearance of these reads. This problem could be attributed to 'index hopping' (index misassignment) as described earlier[37]. Due to the chemistry, essential for the increased speed, the HiSeq 4000 is more prone to index hopping between neighbouring samples. Although the percentage of reads which contributed to the index hopping was very low, this is critical for clinical viral diagnostics, as this is aimed specifically at low abundance targets[37,38].

Bioinformatics classification of metagenomic sequence data with the pipeline Centrifuge required identification of the optimal parameters in order to minimize misclassified and unclassified reads.

Default settings of this pipeline resulted in higher rates of both false positive and false negative results. NCBI's nucleotide database includes a wide variety of unannotated viral sequences, such as partial sequences and (chimeric) constructs, in contrast to the curated and well-annotated sequences in NCBI's Refseq database, which resulted in a higher specificity. In addition to the database, settings for the assignment algorithm were adapted as well. The assignment settings were adjusted to unique assignment in the case of homology to the lowest common ancestor. This modification resulted in higher sensitivity and specificity than the default settings, however the ability to further subtyping diminished. This is likely to be attributed to the limited representation/availability of strain types within NCBI's RefSeq database. In consequence, this leads to a more accurate estimation of the common ancestor for particular viruses, but limited typing results in case of highly variable ones. To obtain optimal typing results, additional annotated sequences may be added or a new database should be built, with a high variety of well-defined and frequently updated virus strain types.

To conclude, this study contributes to the increasing evidence that metagenomic NGS can effectively be used for a wide variety of diagnostic assays in virology, such as unbiased virus detection, resistance mutations, virulence markers, and epidemiology, as shown by the ability to detect SNPs in influenza virus.

These findings support the feasibility of moving this promising field forward to a role in the routine detection of pathogens by the use of mNGS. Further optimization should include the parallel evaluation of adult samples, the inclusion of additional annotated strain sequences to the database, and further elaboration of the classification algorithm and reporting for clinical diagnostics. The importance of both negative non-template control samples[39] and healthy control cases may support the critical discrimination of contaminants and viral 'colonization' from clinically relevant pathogens.

Conclusions

Optimal sample preparation and bioinformatics analysis are essential for sensitive and specific mNGS based virus detection.

Using a high-throughput genome extraction method without viral enrichment, both RNA and DNA viruses could be detected with a sensitivity comparable to PCR.

Using mNGS, all potential pathogens can be detected in one single test, while simultaneously obtaining additional detailed information on detected viruses. Interpretation of clinical relevance is an important issue but essentially not different from the use of PCR based assays and supported by the available information on typing and relative quantities. These findings support the feasibility of a role of mNGS in the routine detection of pathogens.

ACKNOWLEDGEMENTS

We thank our project partners Floyd Wittink, Wouter Suring (Hogeschool Leiden), Danny Duijsings (BaseClear) and Christiaan Henkel (Leiden University). We also like to thank Tom Vreeswijk, Lopje Höcker and Mario van Bussel clinical microbiology laboratory, LUMC) for help with the pre-sequencing experiments, Jeroen Laros (Human Genetics, LUMC) for help with the bioinformatics.

REFERENCES

1. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet (London, England)* 2012; **380**(9859): 2095-128.
2. Nair H, Simoes EA, Rudan I, et al. Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis. *Lancet (London, England)* 2013; **381**(9875): 1380-90.
3. Bates M, Mudenda V, Mwaba P, Zumla A. Deaths due to respiratory tract infections in Africa: a review of autopsy studies. *Current opinion in pulmonary medicine* 2013; **19**(3): 229-37.
4. Jain S, Self WH, Wunderink RG, et al. Community-Acquired Pneumonia Requiring Hospitalization among U.S. Adults. *N Engl J Med* 2015; **373**(5): 415-27.
5. Heikkinen T, Jarvinen A. The common cold. *Lancet (London, England)* 2003; **361**(9351): 51-9.
6. Ieven M, Coenen S, Loens K, et al. Aetiology of lower respiratory tract infection in adults in primary care: a prospective study in 11 European countries. *Clinical microbiology and infection* 2018; **24**(11): 1158-63.
7. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 2012; **367**(19): 1814-20.
8. Prachayangprecha S, Schapendonk CM, Koopmans MP, et al. Exploring the potential of next-generation sequencing in detection of respiratory viruses. *Journal of clinical microbiology* 2014; **52**(10): 3722-30.
9. Wooley JC, Godzik A, Friedberg I. A primer on metagenomics. *PLoS computational biology* 2010; **6**(2): e1000667.
10. Hoffmann B, Scheuch M, Hoper D, et al. Novel orthobunyavirus in Cattle, Europe, 2011. *Emerging infectious diseases* 2012; **18**(3): 469-72.
11. Mongkolrattanothai K, Naccache SN, Bender JM, et al. Neurobrucellosis: Unexpected Answer From Metagenomic Next-Generation Sequencing. *Journal of the Pediatric Infectious Diseases Society* 2017; **6**(4): 393-8.
12. van Boheemen S, de Graaf M, Lauber C, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *mBio* 2012; **3**(6).
13. Kohl C, Brinkmann A, Dabrowski PW, Radonic A, Nitsche A, Kurth A. Protocol for metagenomic virus detection in clinical specimens. *Emerging infectious diseases* 2015; **21**(1): 48-57.
14. Parker J, Chen J. Application of next generation sequencing for the detection of human viral pathogens in clinical specimens. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2017; **86**: 20-6.
15. Zou X, Tang G, Zhao X, et al. Simultaneous virus identification and characterization of severe unexplained pneumonia cases using a metagenomics sequencing technique. *Science China Life sciences* 2017; **60**(3): 279-86.
16. Wylie KM, Mihindukulasuriya KA, Sodergren E, Weinstock GM, Storch GA. Sequence analysis of the human virome in febrile and afebrile children. *PloS one* 2012; **7**(6): e27735.
17. Scheltinga SA, Templeton KE, Beersma MF, Claas EC. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2005; **33**(4): 306-11.
18. Kalpoe JS, Kroes AC, de Jong MD, et al. Validation of clinical application of cytomegalovirus plasma DNA load measurement and definition of treatment criteria by analysis of correlation to antigen detection. *Journal of clinical microbiology* 2004; **42**(4): 1498-504.
19. Wery M, Descrimes M, Thermes C, Gautheret D, Morillon A. Zinc-mediated RNA fragmentation allows robust transcript reassembly upon whole transcriptome RNA-Seq. *Methods (San Diego, Calif)* 2013; **63**(1): 25-31.
20. Joshi NA, Fass JN. Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files. 2011.
21. Martin M. Cutadept removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal* 2011; **17**(10).
22. Kim D, Song L, Breitwieser FP, Salzberg SL. Centrifuge: rapid and sensitive classification of metagenomic sequences. *Genome research* 2016; **26**(12): 1721-9.
23. O'Leary NA, Wright MW, Brister JR, et al. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic acids research* 2016; **44**(D1): D733-45.
24. Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a Web browser. *BMC bioinformatics* 2011; **12**: 385.
25. Vilsker M, Moosa Y, Nooij S, et al. Genome Detective: An Automated System for Virus Identification from High-throughput sequencing data. *Bioinformatics* 2018.

26. Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology* 2012; **19**(5): 455-77.
27. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM; 2013.
28. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)* 2009; **25**(16): 2078-9.
29. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *Journal of molecular biology* 1990; **215**(3): 403-10.
30. Loens K, van Loon AM, Coenjaerts F, et al. Performance of different mono- and multiplex nucleic acid amplification tests on a multipathogen external quality assessment panel. *Journal of clinical microbiology* 2012; **50**(3): 977-87.
31. Orozovic G, Orozovic K, Lennerstrand J, Olsen B. Detection of resistance mutations to antivirals oseltamivir and zanamivir in avian influenza A viruses isolated from wild birds. *PloS one* 2011; **6**(1): e16028.
32. Hsieh NH, Lin YJ, Yang YF, Liao CM. Assessing the oseltamivir-induced resistance risk and implications for influenza infection control strategies. *Infection and drug resistance* 2017; **10**: 215-26.
33. Hasan MR, Rawat A, Tang P, et al. Depletion of Human DNA in Spiked Clinical Specimens for Improvement of Sensitivity of Pathogen Detection by Next-Generation Sequencing. *Journal of clinical microbiology* 2016; **54**(4): 919-27.
34. Ninove L, Nougairede A, Gazin C, et al. RNA and DNA bacteriophages as molecular diagnosis controls in clinical virology: a comprehensive study of more than 45,000 routine PCR tests. *PloS one* 2011; **6**(2): e16142.
35. Schlaberg R, Chiu CY, Miller S, Procop GW, Weinstock G. Validation of Metagenomic Next-Generation Sequencing Tests for Universal Pathogen Detection. *Archives of pathology & laboratory medicine* 2017; **141**(6): 776-86.
36. Bal A, Pichon M, Picard C, et al. Quality control implementation for universal characterization of DNA and RNA viruses in clinical respiratory samples using single metagenomic next-generation sequencing workflow. *BMC infectious diseases* 2018; **18**(1): 537.
37. Sinha R, Stanley G, Gulati GS, et al. Index Switching Causes "Spreading-Of-Signal" Among Multiplexed Samples In Illumina HiSeq 4000 DNA Sequencing. *bioRxiv* 2017.
38. van der Valk T, Vezzi F, Ormestad M, Dalen L, Guschanski K. Estimating the rate of index hopping on the Illumina HiSeq X platform. *bioRxiv* 2018.
39. Naccache SN, Hackett J, Jr., Delwart EL, Chiu CY. Concerns over the origin of NIH-CQV, a novel virus discovered in Chinese patients with seronegative hepatitis. *Proceedings of the National Academy of Sciences of the United States of America* 2014; **111**(11): E976.

CHAPTER 3

The respiratory virome and exacerbations in patients with chronic obstructive pulmonary disease

A.L. van Rijn[a], S. van Boheemen[a], I. Sidorov, E.C. Carbo[a], N. Pappas[b], H. Mei[b], M.C.W. Feltkamp[a], M. Aanerud[c,d], P. Bakke[d], E.C.J. Claas[a], T.M. Eagan[c,d], P.S. Hiemstra[e], A.C.M. Kroes[a], J.J.C. de Vries[a]

[a] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[b] Sequencing Analysis Support Core, Department of Medical Data Sciences, Leiden University Medical Center, Leiden, the Netherlands
[c] Department of Thoracic medicine, Haukeland Unversity Hospital, Bergen, Norway
[d] Department of Cinical Science, University of Bergen, Bergen, Norway
[e] Department of Pulmonology, Leiden University Medical Center, Leiden, the Netherlands

PLoS One 2019 14(10)

ABSTRACT

Introduction: Exacerbations are major contributors to morbidity and mortality in patients with chronic obstructive pulmonary disease (COPD), and respiratory bacterial and viral infections are an important trigger. However, using conventional diagnostic techniques, a causative agent is not always found. Metagenomic next-generation sequencing (mNGS) allows analysis of the complete virome, but has not yet been applied in COPD exacerbations.

Objectives: To study the respiratory virome in nasopharyngeal samples during COPD exacerbations using mNGS.

Study design: 88 nasopharyngeal swabs from 63 patients from the Bergen COPD Exacerbation Study (2006-2010) were analysed by mNGS and in-house qPCR for respiratory viruses. Both DNA and RNA were sequenced simultaneously using an Illumina library preparation protocol with in-house adaptations.

Results: By mNGS, 24/88 samples tested positive. Sensitivity and specificity, as compared with PCR, were 96% and 98% for diagnostic targets (23/24 and 1093/1120, respectively). Additional viral pathogens detected by mNGS were herpes simplex virus type 1 and coronavirus OC43. A positive correlation was found between Cq value and mNGS viral normalized species reads (log value) ($p=0.002$). Patients with viral pathogens had lower percentages of bacteriophages ($p<0.001$). No correlation was found between viral reads and clinical markers.

Conclusions: The mNGS protocol used was highly sensitive and specific for semi-quantitative detection of respiratory viruses. Excellent negative predictive value implicates the power of mNGS to exclude any pathogenic respiratory viral infectious cause in one test, with consequences for clinical decision making. Reduced abundance of bacteriophages in COPD patients with viral pathogens implicates skewing of the virome during infection, with potential consequences for the bacterial populations, during infection.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by exacerbations with high morbidity and mortality, and over 65 million patients suffer from this disease worldwide[1]. A COPD exacerbation is an acute event leading to worsening of the respiratory symptoms and is associated with a deterioration of lung function[2]. Exacerbations are mainly associated with infections, of which a large part is caused by viruses (22-64%)[3-6]. However, in part of the exacerbations an etiologic agent is not detected.

Current routine virus diagnostics is based on polymerase chain reactions (PCR) and inherently the number of detectable pathogens is restricted to the ones included in the assay. Rare, mutated and pathogens with an uncommon clinical presentation will be missed, along with new and currently unknown ones. Over the last decades, several previously unidentified viruses have been discovered as respiratory pathogens, including metapneumovirus[7], middle-east respiratory syndrome coronavirus[8] and human bocavirus[9].

Metagenomic next-generation sequencing (mNGS) is an innovative method, which enables the detection of all genomes in a given sample. Proof of principle studies have shown that mNGS on respiratory samples can confirm and extend PCR results and deliver typing and resistance data at the same time[10-14]. The performance of mNGS in the clinical diagnostic setting, especially the positive and negative predictive value, has not yet been elucidated and is likely to differ per clinical syndrome and sample.

Previous data from reports on 16S rRNA analysis from the respiratory tract have led to increased insight in the microbiome in patients with COPD[15]. Changes in bacterial populations have been associated with exacerbation events and clinical phenotypes[15]. However, these studies are intrinsically limited to analysis of the bacterial part of the microbiome.

So far only a few studies using shotgun metagenomics have focussed on the respiratory virome in children with acute respiratory infections[16,17]. In this study, we analyse the composition of the virome in adult patients with exacerbations of COPD.

Objectives

The aim of this study was to correlate the respiratory virome in COPD patients as found by mNGS with qPCR and clinical data.

MATERIALS AND METHODS

Patients

Patients with COPD were included in the Bergen COPD exacerbation study (BCES) between 2006 and 2010 in Bergen, Norway[18]. All patients lived in the Haukeland University Hospital district. Baseline data taken during the first visit while in the stable state included amongst others exacerbation history, medications, comorbidities, spirometry and Global Initiative for Chronic Obstructive Lung

Disease (GOLD 2007) categorisation. Patients were given a telephone number to a study nurse, whom they would contact in case of an exacerbation. Patients with an exacerbation according to a predefined set of symptoms were scheduled for an appointment with a study physician the next working day. During exacerbations, nasopharynx swabs were sampled and two different markers for the severity of the exacerbation were scored. After an exacerbation a control visit was scheduled. During the study period 154 patients had at least one exacerbation and in total 325 exacerbations were included in BCES, of which 88 exacerbation samples were tested in the current study.

Sample selection

Nasopharyngeal samples were frozen and stored at -80°C. In total 88 nasopharyngeal samples of patients at the time of exacerbation were selected based on the availability of other samples (outside the current focus) and sent to the Leiden University Medical Center (The Netherlands) for further testing.

Lab-developed real-time PCR testing (qPCR)

The viral respiratory panel covered by the multiplex real-time PCR (qPCR) developed in our laboratory consists of coronavirus 229E, coronavirus HKU1, coronavirus NL63, coronavirus OC43, influenza A, influenza B, human metapneumovirus, parainfluenza 1-4 (differentiation with probes), respiratory syncytial virus, and rhinovirus[19].

Total nucleic acids (NA) were extracted directly from 200 µl clinical sample, using the Total Nucleic Acid extraction kit on the MagnaPure LC system (Roche Diagnostics, Almere, the Netherlands) with 100 µL output eluate. Nucleic acid amplification and detection by real-time PCR was performed on a BioRad CFX96 thermocycler, using primers, probes and conditions as described previously[19]. Cq values were normalized using a fixed baseline fluorescence threshold.

Metagenomic next-generation sequencing (mNGS)

The metagenomics protocol used has been described and optimized for simultaneously RNA and DNA detection previously[14]. In short, internal controls, Equine Arteritis virus (EAV) for RNA and Phocid Herpesvirus-1 (PhHV) for DNA (kindly provided by prof. dr. H.G.M. Niesters, the Netherlands), were spiked in 200 µl of the virus transport medium in which the nasopharyngeal swab was stored. Nucleic acids were extracted directly from 200 µl clinical sample using the Magnapure 96 DNA and Viral NA Small volume extraction kit on the MagnaPure 96 system (Roche Diagnostics, Almere, The Netherlands) with 100 µL output eluate (an updated version of the isolation method used for qPCR, tested previously [14]). Extraction buffer was used as negative control (for extraction, library preparation, and sequencing). For library preparation, 7 µl of nucleic acids were used, using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®, with several in-house adaptations to the manufacturers protocol in order to enable simultaneous detection of both DNA and RNA. The following steps were omitted: poly A mRNA capture isolation, rRNA depletion and DNase treatment

step. This resulted in a single tube per sample throughout library preparation containing both DNA and RNA. Metagenomic sequencing was performed on an Illumina NextSeq 500 sequencing system (Illumina, San Diego, CA, USA), and approximately 10 million 150 bp paired-end reads per sample were obtained.

After quality pre-processing, sequencing reads were taxonomically classified with Centrifuge[20] using an index constructed from NCBI's RefSeq and taxonomy databases (accessed February 2019) with reference nucleotide sequences for the viruses, bacteria, archaea, fungi, parasites, and protozoa. Reads with multiple best matches were uniquely assigned to the lowest common ancestor (k=1 Centrifuge setting; previously validated[14]). Both negative and positive results were confirmed using GenomeDetective website[21] version 1.111 (accessed December 2018 - January 2019) and horizontal coverage (%) was determined using GenomeDetective.

Read counts were normalized, dividing the raw read count by the total number of reads in the sample and by the (average) genome size, and multiplied by 10^11 (to achieve comprehensible read counts in the same order of magnitude as the raw read counts).

Virus assembly

For samples with dubious or inconclusive classification results a *de novo* assembly was performed. Pre-processed short reads assigned to a higher taxonomic level of a suspected viral target were extracted and *de novo* assembled with SPAdes version 3.11.1[22] into longer stretches of contiguous sequences (contigs). The resulting contigs were then run against the blast NCBI's nucleotide (nt) database (accessed 2017) using blastn 2.7.1[23]. After identification of a putative target sequence, all the reads from the original sample were mapped against the identified best BLAST hit for further confirmation using BWA 0.7.17 software package[24].

Statistical analysis

Sensitivity, specificity, positive and negative predictive values were calculated based on 24 PCR positive and 1120 PCR negative target results of 88 samples.

Correlation between qPCR Cq value and logarithm of normalized numbers of mNGS viral reads was tested with population Pearson correlation coefficient.

Potential correlations of mNGS data with clinical variables were tested as follows. Cq value/ viral reads and clinical parameters (exacerbation severity, duration of exacerbation or decrease/increase in Forced Expiratory Volume in 1 second (FEV_1, control visit compared to baseline) were tested with one-way ANOVA and Kruskal-Wallis test when appropriate (depending on distribution). Comparison of the percentage of phages of all viral reads (after subtraction of the internal control EAV and PhHV reads) between mNGS virus positive samples and negative samples was tested with Mann-Whitney U test, comparison with clinical parameters with Kruskal-Wallis test. Diversity of the virome in different patient groups was characterized by Shannon Diversity Index (H) and tested with Welch two

sample t-test. Statistical analyses were performed using IBM SPSS Statistics version 25 software for Windows <0.05 were considered statistically significant.

Ethical approval

Prior to inclusion all subjects received written and oral information and signed informed consent. The BCES study was approved by the regional ethical committee in Western Norway (REK-Vest, case-number 165.08). The performance of this study, including mNGS, was approved by the medical ethics review committee of the Leiden University Medical Center (CME number B16.004); no additional consent was necessary.

RESULTS

Patients and samples

In total 63 patients with 88 exacerbations were included with a median of one exacerbation per patient (range 1-5). Baseline patient characteristics and exacerbation characteristics are shown in tables 1 and 2 respectively.

Table 1. Baseline patient characteristics

	Patients (n=63)
Age median years (range)	63.5 (46.6-74.5)
Male sex	40 (64%)
BMI median, kg/m^2 (range)	25 (15-39)
Body composition	
Cachectic	7 (11%)
Normal	24 (38%)
Overweight	22 (35%)
Obese	10 (16%)
Smoking	
Never	0 (0%)
Sometimes	37 (59%)
Daily	26 (41%)
GOLD stage	
II (FEV_1 50-80%)	29 (46%)
III (FEV_1 30-50%)	27 (43%)
IV (FEV_1 <30%)	7 (11%)
FEV_1 in % median (range)	0.49 (0.23-0.74)
>1 exacerbation past 12 months	16 (25%)
Inhalation steroids	50 (79%)

Table 2. **COPD patient and exacerbation characteristics among patients having a viral or non-viral exacerbation.**

	qPCR target virus		
	detected	not detected	
	n=23	n=65	P*
Patient characteristics			
Sex, %			0.21
Women	34.5	65.5	
Men	22.0	78.0	
smoking status, %			0.53
Ex-smoker	23.4	76.6	
Current-smoker	29..3	70.7	
GOLD stage (2007), %			0.35
II (FEV_1 50-80%)	26.3	73.7	
III (FEV_1 30-50%)	30.8	69.2	
IV (FEV_1 < 30%)	9.1	90.9	
Frequent exacerbator, %			0.72
No	25.0	75.0	
Yes	28.6	71.4	
Using inhalation steroids, %			0.55
No	20.0	80.0	
Yes	27.4	72.6	
Age, mean yrs	63.7	64.9	0.10
BMI, mean kg/m²	27.0	25.9	0.92
FEV_1 in % predicted	49.3	47.5	0.48
Exacerbation characteristics			
Exacerbation severity for entire exacerbation			0.75
Mild (not requiring AB or oral steroids or hospitalization)	14.3	85.7	
Moderate (requiring AB or oral steroids)	26.9	73.1	
Severe (Emergency room or hospital admission)	28.6	71.4	
Self-reported exacerbation severity at time of study sampling			0.64
Dyspnea unchanged or increased on errands outside home	36.4	63.6	
Increased dyspnea doing housework	26.5	73.5	
Increased dyspnea at rest	28.6	71.4	
Must sit up at night due to dyspnea	14.3	85.7	
CRP (ng/mL) at time of study sampling†	32.5	34.2	0.27

* Pearson's chi-square test for categorical variables and t-test for continuous variables

† missing data for 4 (1 virus positive, 3 virus negative) exacerbations

Lab developed real-time PCR

Of the 88 samples, 23 (26%) tested positive with in-house PCR: 14 (61%) were rhinovirus positive, three influenza A, two coronavirus NL63, one coronavirus OC43, two parainfluenza 3 and one parainfluenza 4. Cq values ranged from 19-38 (Table 3).

Table 3. qPCR positive samples with respective mNGS results

Samples	qPCR positive (%)	Cq values range	mNGS species positive (%)	mNGS species reads (range)	Coverage (%, range)
All targets	23/88 (26)	19-38	23/88 (26)	0-1,317,490	3-100
Influenza A	3/23 (13)	29-36	3/23 (13)	9-559	3-98
Cov NL63	2/23 (9)	32	2/23 (9)	1,347-127,284	93-100
Cov OC43	1/23 (4)	27	2*/23 (4)	72,644-1,317,490	99-99
PIV3	2/23 (9)	26-36	2/23 (9)	59-288,877	14-99
PIV4	1/23 (4)	24	1/23 (4)	185,235	100-100
Rhinovirus	14/23 (61)	19-38	13**/23 (57)	0-310,491	
			RV-A: 6/13	32-27,096	94-100
			RV-B: 2/13	13,445-18,206	100-100
			RV-C: 5/13	217-310,491	30-100

*Retesting by qPCR confirmed the OC43 finding of mNGS

** Rhinovirus not detected with mNGS had PCR Cq value 38

Metagenomic next-generation sequencing

A median of 11 million (7,522,643-20,906,019) sequence reads per sample were obtained. Of the 11 million reads, approximately 93% were *Homo sapiens* reads, 3 % were bacterial and 0.1% viral (Table 4). A median of 3% of the reads could not be assigned to sequences in the Centrifuge index database (NCBI RefSeq).

Table 4. mNGS read counts

	Median	Min	Max
Total reads	10,764,981	7,522,643	20,906,019
% unassigned reads	3	0.7	22
Homo sapiens reads(% total)	9,470,904 (93)	2,491,763	18,646,521
Bacterial reads (% total)	285,567 (3)	6,289	10,490,131
Viral reads (% total)	15,679 (0.1)	803	1,553,567
PhHV reads (% viral)	3,289 (22)	299	26,623
EAV reads (% viral)	10,152 (72)	197	75,771

Comparison of mNGS to qPCR

Of the 23 qPCR positive samples, 22 tested positive with mNGS, resulting in a sensitivity of mNGS of 96%. Only one sample, that was rhinovirus positive by qPCR (Cq 38), could not be detected by mNGS (Table 3). Coverage of reference genomes was high (93-100%) with the exception of three samples: 30% coverage of rhinovirus C (1,401,120 mapped reads, 88,353-fold depth), 14% coverage of parainfluenza 3 (50 mapped reads, 3-fold depth), and 3% coverage of influenza A virus (single genome segment, 8 mapped reads). Aligning reads with Bowtie confirmed the rhinovirus C and parainfluenza 3 mapping, but not the influenza A mapping. Additional viral pathogens detected by mNGS were herpes simplex virus type 1 (17,031 reads, 82% coverage, 36-fold depth) which was not in qPCR viral respiratory panel (retrospectively confirmed by means of in-house HSV PCR; Cq 24), in the sample with the 8 influenza virus reads, and a betacoronavirus. Since coronaviruses tested negative by means of qPCR, and the mNGS classification was inconclusive , the reads were *de novo* assembled. Of these 83,252 betacoronavirus reads, *de novo* assembly resulted in 3 contigs (size 30743, 274 and 232 bp respectively) with best BLAST hit coronavirus OC43 (reference genome GenBank accession AY391777.1). A coverage plot of all reads against this reference strain (Fig 1) showed good horizontal and vertical coverage (read coverage depth 428). The original OC43 qPCR amplification appeared to have been inhibited, and repeated OC43 qPCR confirmed the positive mNGS result (Cq 25).

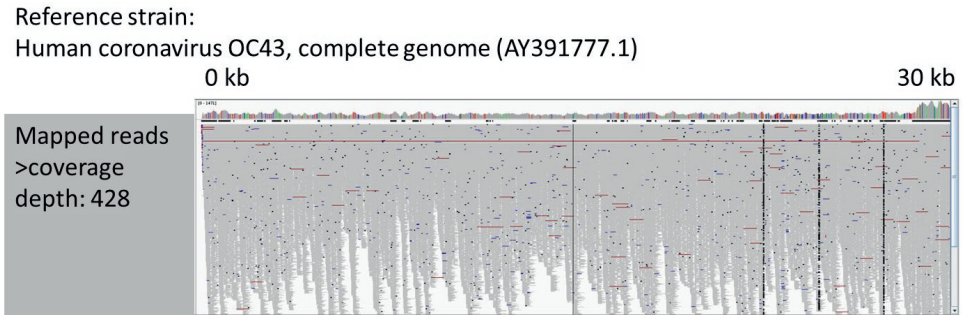


Fig 1. Coverage plot of betacoronavirus reads to coronavirus OC43 reference genome AY391777.1. (depth of coverage: 428)

Sensitivity, specificity and predictive value

The sensitivity, specificity and predictive values of mNGS were calculated based on 24 PCR positive and 1120 PCR negative target results of 88 samples and the normalized read counts (Table 5). Calculations were made using different cut-off values of respectively ≥0, ≥15 and ≥50 normalized read counts. With a cut-off of ≥15, the sensitivity was 92% and specificity 100% and the positive predictive value (PPV) increased to 92%. The negative predictive value (NPV) was 100% for all cut-off levels. A ROC curve (Fig 2), using Youden's index[25] demonstrated that the optimal sensitivity and specificity were achieved using a cut-off of 5 reads (96% (23/24) and 100% (1115/1120) respectively).

Table 5. Sensitivity and specificity of mNGS normalized reads for PCR target viruses.

	Cut-off number of reads		
	0	**15**	**50**
Sensitivity	96% (23/24*)	92% (22/24)	83% (20/24)
Specificity	98% (1093/1120)	100% (1118/1120)	100% (1118/1120)
PPV	46% (23/50)	92% (22/24)	91% (20/22)
NPV	100% (1093/1094)	100% (1118/1120)	100% (1118/1122)

PPV: positive predictive value, NPV, Negative predictive value.

*The sample with positive confirmatory OC43 PCR included.

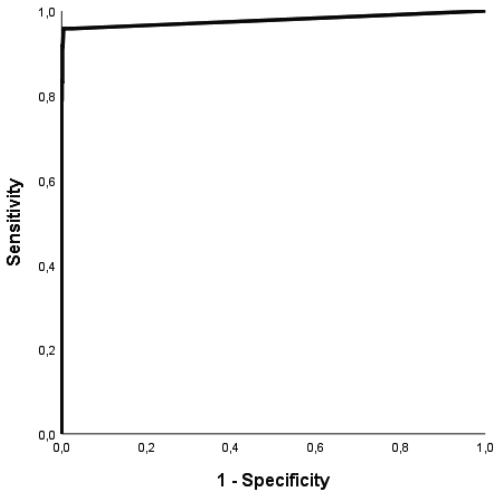


Fig 2. ROC curve of cut-off levels of mNGS normalized reads.

Typing

mNGS provides additional typing data, as compared to qPCR. Of the 13 rhinoviruses detected with mNGS, 6 (46.2%) were rhinovirus A, 2 (15.4%) rhinovirus B and 5 (38.5%) rhinovirus C. The three influenza viruses were assigned to be H3N2 strains by mNGS.

Semi-quantification by means of mNGS read count

In order to analyse the semi-quantitative quality of the mNGS assay, the number of the normalized sequence reads (log) mapping to qPCR target viruses (species level) as obtained with mNGS were compared to the Cq values of qPCR. A significant negative correlation was found (Fig 3; Pearson correlation coefficient ρ=-0.6, p=0.002).

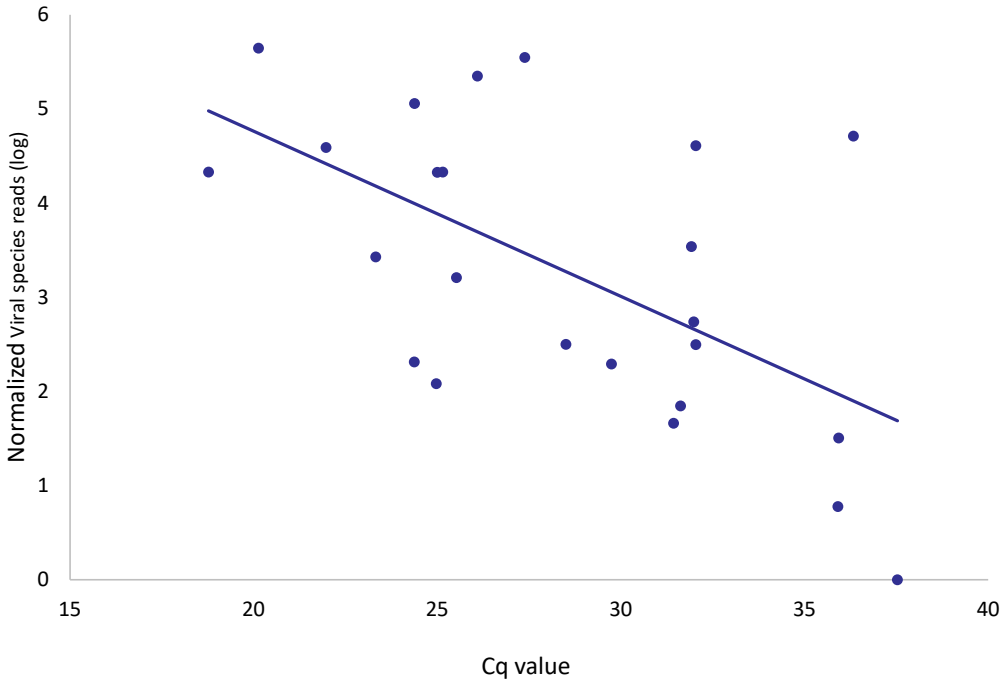


Fig 3. Correlation between mNGS normalized viral species reads (log) and Cq value.
(ρ=-0.6, p=0.002)

Clinical parameters and mNGS pathogen read count

The following markers were tested for potential associations with clinical severity of exacerbation (exacerbation severity, self-reported exacerbation severity), length of exacerbation and a decrease/increase in FEV_1 (control visit compared to baseline): mNGS pathogen positive versus negative exacerbation (qPCR targets), the number of normalized reads (log, cut-off of ≥5 normalized reads) for the different target viruses (species level). No correlation was found between these markers and the different disease severity parameters (results not shown).

The respiratory virome

Overall proportions of normalized read counts of viral families (excluding EAV and PhHV control reads, cut-off of ≥5 normalized reads) detected by mNGS per patient are shown in Fig 4. Patients with viral pathogens (PCR target viruses) had significantly reduced proportions of bacteriophages when compared to patients without viral pathogen: 0% and 79% bacteriophages respectively ($p<0.001$) bacteriophage reads vs. all viral reads, normalized reads excluding EAV and PhHV control reads. The Shannon diversity scores for bacteriophages (normalized reads, cut-off of ≥5 normalized reads) were comparable for COPD exacerbations of viral aetiology in PCR positive versus negative patients (Fig 5). Shannon diversity (normalized reads, excluding internal controls) was significant lower for all viral

reads (P<0.001) and eukaryotic viruses (p=0.028) in patients with viral pathogens (PCR target viruses positive).

No significant association was found between the diversity scores, nor the percentage of bacteriophages, and the following parameters: disease severity, length of exacerbation, number of exacerbations during the study period, difference in FEV_1, GOLD stage, smoking, CRP level, and the virus species (results not shown).

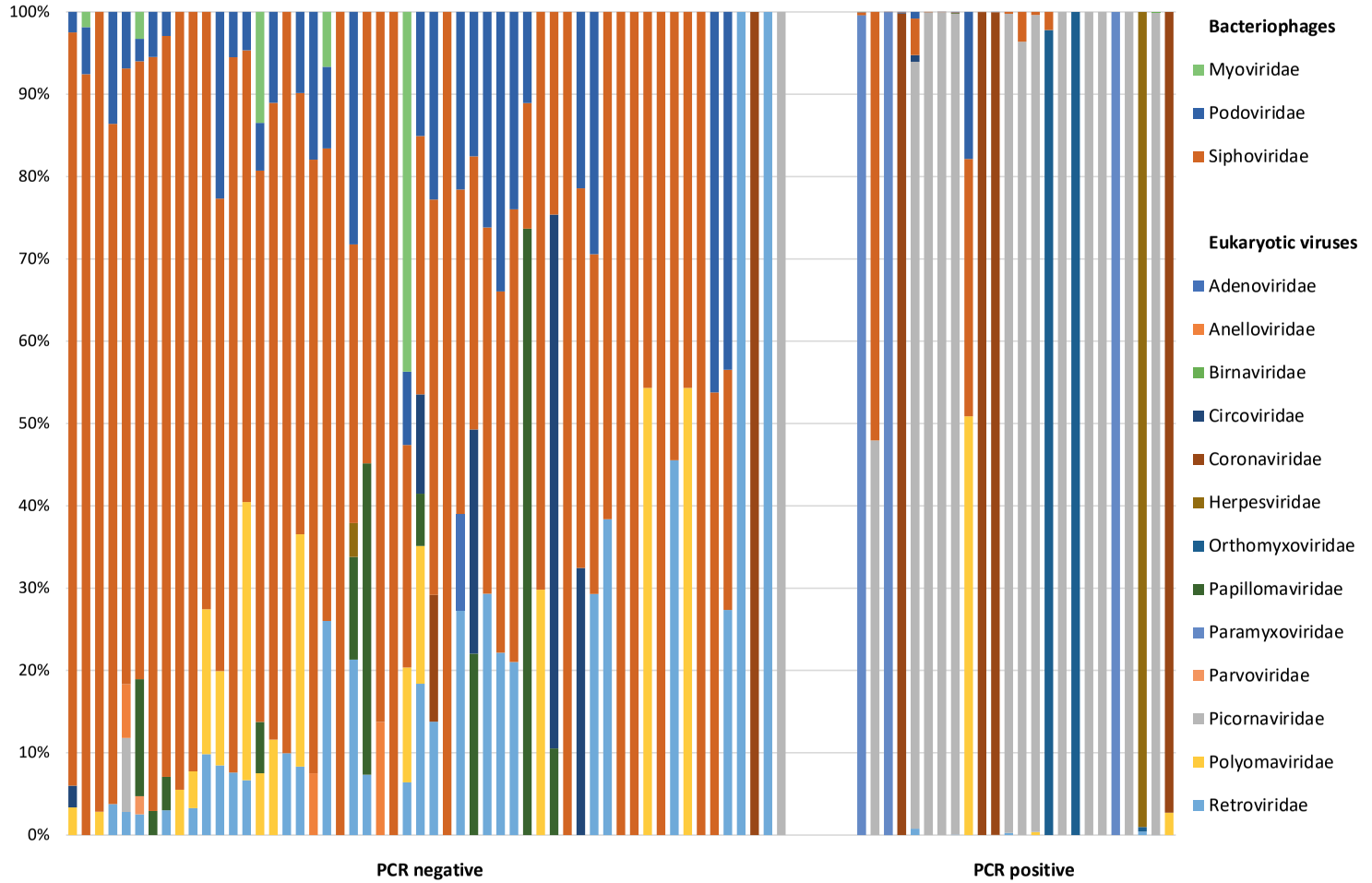


Fig 4. The respiratory virome: proportion of normalized read counts of viral families per patient.
Internal control reads (EAV and PhHV-1) excluded, ten patients without viral reads excluded.

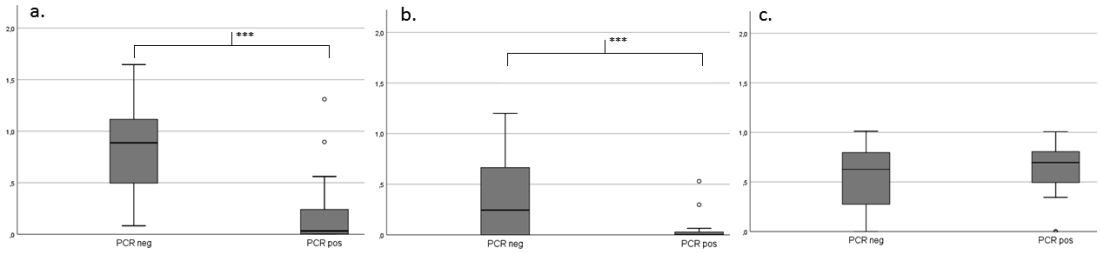


Fig 5. Shannon diversity scores for: (a) viruses, (b) eukaryotic viruses, (c) bacteriophages.
COPD exacerbations of viral etiology had significant lower diversity (b).Boxes span IQR, *** significant (a) all viruses (P<0.001) and (b) eukaryotic viruses (p=0.028), ○ outliers.

The respiratory bacteriome

The most prevalent phyla were Proteobacteria , Firmicutes Actinobacteria and Bacteroidetes, see figure 6.

The normalized bacterial read count of the most prevalent phyla was not significantly different between patients with a PCR-target virus positive and PCR-target negative patients.

Pathogenic bacterial species detected with an abundance of >10% of the bacterial reads were: *H. influenza (five samples)*; *M. catarrhalis (20 samples)*; *S. pneumoniae (one sample)*; and *S. aureus (one sample*). No apparent association with bacteriophages was found, or was a high abundance of bacteriophages associated with COPD exacerbations of viral cause.

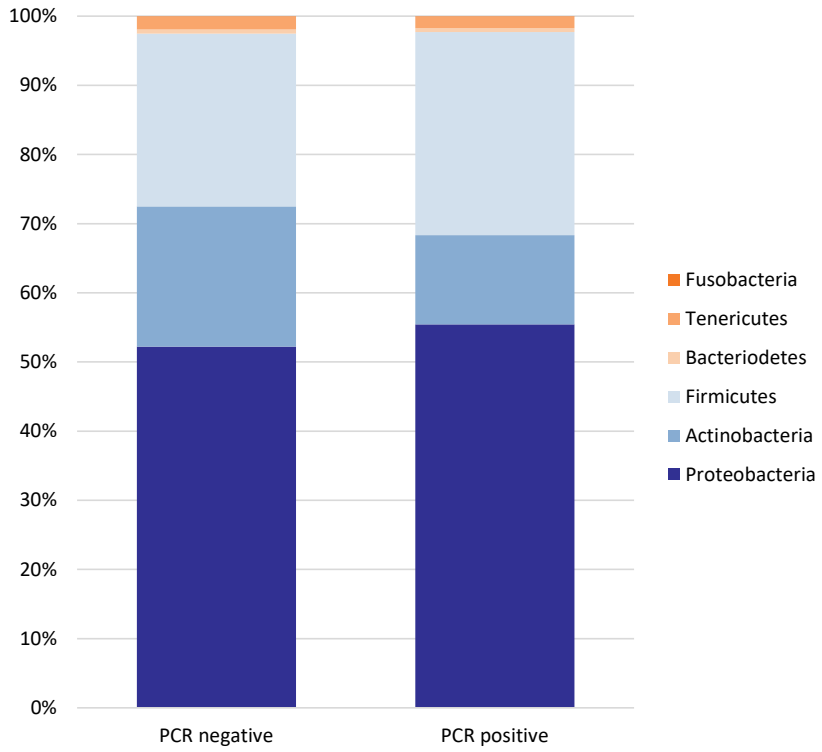


Fig 6. Proportion of normalized bacterial reads per phylum.

Data access

The raw sequence data of the samples, after removal of human reads have been deposited to Sequence Read Archive database (http://www.ncbi.nlm.nih.gov; accession number SRX6713943-SRX6714030).

DISCUSSION

In this study, the respiratory virome in patients with COPD exacerbations was analysed with both mNGS and qPCR, and combined with clinical data. The incidence of viral pathogens was 26% with both mNGS and qPCR. mNGS failed to detect one Rhinovirus with low load (Cq 38) and PCR failed to detect one betacoronavirus OC43 (72644 reads), due to one of the limitations of PCR, *i.e.* inhibition of amplification. One additional viral pathogen, not present in the respiratory PCR panel, was detected: herpes simplex virus 1, found by others to be associated with COPD[26].

The incidence of viral pathogens was comparable to that in previous publications (22-64%)[3,5,6]. The viral pathogen with the highest incidence was rhinovirus, followed by influenza, coronaviruses and para-influenza viruses. Interestingly, subtyping data was readily available by mNGS, accentuating the

high resolution of mNGS, with rhinovirus (RV) species A and C being most frequent, followed by RV-B. RV-C was first identified in 2006 and associated with high symptom burdens in children and asthmatics[27,28]. Recently, an asthma-related cadherin-related family member 3 (CDHR3) gene variant[29] was associated with greater RV-C receptor display on pulmonary cell surfaces of children and adults, and associated with higher susceptibility to severe virus-triggered asthma episodes[30,31]. In line, Romero-Espinoza et al detected predominantly RV-C in children with acute asthma exacerbations by mNGS[32]. The significance of RV-C infection in the adult population is less well studied. Although RV-C has been previously associated with exacerbations of COPD[33,34], to our knowledge, to date, CDHR3 polymorphisms have not yet found to be associated with COPD.

The sensitivity, specificity and positive and negative predictive values of mNGS were high: 96%, 100%, 82% and 100%, respectively, when encountering a cut-off of ≥5normalized reads, with a detection limit of approximately Cq 38. The high negative predictive value implicates the potential of mNGS to exclude the most prevalent viral respiratory infections in one test. The potential to exclude any infectious cause, both viral and bacterial, would have significant consequences for starting and/or continuation of antimicrobial or, at the other end of the spectrum, immune-modulating treatment.

The normalized viral species sequence read count might give an indication of the viral burden and the clinical relevancy of the detected virus. Although in our dataset we could not find any correlation with disease severity, several paediatric studies demonstrated a correlation between virus load and severity in respiratory infections[35-38]. Further analysis with a larger number of infected patients and/or a different spectrum of exacerbation severity will be needed to demonstrate or exclude such an association in COPD patients.

Furthermore, the complete respiratory virome showed a high bacteriophage abundance that could be linked to the absence of viral pathogens. Lower bacteriophage abundance may be the result of viral expansion. Hypothetically, a healthy virome size and diversity fits a certain size and diversity of bacteriophages, while during viral infection, pathogens predominate the virome. Alternatively, others have hypothesized that viral and microbial diversity may play a role in infection susceptibility and the development of acute and chronic respiratory diseases[32]. Our results indicate that virome dysbiosis may be accompanied by bacteriome dysbiosis, though no significant differences were detected in line with other reports[39,40]. However, these studies don't compare between COPD exacerbation with and without viral infections. Others have found a higher phage abundance in a patient with severe COPD when compared with one patients with moderate COPD and healthy controls, DNA sequencing, in line with the hypothesis of a state of dysbiosis that increases with disease progression[26]. In COPD patients, viral infections have been suggested to trigger bacterial overgrowth and infections[41,42], demonstrating the significance of viral-bacterial interactions. Moreover, hypothetically, bacteriophages play a role in the horizontal gene transfer of bacterial virulence factors.

The most abundant bacterial phyla detected in this study were comparable with other reports. Although the percentage of proteobacteria was relatively high when compared to other studies of the nasopharyngeal microbiome, our swabs are sampled during COPD exacerbations[43,44]. Study of the lower airways by means of e.g. protected brushes during bronchoscopy is needed for further

analysis of bacterial and viral (sub)populations including comparison with PCR and culture results. Studies comparing the respiratory virome during stable disease and exacerbations are needed to determine a potential correlation between the virome/bacteriome during stable state and disease progression or exacerbation frequency.

In the current study, most respiratory pathogens detected were RNA viruses. This is in line with previous literature[3,5,6]. However, it must be noted that, despite the fact that a wide range of DNA viruses have been detected with the current protocol (DNA bacteriophages with high abundance, herpes simplex virus, bocavirus, anelloviruses, CMV, KI polyomavirus[14]), we cannot exclude suboptimal detection of some DNA viruses. Furthermore, highly divergent viruses with sequences deviating from their representative NCBI RefSeq sequences may have been missed, as has been described by others[45]. However, bioinformatic classification using alternative databases (both GenomeDetective and local databases) did not result in additional findings.

Though mNGS renders the possibility to detect all viruses in direct respiratory material, this revolutionary method is not yet used as routine accredited diagnostic procedure for pathogen detection. Before mNGS can be implemented as a routine diagnostics, the optimal protocol must be defined and analysis and interpretation of the metagenomic data must be standardized, followed by external quality assessment. This study demonstrates good performance of our mNGS protocol, in line with other studies[36,37,46,47] and seems to overcome some of the current thresholds for implementation in clinical diagnostics.

Conclusions

The mNGS protocol used was highly sensitive and specific for semi-quantitative detection of respiratory pathogenic viruses. Excellent negative predictive value implicates the potential of mNGS to exclude a known viral infectious cause in one test, with consequences for clinical decision making. Reduced abundance of bacteriophages in COPD patients with viral pathogens implicates skewing of the virome, and speculatively the bacterial population, during infection.

ACKNOWLEDGEMENTS

We thank our GENERADE project partners Floyd Wittink, Wouter Suring (Hogeschool Leiden), Danny Duijsings (BaseClear) and Christiaan Henkel (Leiden University). We also like to thank Tom Vreeswijk, Lopje Höcker and Mario van Bussel (KML, LUMC) for help with the pre-sequencing experiments and Jeroen Laros (Human Genetics, LUMC) for help with the bioinformatics. We thank Caroline Brouwer for technical assistance (KML, LUMC).

REFERENCES

1. who. http://www.who.int/respiratory/copd/burden/en/ (accessed 08-10-2018 2018).
2. Aaron SD. Management and prevention of exacerbations of COPD. *BMJ (Clinical research ed)* 2014; **349**: g5237.
3. Ko FW, Chan KP, Hui DS, et al. Acute exacerbation of COPD. *Respirology (Carlton, Vic)* 2016; **21**(7): 1152-65.
4. Wedzicha JA, Seemungal TA. COPD exacerbations: defining their cause and prevention. *Lancet* 2007; **370**(9589): 786-96.
5. Sapey E, Stockley RA. COPD exacerbations . 2: aetiology. *Thorax* 2006; **61**(3): 250-8.
6. Hewitt R, Farne H, Ritchie A, Luke E, Johnston SL, Mallia P. The role of viral infections in exacerbations of chronic obstructive pulmonary disease and asthma. *Therapeutic advances in respiratory disease* 2016; **10**(2): 158-74.
7. van den Hoogen BG, de Jong JC, Groen J, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med* 2001; **7**(6): 719-24.
8. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 2012; **367**(19): 1814-20.
9. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc Natl Acad Sci U S A* 2005; **102**(36): 12891-6.
10. Prachayangprecha S, Schapendonk CM, Koopmans MP, et al. Exploring the potential of next-generation sequencing in detection of respiratory viruses. *Journal of clinical microbiology* 2014; **52**(10): 3722-30.
11. van Boheemen S, de Graaf M, Lauber C, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *mBio* 2012; **3**(6).
12. Yang J, Yang F, Ren L, et al. Unbiased parallel detection of viral pathogens in clinical samples by use of a metagenomic approach. *Journal of clinical microbiology* 2011; **49**(10): 3463-9.
13. Lewandowska DW, Schreiber PW, Schuurmans MM, et al. Metagenomic sequencing complements routine diagnostics in identifying viral pathogens in lung transplant recipients with unknown etiology of respiratory infection. *PloS one* 2017; **12**(5): e0177340.
14. van Boheemen S, van Rijn-Klink AL, Pappas N, et al. Metagenomic Sequencing for Combined Detection of RNA and DNA Viruses in Respiratory Samples from Paediatric Patients. *bioRxiv 2018, Dec 10* 2018: 492835.
15. Wang Z, Bafadhel M, Haldar K, et al. Lung microbiome dynamics in COPD exacerbations. *Eur Respir J* 2016; **47**(4): 1082-92.
16. Wang Y, Zhu N, Li Y, et al. Metagenomic analysis of viral genetic diversity in respiratory samples from children with severe acute respiratory infection in China. *Clin Microbiol Infect* 2016; **22**(5): 458 e1-9.
17. Lysholm F, Wetterbom A, Lindau C, et al. Characterization of the viral microbiome in patients with severe lower respiratory tract infections, using metagenomic sequencing. *PloS one* 2012; **7**(2): e30875.
18. Eagan TM, Ueland T, Wagner PD, et al. Systemic inflammatory markers in COPD: results from the Bergen COPD Cohort Study. *Eur Respir J* 2010; **35**(3): 540-8.
19. Loens K, van Loon AM, Coenjaerts F, et al. Performance of different mono- and multiplex nucleic acid amplification tests on a multipathogen external quality assessment panel. *Journal of clinical microbiology* 2012; **50**(3): 977-87.
20. Kim D, Song L, Breitwieser FP, Salzberg SL. Centrifuge: rapid and sensitive classification of metagenomic sequences. *Genome research* 2016; **26**(12): 1721-9.
21. Vilsker M, Moosa Y, Nooij S, et al. Genome Detective: An Automated System for Virus Identification from High-throughput sequencing data. *Bioinformatics* 2018.
22. Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology* 2012; **19**(5): 455-77.
23. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; **215**(3): 403-10.
24. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009; **25**(14): 1754-60.
25. Akobeng AK. Understanding diagnostic tests 3: Receiver operating characteristic curves. *Acta paediatrica (Oslo, Norway : 1992)* 2007; **96**(5): 644-7.
26. Elbehery AHA, Feichtmayer J, Singh D, Griebler C, Deng L. The Human Virome Protein Cluster Database (HVPC): A Human Viral Metagenomic Database for Diversity and Function Annotation. *Front Microbiol* 2018; **9**: 1110.
27. Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. *Journal of medical virology* 2006; **78**(9): 1232-40.

28. Lamson D, Renwick N, Kapoor V, et al. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004-2005. *The Journal of infectious diseases* 2006; **194**(10): 1398-402.
29. Bonnelykke K, Sleiman P, Nielsen K, et al. A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. *Nat Genet* 2014; **46**(1): 51-5.
30. Bochkov YA, Watters K, Ashraf S, et al. Cadherin-related family member 3, a childhood asthma susceptibility gene product, mediates rhinovirus C binding and replication. *Proc Natl Acad Sci U S A* 2015; **112**(17): 5485-90.
31. Kanazawa J, Masuko H, Yatagai Y, et al. Genetic association of the functional CDHR3 genotype with early-onset adult asthma in Japanese populations. *Allergol Int* 2017; **66**(4): 563-7.
32. Romero-Espinoza JA, Moreno-Valencia Y, Coronel-Tellez RH, et al. Virome and bacteriome characterization of children with pneumonia and asthma in Mexico City during winter seasons 2014 and 2015. *PloS one* 2018; **13**(2): e0192878.
33. Lau SK, Yip CC, Lin AW, et al. Clinical and molecular epidemiology of human rhinovirus C in children and adults in Hong Kong reveals a possible distinct human rhinovirus C subgroup. *The Journal of infectious diseases* 2009; **200**(7): 1096-103.
34. McErlean P, Shackelton LA, Lambert SB, Nissen MD, Sloots TP, Mackay IM. Characterisation of a newly identified human rhinovirus, HRV-QPM, discovered in infants with bronchiolitis. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2007; **39**(2): 67-75.
35. El Saleeby CM, Bush AJ, Harrison LM, Aitken JA, Devincenzo JP. Respiratory syncytial virus load, viral dynamics, and disease severity in previously healthy naturally infected children. *The Journal of infectious diseases* 2011; **204**(7): 996-1002.
36. Houben ML, Coenjaerts FE, Rossen JW, et al. Disease severity and viral load are correlated in infants with primary respiratory syncytial virus infection in the community. *Journal of medical virology* 2010; **82**(7): 1266-71.
37. Zhao B, Yu X, Wang C, et al. High human bocavirus viral load is associated with disease severity in children under five years of age. *PloS one* 2013; **8**(4): e62318.
38. Christensen A, Nordbo SA, Krokstad S, Rognlien AG, Dollner H. Human bocavirus in children: mono-detection, high viral load and viraemia are associated with respiratory tract infection. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2010; **49**(3): 158-62.
39. Gregory AC, Sullivan MB, Segal LN, Keller BC. Smoking is associated with quantifiable differences in the human lung DNA virome and metabolome. *Respiratory research* 2018; **19**(1): 174.
40. Norman JM, Handley SA, Baldridge MT, et al. Disease-specific alterations in the enteric virome in inflammatory bowel disease. *Cell* 2015; **160**(3): 447-60.
41. D'Anna SE, Balbi B, Cappello F, Carone M, Di Stefano A. Bacterial-viral load and the immune response in stable and exacerbated COPD: significance and therapeutic prospects. *Int J Chron Obstruct Pulmon Dis* 2016; **11**: 445-53.
42. Molyneaux PL, Mallia P, Cox MJ, et al. Outgrowth of the bacterial airway microbiome after rhinovirus exacerbation of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2013; **188**(10): 1224-31.
43. Huffnagle GB, Dickson RP, Lukacs NW. The respiratory tract microbiome and lung inflammation: a two-way street. *Mucosal Immunology* 2016; **10**: 299.
44. Ling Z, Liu X, Luo Y, et al. Pyrosequencing Analysis of the Human Microbiota of Healthy Chinese Undergraduates; 2013.
45. Bedarida S, Dussol B, Signoli M, Biagini P. Analysis of Anelloviridae sequences characterized from serial human and animal biological samples. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 2017; **53**: 89-93.
46. Thorburn F, Bennett S, Modha S, Murdoch D, Gunson R, Murcia PR. The use of next generation sequencing in the diagnosis and typing of respiratory infections. *J Clin Virol* 2015; **69**: 96-100.
47. Graf EH, Simmon KE, Tardif KD, et al. Unbiased Detection of Respiratory Viruses by Use of RNA Sequencing-Based Metagenomics: a Systematic Comparison to a Commercial PCR Panel. *J Clin Microbiol* 2016; **54**(4): 1000-7.

CHAPTER 4

Clinical implications of rapid eplex® respiratory pathogen panel testing compared to laboratory developed real-time pcr

A.L. van Rijn[a], R.H.T. Nijhuis[a], V. Bekker[b], G.H. Groeneveld[c], E. Wessels[a], M.C.W. Feltkamp[a], E.C.J.Claas[a]

[a] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[b] Department of Paediatrics, Leiden University Medical Center, Leiden, the Netherlands
[c] Department of Internal Medicine, Leiden University Medical Center, Leiden, the Netherlands

Eur J CLin Microbiol Infect Dis 2018 37 (3): 571-577

ABSTRACT

Introduction: Rapid diagnosis of respiratory infections is of great importance for adequate isolation and treatment. Due to the batch-wise testing, lab developed real-time PCR assays (LDT) often result in a time to result of one day. Here, LDT was compared with rapid ePlex® respiratory pathogen (RP) panel testing of GenMark Diagnostics (Carlsbad, CA, USA) with regard to time to result, installed isolation precautions and antibacterial/antiviral treatment.

Methods: Between January and March 2017, 68 specimens of 64 patients suspected of an acute respiratory infection were tested with LDT and ePlex® RP panel. Time to result was calculated as time between sample reception and result reporting. Information regarding isolation and antibacterial/antiviral treatment was obtained from the patient records.

Results: Thirty specimens tested LDT positive (47%) and 29 ePlex® RP panel positive (45%). The median time to result was 27.1 hours (range 6.5-96.6) for LDT vs. 3.4 hours (range 1.5-23.6) for RP panel, *P*-value <0,001. In 14 out of 30 patients, isolation was discontinued based on ePlex® RP panel results, saving 21 isolation days. ePlex® RP panel test results were available approximately one day ahead of LDT results in the 19 patients receiving antiviral/ antibacterial treatment. In addition, two bacterial pathogens, not requested by the physician, were detected using RP panel.

Conclusions: Analysis of respiratory infections with the ePlex® RP panel resulted in a significant decrease in time to result, enabling a reduction in isolation days in half of the patients. Furthermore, syndromic RP panel testing increased identification of causative pathogens.

INTRODUCTION

Respiratory tract infections are a leading cause of hospital admission, morbidity, and mortality[1-4]. At presentation aetiological agents of the respiratory tract infection cannot be identified solely based on clinical signs and symptoms. Therefore, and awaiting microbiological confirmation, empirical antibiotic and antiviral treatment is initiated based on severity score and the influenza season[5]. Since only a minority of the infections is being caused by bacteria, this empiric antibiotic treatment approach is redundant and can lead to an increase in antibiotic resistance. Moreover, empiric isolation precautions are installed to protect other patients and health care workers from a possible (viral) infection. Altogether, there is a need for rapid identification or exclusion of a viral respiratory tract infection to reduce inappropriate (unnecessary) hospital hygienic interventions and focus (shorten) antibacterial/antiviral treatment.

Currently, the diagnosis of respiratory infections is usually based on (a combination of) molecular amplification methods and bacterial culture. In our laboratory, lab developed real-time PCR multiplex assays (LDTs) are used that show excellent sensitivity and specificity. However, this approach is limited by the number of targets per multiplex reaction and the need for batch-wise testing. The assays are performed once daily, with a time to result of approximately 20 hours.

Recently, the Respiratory Pathogen (RP) Panel of GenMark Diagnostics (Carlsbad, CA, USA) has become available for detection of an extensive panel of respiratory pathogens (21 respiratory viruses, 3 bacterial species, see Methods) using eSensor technology[6]. This test is a cartridge based molecular assay to be used on the ePlex® platform with a time to result of approximately 90 minutes that showed an concordance of >97% compared to LDT[7]. Hypothetically ePlex® RP panel testing represents a considerable reduction in time to diagnosis, as compared to LDT, which could have significant clinical benefits. In this paper, a pilot study is reported that analysed the implications of using the ePlex® RP panel for the detection of respiratory infections compared to LDT regarding time to result, isolation precautions, and antibacterial/antiviral therapy.

METHODS

Inclusion of patients

This prospective, single centre study in the Leiden University Medical Center (LUMC) included patients from January to March 2017. Patients with symptoms of an acute respiratory infection were included upon request of the physician of the acute ward, intensive care unit, and paediatric department. Specimens included were obtained during weekdays and tested with both the RP panel and the LDTs after consulting the microbiologist. Information regarding baseline characteristics, infection parameters, admittance, isolation and treatment was obtained from the electronic patient records. Additional information about cultures was retrieved from the laboratory information system (GLIMS, MIPS, Belgium). The medical ethics review committee of the LUMC approved the study.

Primary outcome measure

The primary endpoint of this study was the time to result of the ePlex® RP panel compared to the LDT.

Secondary outcome measures

The ePlex® RP panel was offered as a pilot to elevate the pressure on droplet isolation rooms, thus isolation was discontinued based on the ePlex® RP panel results. Due to the pilot nature of this study antibacterial and antiviral treatment were not adjusted based on the ePlex® RP panel results, therefore only the theoretical time reduction in treatment was calculated using the time to results of the ePlex® RP panel and the LDT. Secondary outcome measures were the reduction of isolation days based on ePlex® RP panel ahead of LDT results, the theoretical reduction in hours in oseltamivir and atypical pneumonias treatment calculated with the time to results of LDT and ePlex® RP panel, and possible additional diagnosis found with the ePlex® RP panel.

Laboratory-developed test (LDT)

LDT viral testing and testing for *Mycoplasma pneumoniae, Chlamydia pneumoniae and Chlamydia psittaci* was performed the same day on all samples that arrived at the laboratory before 8:15 A.M. Samples arriving at the laboratory before 3:30 P.M., were tested for *Legionella pneumophila* and *Bordetella parapertussis* the following day. These assays were performed daily from Monday till Friday and on request on weekend days. The viral respiratory panel of LDT consists of adenovirus, bocavirus, coronavirus 229E, coronavirus HKU1, coronavirus NL63, coronavirus OC43, influenza A, influenza B, human metapneumovirus, parainfluenza 1-4 (differentiation with differently labelled probes), respiratory syncytial virus, and rhinovirus. In addition, testing for bacterial pathogens could be requested: Legionella species, *Legionella pneumophila*, *Mycoplasma pneumoniae, Chlamydia pneumoniae, Chlamydia psittaci, Bordetella pertussis,* and *Bordetella parapertussis.*

All sputa samples were 1:5 diluted in PBS and homogenized by bead-beating prior to extraction. Then, 200 µl of each respiratory sample was used to extract 100 µl total nucleic acids using the Total Nucleic Acid extraction kit on the MagnaPure LC system (Roche Diagnostics). Nucleic acid amplification and detection by real-time PCR was performed on a BioRad CFX96 thermocycler, using primers, probes and conditions as described previously[8-10]. For the detection of *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and *Chlamydia psittaci* the b-CAP assay (Biolegio, Nijmegen, the Netherlands) developed for the BD-max system was used by testing 200 µl of each respiratory sample according to the manufacturer's instructions[11]. LDT test results were reported in the electronic patient record. Time to result for the LDT was calculated as time of receipt of the sample in the laboratory to the time results were available in the electronic patient record.

ePlex® RP panel

Specimens for diagnosis using the CE-IVD cleared RP panel were accepted on weekdays between 8:15 A.M. and 3:00 P.M. and tested during the day, as soon as possible. The ePlex® respiratory panel was not offered during the weekend, while treatment was not adjusted based on the results. The RP panel as used in the study was able to detect: adenovirus, bocavirus, coronavirus 229E, coronavirus HKU1, coronavirus NL63, Coronavirus OC43, influenza A H1, influenza A 2009 H1N1, influenza A H3, influenza B, metapneumovirus, Middle East respiratory syndrome coronavirus, parainfluenza 1-4, respiratory syncytial virus A and B, rhinovirus/ enterovirus, *Bordetella pertussis, Legionella pneumophila,* and *Mycoplasma pneumoniae.* As with LDT, sputa samples were diluted in an 1:5 dilution using PBS. According to the manufacturer's instructions 200 µl of the respiratory sample was pipetted in a buffer tube and after vortexing transferred to the ePlex cartridge and subsequently to the ePlex tower. If the test gave an invalid result, the run was repeated. Results were reported by telephone to the requesting physician, since the results were not reported in the electronic patient record. Time to result was calculated as time of receipt of the sample to the time results were reported by telephone.

Statistics used for comparison

Time to result was compared with Wilcoxon Signed Rank Test, using IBM SPSS Statistics version 23 software for Windows. A P-value <0.05 was considered statistically significant.

RESULTS

LDT and ePlex® RP panel results

Between January and March 2017 64 patients were included with symptoms of acute respiratory infection whose characteristics are summarized in Table 1. A total of 68 samples were tested, comprising 40 throat swabs, 13 sputum samples, 11 nasal lavages, and four nasopharyngeal swabs. Thirty-four tested positive for a respiratory pathogen in one or both assays. Six samples failed in the ePlex® RP panel, of which two gave a valid result upon retesting. The other four were not retested, two because of insufficient remaining sample volume. The failed samples, if not retested, were excluded from further analysis, leaving 64 samples of 61 patients for further analysis. None of the samples failed in the LDT.

Of the 64 samples 31 tested positive for a total of 37 pathogens with LDT or ePlex® RP panel (Table 2). Using LDT, 30 tested positive and 34 negative, whereas this was 29 and 35 using the ePlex® RP panel. As shown in Table 3A, a discordant result was found in five samples.

In three patients, different sample types were tested (Table 3B). From the first a sputum and a throat swab were collected, of which only the first tested LDT positive for influenza A. The second tested rhinovirus positive in a nasal lavage, with LDT only, and negative in sputum. Of the third patient a sputum and a throat swab were tested, of which only the sputum tested coronavirus 229E positive.

Table 1. patient characteristics

	Patients n=64	Range/%
Demographics		
Age, median years (range)	60	0-93
Male sex (%)	33	52
Clinical features		
Diagnosis		
Pneumonia (%)	25	39
COPD/ asthma exacerbation (%)	7	11
RTI other than pneumonia (%)	12	19
Other diagnosis (%)	20	31
Leukocytes, median x10^9/L (range)	11.4	0.44-49.16
C-reactive protein level, median mg/L (range)	62	2-360
Cough (%)	49	77
Sputum (%)	26	41
Previous antibiotic treatment (%)	20	31
Duration of symptoms, median days (range)	2	1-21
Comorbidity		
COPD/asthma (%)	17	27
Diabetes (%)	7	11
Malignancy (%)	6	9
Transplantation (%)	12	19
Auto-immune disease (%)	8	13
Admission ward		
Acute ward	32	50
Intensive care (including children)	8	13
Paediatric department	8	13
Other departments	15	23
Not admitted	1	1

Abbreviations: n, number; COPD, chronic obstructive pulmonary disease; RTI, respiratory tract infection

Table 2. Respiratory pathogens found in clinical samples with lab developed real-time PCR assay or ePlex® RP panel

Pathogens	LDT	ePlex® RP panel
Coronavirus 229E	2	2
Coronavirus HKU1	1	1
Human bocavirus	1	1
Human metapneumovirus	5	4
Influenza A	10	9 (all H3)
Influenza B	1	1
Parainfluenza virus type 3	1	1
Respiratory syncytial virus	4	0
Respiratory syncytial virus type A		2
Respiratory syncytial virus type B		2
Rhinovirus/enterovirus	8	9
Bordetella pertussis	1	1
Mycoplasma pneumoniae	1	1

Abbreviation: LDT, Lab developed real-time PCR

Table 3A. Discrepant results of lab developed real-time PCR assay compared to ePlex® RP panel

	LDT	ePlex® RP panel
Throat swab	Negative (retesting negative)	RV/EV
Nasopharyngeal swab	InfA (Cq 26) (enterovirus negative)	InfA- RV/EV
Nasal lavage	RV (Cq 39.1)	Negative
Sputum	MPV (Cq 30.3)	Negative (retesting MPV pos)
Sputum	infA (Cq 33.1)	Negative (retesting negative)

Abbreviations: LDT, Lab developed real-time PCR; RV, rhinovirus; EV, enterovirus; InfA, influenza A; MPV, metapneumovirus; Cq,quantification cycle

Table 3B. Different sample types tested

Patient	Material	LDT	ePlex® RP panel
1	**Sputum**	infA (Cq 33.1)	Negative
	Throat swab	Negative	Negative
2	**Sputum**	Negative	Negative
	nasal lavage	RV (Cq 39.1)	Negative
3	**Sputum**	CoV 229E (Cq 33.4)	CoV229E
	Throat swab	Negative	Negative

Abbreviations: LDT, Lab developed real-time PCR; RV, rhinovirus; InfA, influenza A; CoV,coronavirus; Cq,quantification cycle

Primary outcome measure:

Difference in time to result

For 62 of the 64 samples, both the time of acceptance and the time of result was recorded. The calculated time to result was significantly shorter, approximately 24 hours, for the ePlex® RP panel than for LDT (*P*<0.001) (Table 4). A time to result of over 35 hours was seen with LDT testing in 15 samples, of which 13 had arrived on Friday and were tested on Monday. In the two remaining samples there was a delay in requesting and authorisation of the test subsequently. In the ePlex® RP panel, four samples had a time to result of more than 18 hours. Two of these samples were already at the laboratory for several hours before the ePlex® RP panel testing was requested, while the testing of two sample was requested after 3:00 P.M. and therefore performed the next day (one due to failure of the initial sample).

Table 4. Time to result in hours of lab developed real-time PCR assay compared to ePlex® RP panel

Time to result	LDT	ePlex® RP panel	P-value*
Median (hours)	27.11	3.35	<0,001
Range (hours)	6.52-96.57	1.45-23.56	

Abbreviation: LDT, Lab developed real-time PCR

* P-value calculated with Wilcoxon signed rank test

Secondary outcome measures

Consequences for patient isolation

Of the 61 patients included in the analysis, 60 were admitted to the hospital at the time respiratory testing was requested. Fifty-one of these hospitalised patients were isolated while awaiting test results, whereas nine patients were not admitted in isolation. In these cases isolation was not installed mainly because of low clinical suspicion of a pathogen requiring isolation. One of these nine patients needed isolation, since the ePlex® RP panel tested positive for influenza A (three days ahead of LDT).

The tests showed that 19 out of 51 patients admitted in isolation had a respiratory pathogen requiring isolation. Of the remaining 32 patients, one died before test results became available and for one patient the duration of isolation was unknown, leaving 30 patients for further analysis. In 14 of these isolation was discontinued based on ePlex® RP panel results ahead of LDT results. This resulted in a total reduction of 21 isolation days, with a median reduction of 2 days (range 1-4 days) per patient. In eight of the remaining patients, isolation was discontinued when LDT results became available. In the other eight patients, of which three children, isolation was not withdrawn at the moment LDT results were reported.

Theoretical consequences for antiviral and antibacterial treatment

A total of 50 out of the 61 patients received antiviral or antibacterial treatment during hospitalization. Oseltamivir treatment was initiated in 19 patients awaiting test results, of which five tested positive for influenza A. In the 14 influenza ePlex® RP panel negative patients, oseltamivir could have been stopped approximately one day earlier (median of 22.59 hours, range 5.33-72.03) based on ePlex® RP results compared to LDT (Table 5). Of the in total 11 patients who tested influenza positive, the remaining six did not receive oseltamivir at the time of diagnosis. In one patient, oseltamivir treatment was started as soon as ePlex® RP panel showed influenza A, one day prior to LDT results, and one patient started when LDT was positive. Four patients did not receive any antiviral treatment, of which two were already dismissed at the time of definite LDT diagnosis.

Awaiting test results, 19 patients received antibiotic treatment for bacteria causing atypical pneumonias. In none of these patients, either the ePlex® RP panel or the LDT (eight were tested) was positive for *Bordetella pertussis, Legionella pneumophila,* and *Mycoplasma pneumoniae.* In theory, in these 19 patients, a median duration of 23.35 hours (range -0.43- 75.28 hours) antibiotic treatment for atypical pneumonia could have been saved, if treatment was stopped when ePlex® RP panel tested negative.

Table 5. Theoretical median time in hours of isolation and treatment calculated based on time to results

	No.	LDT (range)	ePlex® RP panel (range)	Difference (range)
Oseltamivir, h	14	27.08 (10.10-75.15)	3.38 (2.00-23.56)	22.59 (5.33-72.03)
Antibiotics atypical pneumonias, h	19	27.12 (8.27-81.11)	3.38 (1.52-23.56)	23.35 (-0.43-75.28)

Abbreviations: No, number of patients; LDT, Lab developed real-time PCR; h, hours

Additional diagnoses

Of the 61 patients, two tested positive by ePlex® RP panel for a bacterial agent, one *Bordetella pertussis* and one *Mycoplasma pneumoniae.* In both patients, testing for these pathogens was not requested for by the clinician and as a consequence not included in the routine diagnostic LDT workflow. The positive ePlex® RP panel results were confirmed by LDT with Cq-values of 25.6 and 34.6 for *B. pertussis* and *M. pneumoniae*, respectively. LDT testing for atypical bacterial pathogens (*Mycoplasma pneumoniae, Chlamydia pneumoniae, Chlamydia psittaci)* was requested in only 16 patients. Legionella LDT testing was requested in only 10 patients.

DISCUSSION

As hypothesized, diagnosis with the ePlex® RP assay significantly reduced the time to result (median 23.34 hours) as compared to batch wise LDT testing. Consequently, a total of 21 isolation days were saved and three days of influenza A exposure prevented. Unnecessary oseltamivir treatment could have been shortened at least 20 hours in 14 patients and antibiotic treatment for atypical pneumonias by a median of 23.35 hours days in 19 patients. Proper therapeutic and isolation measurements could be installed in two patients for bacterial pathogens based on ePlex® RP panel detection that were not considered by the treating physicians and therefore not analysed by routine LDT.

To our knowledge this study is the first to report the use of the ePlex® respiratory panel in a clinical setting. It demonstrated a significant time reduction, reflecting previous clinical studies implementing rapid molecular testing[12-15], and significantly reduced the number of isolation days. Furthermore, confirmation of a single viral cause of infection in a cohort of patients enabled cohort nursing, which increased the number of isolation rooms available to patients awaiting identification of their respiratory pathogen. Efficient use of isolation rooms is essential during influenza season when the demand for these rooms is high.

The rapid ePlex® RP panel results could have resulted in a reduction of oseltamivir usage, which is in line with previous studies[14].Results regarding reduction in antibiotic treatment for atypical pneumonias should be interpreted with care, while they are, according to the Dutch guidelines, only indicated for *Legionella pneumophila* in high risk populations and can also been stopped based on negative urine-antigen testing. The lack of routine testing for atypical respiratory bacterial pathogens (mostly *Legionella pneumophila*) and the finding of additional respiratory pathogens, initially not considered by the clinicians, underline the importance of syndromic respiratory testing.

Our study has a number of limitations. First, the clinical impact of our pilot study was hampered by its design. Since the ePlex® RP assay was readily offered to reduce the quest for isolation rooms during the coinciding influenza and RSV epidemics early 2017, its test results were not shown yet in the hospital information system but reported by phone, creating a bias. Moreover, the ePlex® RP panel result was reported as a provisional result awaiting routine LDT confirmation. The delay in showing the test results in the electronic patient record, might have withheld clinicians to discontinue isolation and therefore created an underestimation of the true clinical potential. Furthermore, the findings of this study cannot be extrapolated readily, since this was a single centre study during just a part of one winter season. The benefits of rapid diagnostics might be more pronounced when assessing complete respiratory seasons.

So far, the ePlex® RP panel has been CE-IVD cleared for nasopharyngeal swabs only. However, especially samples from the lower respiratory tract as sputum and bronchoalveolar lavage can be important to include in the CE-IVD clearance, since our study shows that these samples might have a higher diagnostic yield. However, both in our previous and current study several different sample types were tested with good results[7]. Nevertheless, the ePlex® RP panel had a failure rate of nearly 10%, in two cases due to internal control failure, none of the LDT tests failed. Overall, the ePlex® RP

panel results showed excellent concordance with our LDT, only three LDT positives (all with Cq-values >30) could not be detected using the ePlex® RP panel. This is in line with our previous findings reported by Nijhuis et al.[7]. The ePlex® RP panel is based on syndromic testing and has a standard panel containing most common respiratory pathogens that are requested by the physician. However, the ePlex® RP panel is not complete, especially when caring for in immunocompromised patients. In that case additional LDT testing for Legionella species, cytomegalovirus, herpesvirus, toxoplasmosis and fungal pathogens would still be necessary. Compared to LDT, ePlex® RP panel testing is more expensive regarding reagents and consumables but cheaper with respect to hand-on-time.In addition, rapid diagnostics will result in a cost reduction in the clinical departments as demonstrated previously[16].

In conclusion, diagnosis of respiratory infections with the ePlex® RP assay resulted in a significant reduction in time to result compared to LDT, which causes a reduction in isolation days and theoretically improved treatment regimens. Because of these advantages, we assume this rapid diagnostic molecular assay will be of added value for ongoing improvement in patient care.

ACKNOWLEDGEMENTS

We thank Mario van Bussel and Wilfred Rijnsburger of the Department of Medical Microbiology for the molecular testing. We thank Martijn Bauer of the acute ward and Dagmar Berghuis and Robbert Bredius of the paediatric department for including patients. And we thank the medical virologists on call for including the cases and reporting the ePlex® RP assay results by telephone.

REFERENCES

1. Bates M, Mudenda V, Mwaba P, Zumla A. Deaths due to respiratory tract infections in Africa: a review of autopsy studies. *Current opinion in pulmonary medicine* 2013; **19**(3): 229-37.
2. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet (London, England)* 2012; **380**(9859): 2095-128.
3. Lim SS, Vos T, Flaxman AD, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet (London, England)* 2012; **380**(9859): 2224-60.
4. Anne Pfuntner LMW, M.P.H., and Carol Stocks, R.N.,, M.H.S.A. Most Frequent Conditions in U.S. Hospitals, 2010. Accessed 17 March 2017 (accessed 17-03-2017 2017).
5. Fine MJ, Auble TE, Yealy DM, et al. A prediction rule to identify low-risk patients with community-acquired pneumonia. *N Engl J Med* 1997; **336**(4): 243-50.
6. https://www.genmarkdx.com/int/solutions/panels/eplex-panels/respiratory-pathogen-panel/. Accessed 07 June 2017.
7. Nijhuis RH, Guerendiain D, Claas EC, Templeton KE. Comparison of the ePlex(R) Respiratory Pathogen Panel with Laboratory Developed Real-Time PCR for the Detection of Respiratory Pathogens. *Journal of clinical microbiology* 2017; **55**(6): 1938-45.
8. Loens K, van Loon AM, Coenjaerts F, et al. Performance of different mono- and multiplex nucleic acid amplification tests on a multipathogen external quality assessment panel. *Journal of clinical microbiology* 2012; **50**(3): 977-87.
9. Templeton KE, Scheltinga SA, van der Zee A, et al. Evaluation of real-time PCR for detection of and discrimination between Bordetella pertussis, Bordetella parapertussis, and Bordetella holmesii for clinical diagnosis. *Journal of clinical microbiology* 2003; **41**(9): 4121-6.
10. Templeton KE, S.A. Scheltinga, P. Sillekens, J.W. Crielaard, A.P. van Dam, H. Goossens, and E.C.J. Claas. . Development and clinical evaluation of an internally controlled, single tube multiplex real-time PCR for the diagnosis of Legionella pneumophila and Legionella species. *Journal of clinical microbiology* 2003; **41**: 4016-21.
11. I.O.M. Op den Buijs. Development of a multiplex qPCR for the detection of atypical pneumonia using the automated MAX™ system. *NVMM Spring meeting* 2014.
12. Brendish NJ, Malachira AK, Armstrong L, et al. Routine molecular point-of-care testing for respiratory viruses in adults presenting to hospital with acute respiratory illness (ResPOC): a pragmatic, open-label, randomised controlled trial. *The Lancet Respiratory medicine* 2017; **5**(5): 401-11.
13. Muller MP, Junaid S, Matukas LM. Reduction in total patient isolation days with a change in influenza testing methodology. *American journal of infection control* 2016; **44**(11): 1346-9.
14. Chu HY, Englund JA, Huang D, et al. Impact of rapid influenza PCR testing on hospitalization and antiviral use: A retrospective cohort study. *Journal of medical virology* 2015; **87**(12): 2021-6.
15. Rogers BB, Shankar P, Jerris RC, et al. Impact of a rapid respiratory panel test on patient outcomes. *Archives of pathology & laboratory medicine* 2015; **139**(5): 636-41.
16. Poelman R, van der Meer J, van Leer-Buter C, Riezebos-Brilman A, Niesters HGM. Point-of-impact testing in the emergency department: Rapid diagnostics for respiratory viral infections. *Journal of Clinical Virology*; **70**: S48.

CLINICAL IMPLICATIONS OF RHINOVIRUS

PART II

CHAPTER 5.1

The impact of rhinovirus infections in paediatric cardiac surgery (risk): study protocol for a prospective cohort study

A.L. van Rijn[a], P.P. Roeleveld[b], R.B.P. de Wilde[b], E.W. van Zwet[c], M.G. Hazekamp[d], J. Wink[e], J. Calis[f], A.C.M. Kroes[a], J.J.C. de Vries[a]

[a] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[b] Department of paediatric Intensive care, Leiden University Medical Center, Leiden, the Netherlands
[c] Department of Statistics, Leiden University Medical Center, Leiden, the Netherlands
[d] Department of thoracic surgery, Leiden University Medical Center, Leiden, the Netherlands
[e] Department of anaesthesiology, Leiden University Medical Center, Leiden, the Netherlands
[f] Department of paediatric Intensive care, Academic Medical Center Amsterdam, Amsterdam, The Netherlands

J Clin Trials 2015 5:246

ABSTRACT

Background: Respiratory infections are considered a potential risk of adverse events in children undergoing surgery. Rhinovirus is a common cause of respiratory infections and congenital heart disease is a risk factor for severe rhinovirus infection. However, we do not know what the impact of, clinical or subclinical, rhinovirus infections is on postoperative course following congenital heart surgery in children.

Based on our clinical experience, one case-controlled study, and a case reported in the literature, we hypothesize that paediatric patients with per-operative rhinovirus positive Polymerase Chain Reaction testing have a longer paediatric intensive care unit admission, compared to children who test negative.

Methods/ Design: This is a prospective single-center observational study in the Leiden University Medical Center with approximately 250 children (<12 years) undergoing elective cardiac surgery, for congenital heart disease.

The parents/guardians of the children will be asked to fill out a questionnaire, to asses respiratory symptoms in the last weeks, before the operation of their child. In the operating theatre, a nasopharyngeal swab will be collected. Clinical data will be collected daily during paediatric intensive care admission and paediatric intensive care unit and hospital length of stay will be recorded. If children are still intubated at day 4, a second nasopharyngeal swab and residual blood will be collected. The samples will be tested for rhinovirus with polymerase chain reaction. Primary outcome is the paediatric intensive care unit length of stay in per-operative rhinovirus – positive compared to rhinovirus-negative patients.

Discussion: This is the first study to screen children for rhinovirus before undergoing cardiac surgery and to study the effects on paediatric intensive care unit length of stay. Furthermore, we aim to identify children at risk for prolonged paediatric intensive care admission after cardiac surgery.

Trial registration: ClinicalTrials.gov Identifier NCT02438293; registration date 5 May 2015.

BACKGROUND

Rhinovirus (RV), a Ribonucleic Acid (RNA) virus of the family *Picornaviridae* and genus *Enterovirus* is a major cause of upper respiratory tract infections. The clinical spectrum ranges from asymptomatic to severe, life-threatening-pneumonia[1].

Asymptomatic shedding of RV is very common, especially in children, and tends to be higher in the younger age groups. In a study in the Netherlands, the prevalence of rhinovirus amounted to 20% for children under 2 years of age without nasal symptoms[2]. Risk factors for severe rhinovirus infections (lower respiratory tract infections, infections needing hospitalisation) in children are prematurity, congenital heart disease, respiratory syncytial virus (RSV) co-infections and non-infectious respiratory disease[3].

Upper respiratory tract infections are considered to carry a potential risk of adverse events in children undergoing surgery. Surgery in children with symptomatic upper respiratory tract infection is commonly postponed, because of the increased risk of complications of anaesthesia in these patients[4]. Adverse events (AE) related to respiratory tract infections are: laryngo-bronchospasm, breath holding spells, atelectasis, arterial oxygen desaturation, bacterial pneumonia and unplanned hospital admission[5,6]. Age below 6 years and cardiac surgery are additional specific risk factors for postoperative complications. The risk of peri-operative adverse events is increased up to 6 weeks after upper respiratory tract infections[4]. Patients undergoing cardiac surgery might have an additional risk, because of the immune-modulatory effect of cardiopulmonary bypass[7-9]. Children with a clinically apparent upper respiratory tract infection at the time of cardiac surgery have higher incidences of respiratory complications, multiple complications, postoperative infections and significantly longer paediatric intensive care unit (PICU) admission[10]. Paediatric patients with postoperative symptomatic rhinovirus infection appear to have a more complicated and prolonged post-operative course compared to other patients undergoing cardiac surgery (unpublished Leiden University Medical Center (LUMC) data). Especially PICU length of stay and duration of mechanical ventilation seem to be longer in patients with a rhinovirus infection. Every year approximately 20 paediatric patients in the LUMC have a complicated post-operative course in combination with a rhinovirus infection (unpublished LUMC data). Little has been published about the impact of rhinovirus infections on the post-operative course after cardiac surgery in paediatric patients. Simsic et al. described a case of a nine months old boy with rhinovirus infection and a complicated course after cardiac surgery. Based on this case the authors screen all their paediatric patients with single-ventricle congenital heart disease by polymerase chain reaction (PCR) for respiratory viruses. In case of a positive PCR result, they postpone the operation for 4-6 weeks[11]. Delgado-Corcoran et al. recently published a case-control study of 19 cases of infants who tested positive for rhinovirus after cardiac surgery compared to 56 matched controls, untested symptom free patients. In this study the rhinovirus positive patients had a significantly longer intubation time and a three times longer PICU length of stay[12].

Ideally, these complications may be prevented by screening patients for the presence of respiratory infections with a clinical assessment in combination with PCR on a nasopharyngeal swab prior to their elective surgery.

Based on these retrospective data, we designed a single center prospective observational cohort study to analyse the impact of per-operative rhinovirus infection on the post-operative course in children undergoing elective cardiac surgery, with PICU length of stay as primary outcome. The subsequent goal is to develop an algorithm to identify children with increased risk for prolonged respiratory support after cardiac surgery. We named it the RISK study: Rhinovirus Infection & cardiac Surgery in Kids.

METHODS/ DESIGN

Design

This is a prospective, single-center, observational cohort study of the effects of rhinovirus in children undergoing cardiac surgery in the LUMC.

The LUMC is an academic hospital in Leiden, the Netherlands, and a top referral center for congenital heart disease and congenital cardiac surgery with approximately 250 operations each year. Combined with the two university medical centers of Amsterdam it constitutes the center for congenital heart disease Amsterdam - Leiden, in Dutch CAHAL[13].

Current standard procedures regarding elective cardiac surgery include the following:

The day before the planned operation patients are assessed by the cardiologist, cardiac surgeon and anaesthesiologist who decide if the surgery can go ahead or if it will have to be postponed because of possible clinically relevant (respiratory tract) infection.

After surgery, patients are admitted to the PICU, where ventilation and circulatory support are weaned depending on the type of operation, bypass times and clinical stability. If symptoms of respiratory tract infection become apparent during the postoperative PICU stay, patients are routinely tested for either bacterial infections (culture) and/or viral infections by PCR for respiratory viruses on nasal lavage at the discretion of the treating intensive care physician.

In this study we will test all children for rhinovirus who are < 12 years of age with congenital heart disease undergoing elective cardiac surgery in the LUMC. The majority of the children will be 0-1 years old, since cardiac surgery is preferably performed at (very) young age.

The primary endpoint of this study is post-operative PICU length of stay in rhinovirus positive compared to rhinovirus negative patients.

Secondary endpoints are:

- Duration of ventilatory support
- mechanical ventilation conditions (mean airway pressure, Fraction of inspired oxygen (FiO_2))
- Antibiotic free days (alive at PICU discharge)
- Inotrope requirement
- Infection parameters
- Hospital length of stay (LOS)
- Secondary infections

Alternative predictors of the endpoints are viral load (strength of PCR value), duration of respiratory shedding, genotype of the different rhinoviruses and rhinovirus viremia.

Inclusion criteria

In order to be eligible to participate in this study, subjects must meet all of the following criteria:

- Children (<12 year) with a congenital heart disease undergoing elective cardiac surgery who were not admitted pre-operatively
- Written informed consent by parents or guardian

Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- No informed consent from one of the parents (or the legal representative if applicable)
- Emergency surgery (postponing of surgery not an option)
- Children not admitted to the intensive care unit after cardiac surgery (negligible amount of children)
- Children who will certainly have a prolonged PICU length of stay regardless of a possible rhinovirus infection:
    - Children undergoing a second elective cardiac operation during the same intensive care stay
    - Children with duct-dependent physiology who remain prostaglandin-dependent after the heart operation. For example: hypoplastic left heart syndrome following pulmonary artery banding who will remain on prostaglandins until the next staged operation.

Sample size calculation

This study will include approximately 250 children. The sample size calculation is based on the expected percentage of rhinovirus positive children and the expected difference in duration of PICU admission, our primary end point.

The estimated prevalence of preoperative children with rhinovirus of 20% was based on a prospective birth cohort study in the Netherlands among healthy children followed until the age of 2 years. In that study, the prevalence of rhinovirus among children without nasal symptoms was 20% (range 14-28%) (and 40% among children with rhinitis/upper respiratory tract infection)[2].

The mean duration of PICU admission following cardiac surgery in our PICU is 3.6 days. The study of Delgado-Corcoran et al., demonstrated almost a tripling of intensive care (IC) length of stay in rhinovirus infected children after surgery for congenital heart disease (up to one year of age), 2,14 (IQR 2.00-3.22) versus 6,03 days (IQR 2.98-14.22, p<0.0001). Since the control patients in this study were not tested for rhinovirus and therefore could be (asymptomatic) rhinovirus positive, the difference within our study might turn out to be smaller[12]. Combining these published data with a clinically relevant difference of 1 day and more, we aim to distinguish a difference between the two groups of 2 days. We base our sample size calculation on the comparison of two independent exponential means (PICU LOS in both groups).

Given these data for a power of 80% ((β = 0.20), tested with a significance level of 5% (α = 0.05), we calculated n 49 (sample size rhinovirus positive group and n=196 (sample size rhinovirus negative group) (ratio 1:4).

Data collection

Study procedure

If a patient is eligible for the study, the parents/ guardians will receive the information folder and a questionnaire by mail (see additional file 1) (asking for signs and symptoms of current and/or recent respiratory infections) approximately one week prior to the date of operation.

On the day of admission (day 0) all parents of eligible children will be asked to participate in this study and fill out a written informed consent form.

At day 1, the operation day, in the operating theatre, a nasopharyngeal swab will be collected following anaesthetic induction and tested for rhinovirus. All clinicians will be blinded for the PCR results which will only be accessible for the investigator from the virology department.

Clinical and laboratory data will be collected from all patients until discharge from the PICU. Of the patients still on mechanical ventilation at day 4, an additional rhinovirus PCR will be tested on a nasopharyngeal swab and residual blood will be used to determine rhinovirus viremia in the nasopharyngeal rhinovirus positive patients.

Collection of clinical data

On admission (i.e. day 0)

Baseline and demographic characteristics will be collected, including:

- Age, gender, cardiac diagnosis and medical history, including vaccinations, medication, respiratory conditions (asthma) and family medical history of atopic diseases.
- History of respiratory infections: amount, prior viral testing, symptoms, medication use and symptoms of family members.
- Passive smoking
- Physical examination: temperature – blood pressure- hearts rate- respiratory rate- cardiac sounds – breath sounds – rhinorrhoea (all part of routine preoperative medical screening/ care)
- Additional examination: Chest x-ray- Electrocardiogram (ECG)- Blood tests (C-reactive protein (CRP), Leukocytes) (all part of routine preoperative medical screening/ care)

On day of operation (i.e. day 1)

Collection of operating conditions, including:

- Anaesthesia: dexamethasone given because of bypass yes/no - type of induction - endotracheal tube size (ETT) - redness or swelling during laryngoscopy – secretions (pus) seen during intubation - high pressures needed during mechanical ventilation in the operation room (OR) - duration of anaesthesia-administration of ketamine yes/no-continuous administration of ketamine yes/no - amount of red blood cells, fresh frozen plasma and platelet concentrate transfused.
- Operation: Type of operation, risk adjustment congenital heart surgery score (RACHS-II)[14]- duration of cardiopulmonary bypass (CPB) - duration of aortic-cross clamping - antegrade cerebral perfusion

Daily until PICU discharge

Collection of clinical condition, including:

- Administered steroids.
- Infection: Infection parameters (routinely performed) - use of medication (antiviral/ antibacterial)- recording of any bacterial/ viral testing of respiratory tract- temperature (highest recorded per day)
- Ventilation: settings (mean airway pressure, fraction of inspired oxygen (FiO2), Peak inspiratory pressure and positive end expiratory pressure (PEEP) (maximal values))-use of non-invasive ventilation (continuous positive airway pressure (CPAP), optiflow, face mask ventilation)- use of nitric oxide- failure of extubation (reintubation within 24 hours after extubation)
- Inotropes, vasoactive medication (milrinone, adrenaline, noradrenaline, dobutamine, dopamine, levosimendan)

Date of PICU and hospital discharge.

Collection of samples see figure 1

On day of operation (i.e. day 1): nasopharyngeal swab

On day 4, if still intubated: nasopharyngeal swab and blood from the biochemical laboratory (residual material)

After collection, respiratory specimens (nasopharyngeal swabs) will be transported to the Clinical Microbiology Laboratory for further processing and storage. Respiratory specimens will be processed daily (with routine diagnostics) and stored at –80°C. Blood samples will be stored at –80°C until testing. After the first testing is done, all samples will be stored 15 years after the study inclusion has ended.

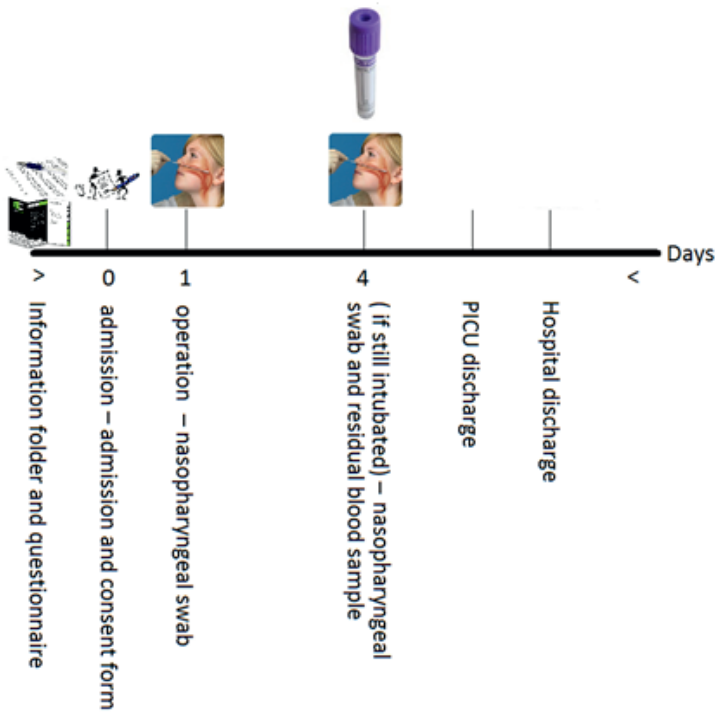


Figure 1. Schematic overview of collection of clinical data and samples

Viral testing

Testing of rhinovirus in specimens (nasopharyngeal swab, blood) will be performed at the Clinical Microbiological Laboratory of the LUMC using a validated internally controlled real-time rhinovirus PCR, yielding semi–quantitative results based on Cycle threshold (Ct) values[15]. Rhinovirus genotyping will be performed by PCR and/or amplification and sequence-analysis of the capsid proteins VP3/VP1, VP4/VP2 or the 5'UTR genome regions previously described by Zlateva et al.[16].

Other respiratory viral pathogens

In case of suspected clinical infectious respiratory problems during PICU admission, clinical routine microbiological diagnostics will be performed for respiratory viral pathogens at the discretion of the treating physician. The results of these clinical tests will also be evaluated in the final data analysis to help establish the role of potential co-infections in rhinovirus positive patients and the role of other viral infections in rhinovirus negative patients.

In rhinovirus negative patients with a prolonged duration of mechanical ventilation and/or PICU length of stay (> 3 days), at final analysis of the study results, the initial per-operative nasopharyngeal swabs will be re-tested for other viral pathogens with in-house real-time multiplex PCR (eg RSV, influenza, parainfluenza, etc) to analyse the effect of possible other viral pathogens on the prolonged LOS[15].

Statistical analysis

Dichotomous variables will be expressed in numbers and percentages.

Continuous variables will be expressed as (exponential) means with standard deviations (SD) or medians with interquartile ranges [IQR], where appropriate.

Outcome measures, viral load and demographic features will be expressed for the total study population. In addition, these data will be shown for the following subgroups: rhinovirus infection (overall), symptomatic rhinovirus infection, asymptomatic rhinovirus infection and no rhinovirus infection at admission.

Multivariate analysis

The primary and secondary outcome measures will be compared between the rhinovirus PCR positive patients versus the rhinovirus PCR negative groups.

Because we expect the distribution of our primary outcome (IC length of stay) to be skewed, we will use exponential regression for our primary analysis. We will correct for potential confounders: gender and perfusion time. Numerical outcomes that do not show strong deviation from normality will be analysed with multivariate linear regression. Logistic regression or log-binomial regression where appropriate, will be used for all dichotomous or categorical outcome measures, corrected for potential confounders.

In children with rhinovirus infection investigate the association between viral load (strength of PCR), duration of shedding, symptomatic or asymptomatic infection, genotype, viremia and the primary and secondary outcome measures shall be investigated.

Adjustment and stratification

Variables that can affect both LOS and peroperative RV PCR positivity (confounders) are adjusted for (age, see figure 2) .

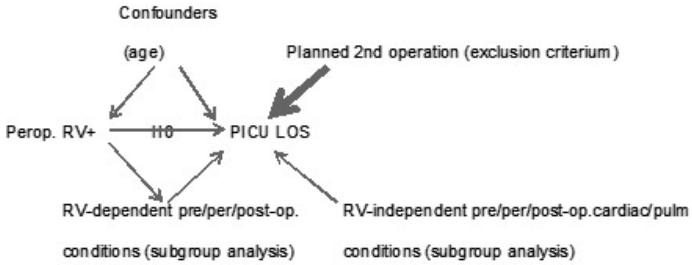


Figure 2. Flow chart of variables affecting LOS after elective cardiac surgery, in relation to RV infection.

Variables that can affect LOS without affecting RV PCR positivity are either implemented as

a) exclusion criteria, when having an absolute effect on LOS, independent of RV PCR positivity, e.g.:

-planned 2nd operation for single ventricle physiology,

-planned prolonged intubation because of (MRI) procedure or transport to other hospital

-unplanned 2nd operation (exclusion retrospectively)

b) subgroup, when having a less absolute effect on LOS, e.g.:

-pre-operative cardiac condition

-pre-operative viral co-infection

Here, subgroup analysis might indicate patients vulnerable for enhanced LOS when per-operative RV positive.

c) other predictors for LOS that are either theoretically independent of RV or potentially resulting from RV positivity: no adjustment:

-Per-operative perfusion time (cardio-pulmonary-bypass-time)

-Post-operative cardiac condition e.g. heart failure leading to prolonged inotrope support and/or respiratory support

-Pre /post-operative pulmonary condition (eg tracheabronchomalacia)

Number of patients excluded from the study will be presented in a Consolidated Standards of Reporting Trials (CONSORT)- flow-chart.

DISCUSSION

This is, to our knowledge, the first study to screen all children undergoing elective cardiac surgery for rhinovirus and to monitor postoperative outcome. It is important to determine whether rhinovirus is a risk factor for prolonged LOS in children undergoing heart surgery as it might have important implications in timing of the operation.

We expect that the effect of rhinovirus on the difference in PICU LOS is the largest in children undergoing open heart surgery using cardiopulmonary bypass compared to operations without cardiopulmonary bypass (eg repair of coarctation of the aorta, placement of a modified Blalock-Taussig shunt).

Secondary aim of the study is to develop an algorithm, based on clinical parameters and nasopharyngeal swab results, to help identify asymptomatic children with an increased risk of prolonged PICU LOS after cardiac surgery, in the pre-operative stage. The ultimate goal is to prevent prolonged PICU admission due to respiratory complications..

In this study the children will be tested for rhinovirus at the moment of operation.

Though this moment may not exactly represent the moment of admission (one day earlier), we have chosen this moment to minimize the burden for the participating children.

It must be noted that if rhinovirus positivity is indeed predictive for prolonged PICU LOS, this study is not designed to determine whether postponing the operation is effective in preventing prolonged PICU LOS, which would require a specific future trial randomizing between different postponement times (eg 2, 4 or 6 weeks). We chose not to perform this trial immediately because we think the effect of rhinovirus needs to be determined on forehand. Furthermore the organisation and costs, as well as the emotional burden for the children and their family of postponing an operation are considerable and can only be warranted if rhinovirus actually is a risk factor.

While duration of mechanical ventilation seems to be more directly related to rhinovirus infection than PICU length of stay, we selected PICU length of stay as primary endpoint. Duration of mechanical ventilation as an endpoint is more difficult to define, given the several types of respiratory support (invasive and non-invasive) and less clear-cut situations like extubation failure and or re-intubation. Because PICU length of stay is a more clear endpoint, easier to compare between studies, and of clinical and economical relevance, we chose this as primary endpoint.

Rhinovirus will be the only respiratory pathogen that is routinely tested on the pre-operative nasopharyngeal swabs in this study. However, all nasopharyngeal swabs will be stored and can be retested for an entire respiratory viral panel in rhinovirus negative patients with prolonged LOS (>4 days) to exclude patients with prolonged length of stay due to another virus.

Conclusion

We have designed a prospective observational study to determine the effect of rhinovirus on the postoperative course in children who are admitted from home to undergo elective congenital heart surgery. The primary endpoint is PICU LOS.

REFERENCES

1. Jacobs SE, Lamson DM, St George K., Walsh TJ: Human rhinoviruses. *Clinical microbiology reviews.* 2013, **26**: 135-62.
2. van Benten I, Koopman L, Niesters B, Hop W, van Middelkoop B, de Waal L, van Drunen K, Osterhaus A, Neijens H, Fokkens W: Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants. *Pediatric Allergy and Immunology.* 2003, **14**: 363-70.
3. Costa LF, Queiroz DA, Lopes da Silveira H, Bernardino Neto M, de Paula NT, Oliveira TF, Tolardo AL, Yokosawa J: Human rhinovirus and disease severity in children. *Pediatrics.* 2014, **133**: e312-21.
4. Becke K: Anesthesia in children with a cold. *Current Opinion in Anaesthesiology.* 2012, **25**: 333-9.
5. Tait AR, Malviya S, Voepel-Lewis T, Munro HM, Seiwert M, Pandit UA: Risk factors for perioperative adverse respiratory events in children with upper respiratory tract infections. *Anesthesiology.* 2001, **95**: 299-306.
6. von Ungern-Sternberg BS, Boda K, Schwab C, Sims C, Johnson C, Habre W: Laryngeal mask airway is associated with an increased incidence of adverse respiratory events in children with recent upper respiratory tract infections. *Anesthesiology.* 2007, **107**: 714-719.
7. Li YP, Huang J, Huang SG, Xu JG, Xu YY, Liao JY, Feng X, Zhang XG, Wang JH, Wang J: The compromised inflammatory response to bacterial components after pediatric cardiac surgery is associated with cardiopulmonary bypass-suppressed Toll-like receptor signal transduction pathways. *J Crit Care* 2014, **29**: 31. e7-13.
8. Allen ML, Hoschtitzky JA, Peters MJ, Elliott M, Goldman A, James I, Klein NJ: Interleukin-10 and its role in clinical immunoparalysis following pediatric cardiac surgery. *Crit Care Med.* 2006, **34**: 2658-65.
9. Cornell TT, Sun L, Hall MW, Gurney JG, Ashbrook MJ, Ohye RG, Shanley TP: Clinical implications and molecular mechanisms of immunoparalysis after cardiopulmonary bypass. *J.Thorac Cardiovasc surg*. 2012, **143**: 1160-1166.
10. Malviya S, Voepel-Lewis T, Siewert M, Pandit UA, Riegger LQ, Tait AR: Risk factors for adverse postoperative outcomes in children presenting for cardiac surgery with upper respiratory tract infections. *Anesthesiology* 2003, **98**: 628-32.
11. Simsic J, Phelps C, Yates A, Galantowicz M: Management strategies after cardiac surgery in an infant with human rhinovirus. *Pediatric Cardiology*. 2013, **34**: 1922-4.
12. Delgado-Corcoran C, Witte MK, Ampofo K, Castillo R, Bratton SL: The Impact of Human Rhinovirus Infection in Pediatric Patients Undergoing Heart Surgery. *Pediatric Cardiology* . 2014, **35**: 1387-94.
13. Medweb Development lab. http://www.cahal.nl (2015). Accessed 20 May 2015.
14. Jacobs JP, Jacobs ML, Lacour-Gayet FG, Jenkins KJ, Gauvreau K, Bacha E, Maruszewski B, Clarke DR, Tchervenkov CI,Gaynor JW, Spray TL, Stellin G, O'Bien SM, Elliott MJ, Mavroudis C: Stratification of complexity improves the utility and accuracy of outcomes analysis in a Multi-Institutional Congenital Heart Surgery Database: Application of the Risk Adjustment in Congenital Heart Surgery (RACHS-1) and Aristotle Systems in the Society of Thoracic Surgeons (STS) Congenital Heart Surgery Database. *Pediatric Cardiology*. 2009, **30**: 1117-30.
15. Loens K, van Loon AM, Coenjaerts F, van Aarle Y, Goossens H, Wallace P, Claas EJC, Ieven M, GRACE Study Group: Performance of Different Mono- and Multiplex Nucleic AcidAmplification Tests on a Multipathogen External Quality AssessmentPanel. *Journal of Clinical Microbiology.* 2011, **50**: 977-87.
16. Zlateva KT, de Vries JJ, Coenjaerts FE, van Loon AM, Verheij T, Little P, Butler CC, Goossens H, Ieven M, Claas ECJ, GRACE Study Group: Prolonged shedding of rhinovirus and re-infection in adults with respiratory tract illness. *European Respiratory journal.* 2014, **44**: 169-77.

SUPPLEMENTARY FILE

File 1: Questionnaire, questionnaire, which will be sent to all patients, asking for signs and symptoms of current and/or recent respiratory infections.

The impact of rhinovirus in children undergoing cardiac surgery

1. After how many weeks of pregnancy was your child born?

2. Does your child have any problems concerning his/ her airways? If yes please specify (for example asthma, cystic fibrosis)?

3. Does your child use any medication, if so which one?

4. Does your child often suffer from respiratory infections? How many episodes a year?
   - □ 1
   - □ 2 to 4
   - □ 5 to 8
   - □ More than 8
   - □ No, my child does not have respiratory infections

5. Did your child suffer from any of these symptoms in the past 6 weeks? (Tick if applicable, multiple answers possible):

	Never	1X	2-4X	Every week	2-5 days a week	Every day
Runny nose	☐	☐	☐	☐	☐	☐
Nasal congestion	☐	☐	☐	☐	☐	☐
Sneezing	☐	☐	☐	☐	☐	☐
Wheezing	☐	☐	☐	☐	☐	☐
Cough	☐	☐	☐	☐	☐	☐
Mucous expectorate	☐	☐	☐	☐	☐	☐
Hoarseness	☐	☐	☐	☐	☐	☐
Sore throat	☐	☐	☐	☐	☐	☐
Earache	☐	☐	☐	☐	☐	☐
Otorrhoea	☐	☐	☐	☐	☐	☐
Fever	☐	☐	☐	☐	☐	☐
Being unwell	☐	☐	☐	☐	☐	☐

If your child did not suffer from any of the above symptoms, you can continue to question 12.

6. If your child had a fever in the past 6 weeks, what was the maximum temperature?
   - ☐ 37 – 37.5
   - ☐ 37.5 – 38.0
   - ☐ 38.0 – 38.5
   - ☐ 38.5 – 39.0
   - ☐ 39.0 – 39.5
   - ☐ 40 degrees or more

7. On what date were the symptoms at their maximum?

8. Did your child use any medication (for example nose drops/ antibiotic/ analgesics) for the symptoms mentioned above, if so which?

9. Did you visit a doctor concerning these symptoms?
   - □ Yes
   - □ No *(go to question 11)*

10. Did your doctor order any laboratory tests to determine the cause of the symptoms. If so, do you know the results?

11. Does your child have any symptoms at this moment, if so which? If not, what was the last time your child did have symptoms?

12. Are there family members who have had a respiratory infection in the past 6 weeks, if so what is their family relation with your child?

13. Are there lung diseases in the family, for example asthma or cystic fibrosis? If so, which disease and what is the family relation with your child?

14. Are there other diseases/ disorders in the family?

15. Do people smoke in the presence of your child?
   - □ Yes
   - □ No

CHAPTER 5.2

Rhinovirus detection in the nasopharynx of children undergoing cardiac surgery is not associated with longer PICU length of stay; results of the RISK study

P.P. Roeleveld[a#], A.L. Van Rijn[b#], R.B.P. de Wilde[a], E.W. van Zwet[c], J. Wink[d], L. Rozendaal[e], K. Hogenbirk[a], M.G. Hazekamp[f], W.H. Man[g], I.Sidorov[b], M.E.M. Kraakman[b], E.C.J. Claas[b], E.de Jonge[h], A.C.M. Kroes[b], J.J.C. de Vries[b]

[a] Department of Pediatric Intensive Care, Leiden University Medical Center, The Netherlands
[b] Department of Medical Microbiology, Leiden University Medical Center, The Netherlands
[c] Department of Statistics, Leiden University Medical Center, The Netherlands
[d] Department of Anesthesiology, Leiden University Medical Center, The Netherlands
[e] Department of Pediatric Cardiology, Leiden University Medical Center, The Netherlands
[f] Department of Thoracic Surgery, Leiden University Medical Center, The Netherlands
[g] Department of Pediatrics, Leiden University Medical Center, The Netherlands
[h] Department of Intensive Care, Leiden University Medical Center, The Netherlands
[#] These authors contributed equally to this work

ABSTRACT

Objectives: To determine whether children with asymptomatic carriage of rhinovirus in the nasopharynx before elective cardiac surgery have an increased risk of prolonged pediatric intensive care (PICU) length of stay.

Study Design: Prospective, single-center, blinded observational cohort study.

Setting: PICU in a tertiary hospital in the Netherlands.

Patients: Children under 12 years of age undergoing elective cardiac surgery were enrolled in the study after informed consent of the parents/guardians.

Interventions: The parents/guardians filled out a questionnaire regarding respiratory symptoms. On the day of the operation a nasopharyngeal swab was obtained. Clinical data was collected during PICU admission, and PICU/hospital length of stay were reported. If a patient was still intubated 3 days after operation an additional nasopharyngeal swab was collected. Nasopharyngeal swabs were tested for rhinovirus and other respiratory viruses with PCR.

Measurements and Main Outcomes: Of the 163 included children, 74 (45%) tested rhinovirus positive. Rhinovirus positive patients did not have a prolonged PICU LOS (median 2 days each, p=0.104). Rhinovirus positive patients had a significantly shorter median hospital length of stay compared to rhinovirus negative patients (8 versus 9 days, respectively, p=0.002).

Overall, 97 (60%) of the patients tested positive for one or more respiratory virus. Virus positive patients had significantly shorter PICU and hospital length of stay, ventilatory support, and non-mechanical ventilation. Virus negative patients had respiratory symptoms suspected for a respiratory infection more often.

In 31% of the children the parents reported mild upper respiratory complaints a day prior the cardiac surgery, this was associated with post extubation stridor, but no other clinical outcome measures.

Conclusions: Preoperative rhinovirus PCR positivity is not associated with prolonged PICU LOS. Our findings do not support the use of routine PCR testing for respiratory viruses in asymptomatic children admitted for elective cardiac surgery.

Trial registration: ClinicalTrials.gov Identifier NCT02438293; registration date 5 May 2015.

INTRODUCTION

Symptomatic respiratory infections have been shown to increase the duration of mechanical ventilation, intensive care and hospital length of stay (LOS), and increase the risk of postoperative complications in children following cardiac surgery[1-5]. Previous reports have mainly focused on respiratory syncytial virus (RSV)[1,5-11], but rhinoviruses (RV) may also impact postoperative outcomes[2-4,12-14]. Rhinoviruses in humans worldwide cause more than 50% of upper respiratory tract infections (URTI), such as common cold[12,13,15,16]. They are the leading cause of viral bronchiolitis in infants, the most common virus associated with wheezing in infants[17], prolonged shedding in specific patient groups, and can cause major morbidity and mortality[15,18,19].

Current anesthetic recommendations suggest that children with mild viral respiratory tract infections can safely be operated, but in children with wheezing, purulent secretions, fever, and altered general condition, surgery is recommend to be postponed[20]. Although RV infections, both symptomatic and asymptomatic, are very prevalent, there are no clear markers to help decide to postpone surgery as the evidence is scarce and based on small retrospective perioperative studies with variable symptomatology or outdated diagnostic tests[1,3,20,21]. Children with congenital heart diseases often have chronic and mild upper respiratory tract symptoms that may disappear after surgery. Postponing the operation is not in the best interest of these patients and might also result in empty operating rooms, leading to increased medical costs and waiting lists. Therefore, more evidence is needed to better ascertain which patients are at risk of perioperative complications and a protracted postoperative course.

We designed a single center prospective cohort study to determine whether asymptomatic children, clinically cleared for elective cardiac surgery, who test PCR positive for RV preoperatively, have an increased risk of a prolonged postoperative pediatric intensive care (PICU) LOS compared to those who test negative[22]. We hypothesized that RV positive children would have a prolonged postoperative PICU LOS.

MATERIALS AND METHODS

Design

A detailed RISK study protocol is previously published[22]. This prospective, single-center, blinded observational cohort study was designed to determine the association of RV with PICU LOS in children <12 years undergoing elective cardiac surgery in the Netherlands. Secondary endpoints were hospital LOS, duration of mechanical ventilation (MV), oxygenation index on admission, clinical suspicion of infection post-surgery, and development of adverse events. We also analyzed RV PCR quantification cycle (Cq)-values, RV genotypes, a parental questionnaire, and the occurrence of abnormal findings during intubation with the primary and secondary outcome measures.

Excluded were children admitted to hospital prior to surgery, who required emergency surgery, were not admitted to the PICU after operation (negligible amount), would have a planned prolonged PICU stay (e.g. duct-dependent lesions requiring prolonged prostaglandin infusion), or the lack of informed consent.

Study procedure

The parents/guardians of the eligible children received the information folder and a questionnaire (see supplement 1). The questionnaire consisted of questions regarding respiratory symptoms during the six weeks prior to surgery (e.g. fever, runny nose, coughing, wheezing, etc.), underlying pulmonary disease (of the children and their family), medication use, prematurity, and passive smoking.

As per local protocol, children were admitted one day before the operation (day 0), and clinically assessed and cleared for the operation by the anesthesiologist, cardiologist and cardiac surgeon when no signs of active infection other than rhinorrhea or nasal congestion were present. Written informed consent was then asked by the independent researchers. On the day of surgery (day 1) a nasopharyngeal swab for viral testing was obtained at the induction of anesthesia. Anesthesia was induced with propofol or sevoflurane and maintained with propofol and either sufentanil or remifentanil at the discretion of the anesthesiologist. The anesthesiologist filled out a study form (see supplement 2) detailing findings at direct laryngoscopy (secretions, redness, pus) and other details regarding the induction, the use of steroids, type of anesthesia, and operation conditions. Also, cardiopulmonary bypass times, type of operation, and the Risk Adjustment for Congenital Heart Surgery Score (RACHS) score were collected [23]. After the operation, children were admitted to the PICU, and clinical and laboratory data (blood gas analysis, inflammatory markers, inotrope dose, respiratory conditions, medication and infection) were prospectively collected until PICU discharge, and date of hospital discharge. In case of prolonged PICU admission, a follow-up swab was taken at day 4 of the patients with respiratory support.

All children received 24 hours of peri-operative cefazolin prophylaxis. In the case of postoperative open chest management, cefazolin was switched to flucloxacillin after 24 hours and continued until 24 hours after delayed chest closure. Children were weaned from MV at the discretion of the treating pediatric intensivist.

Definitions

A ‘positive’ questionnaire was defined as any respiratory symptoms in the six weeks prior to surgery, as reported by the parents. Respiratory ‘complaints on admission’ were defined as rhinorrhea and/or nasal congestion present as reported by parents. Hospital LOS was the LOS in the hospital from the day of admission prior to operation until discharge. An ‘adverse event’ included reintubation, readmission, post-extubation stridor, suspected clinical infection, cardiac arrest requiring resuscitation, or arrhythmia requiring treatment. Reintubation was defined as intubation within 48 hours of extubation and ‘readmission’ as readmission within 48 hours of PICU discharge. Post-extubation stridor was defined as stridor within 48 hours of extubation requiring treatment with inhaled steroids, inhaled adrenaline, or systemic steroids. Clinical suspicion of infection was defined as clinical symptoms leading to microbiologic testing and/or antibiotic treatment at the discretion of the treating intensivist. We defined ‘abnormal laryngoscopy’ as redness and/or (purulent) secretions of the larynx, identified by direct laryngoscopy at the time of intubation. No indirect (fiberoptic) laryngoscopy was performed. Chest x-ray on PICU admission was considered abnormal if an

atelectasis and/or a consolidation was present. Non-invasive respiratory support was defined as nasal or mask with continuous positive airway pressure, mask ventilation, or high-flow nasal cannula.

Respiratory virus testing

After publication, our protocol was amended, and all viral respiratory pathogens in our assay were tested[22]. The nasopharyngeal swabs (day 1) were tested for respiratory viruses by means of in-house PCR [24] targeting adenovirus, bocavirus, RV, influenza A/B, RSV, metapneumovirus, para-influenza 1-4, human coronaviruses OC43, HKU1, NL63, and 229E. The day four samples were stored at -80°C, and tested retrospectively. PCR results were blinded for the clinicians and (research) nurses. Genotyping of RV was initially performed by PCR amplification and Sanger sequencing of the VP4/2 region as previously described by Zlateva et al.[25]. Later, bulk sequencing of the same amplicons was performed by next-generation sequencing (NovaSeq6000, Illumina, San Diego, CA, USA). Sequence reads were assembled using SPAdes, version 3.11.1[26]. The reconstructed genome fragments were blasted (BLAST version 2.2.31), against a database of complete genomes of Picornaviridae (database version as of 25 October 2019, prepared with HAYGENS tool, https:// veb.lumc.nl/HAYGENS). For blasting, contigs with a length ranged from 600 to 700 nt concordant to the genome region of rhinoviruses consisting of VP4/2 genes only were used. Both nucleotide and amino acid searches for these regions and scaffolds were performed.

Statistical Analysis

Sample size was initially calculated based on the estimated percentage of RV positive children (20%) and a difference in PICU LOS of 2 days, to be approximately 250 children (ratio 1:4) [22]. However, 11 months after initiation, the percentage of RV positive children turned out to be nearly 50%, therefore the sample size was adjusted to 162 (ratio 1:1) and the protocol was amended accordingly. All continuous data were tested for distribution using the Kolmogorov-Smirnov test. Normally distributed data were presented as means with standard deviations and not-normally distributed data as medians with interquartile range (IQR).

Significant differences between the different groups for the study endpoints were tested with Mann-Whitney U test, t-test, Chi-square or Fisher's exact test where appropriate. Multivariate linear regression analysis was used to adjust for potential confounders, and to identify risk factors. To compare the Cq values at the day of operation with Cq values during the PICU stay, a paired t-test was used. Statistical analyses were performed using IBM SPSS Statistics version 25 software. A p-value of <0.05 was considered statistically significant.

Ethical approval

This study was approved by the medical ethics review committee of the Leiden University Medical Center research file NL51483.058.14 (RV-MM-PED-1), protocol number P14.303.

RESULTS

During the study period, June 2015 – June 2018, 814 children (< 18 years) underwent elective cardiac surgery of which 359 children were eligible for inclusion. One hundred and eighty one parents/guardians were asked for informed consent of which 15 refused (8%), leaving 166/356 (46%) to be included in the study (figure 1). The main reason for exclusion was due to inability to ask informed consent (language barrier, admission during weekend, and staffing constraints). An additional two patients were excluded because of expected prolonged PICU stay (one for mechanical ventilation dependency due to hypotonia and one because of planned reoperation). In 164/166 (99%) patients a nasopharyngeal swab was obtained. For one sample the PCR failed, this patient was excluded from further analysis.

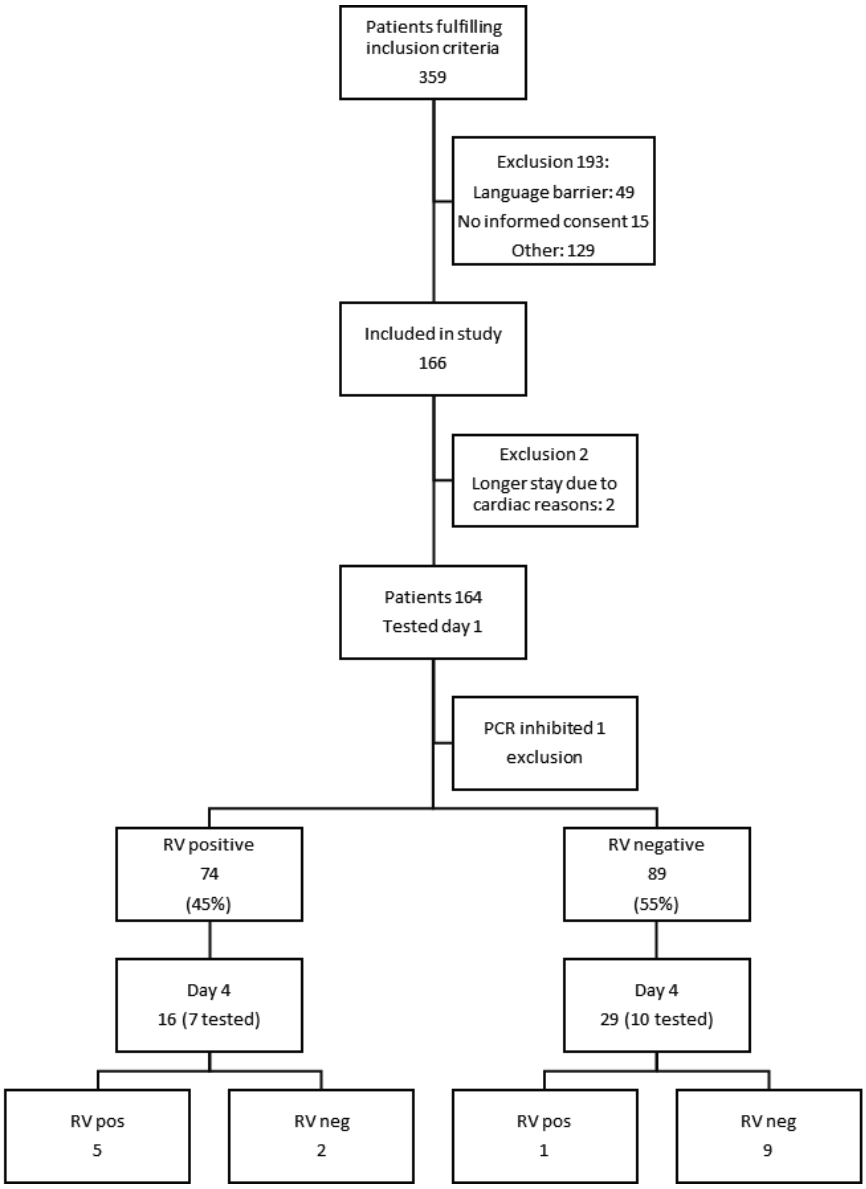


Figure 1. Flowchart of study inclusion.

The median age was 15 months and half of the children were male. Of the 163 children included, 74 (45%) tested RV positive and 89 (55%) RV negative. There were no statistically significant differences in baseline demographics between RV positive and RV negative patients (table 1) with the exception that RV positive patients had more often received steroids during the operation (p=0.026), and a tendency towards current respiratory complaints in the RV positive patients (p=0.070). The most frequent operation indication was biventricular repair (91%). There was no difference in complexity of surgery (RACHS, CPB duration, cross clamp time, delayed sternal closure), anesthetic management (blood products, cumulative fluid) between the groups, or expected mortality (PRISM/PIM) [27,28].

Out of the 74 RV positive patients, 25 (34%) had a coinfection with another virus, of which five patients tested positive for more than two viruses. Of the 89 RV negative patients, 23 tested positive for another virus, of which five patients tested positive for two viruses. After RV, adenovirus was the most prevalent virus (12%), followed by bocavirus (5%). Two patients (1%) were influenza A positive, one influenza B (0.4%) and three patients (2%) tested positive for both rhinovirus and RSV. Respiratory viruses were found throughout the year, with the highest percentage of virus positive patients in June (93%; figure 2).

The outcome variables of RV positive and RV negative patients are listed in table 2. Before and after correction for age (per-protocol) and steroid use during operation, RV positive patients had similar PICU-LOS compared to RV negative patients (median, 2 days each, p=0.104). RV positive patients had a significantly shorter hospital length of stay compared to the RV negative patients (median, 8 versus 9 days, respectively; p=0.002) and were suspected of clinical infection after surgery twice less often than RV negative patients which approached statistical significance (10% versus 21% respectively, p=0.068).

Table 1. Baseline characteristics of included patients.

	all patients n=163	rhinovirus positive n=74	rhinovirus negative n=89	p-value
Demographics				
Age at surgery (months) (median, IQR)	15 (5-47)	12.5 (5.75-44)	16 (4.5-49)	0.987
Male (%)	81 (50)	35 (47)	46 (52)	0.577
Weight at surgery (kg) (median, IQR)	9 (6.3-15.0)	9.1 (6.575-15)	9 (6.1-15)	0.678
Underlying respiratory conditions (%)	22/160 (14)	9/71 (13)	13/89 (15)	0.751
Asthma	4 (25)	0	4 (31)	
Bronchitis	2 (13)	1 (14)	1 (8)	
Tracheomalacia	2 (13)	2 (28)	0	
Pleural fluid	1 (6)	0	1 (8)	
Multiple airway infections	3 (19)	3 (42)	0	
Other	10 (63)	3 (42)	7 (54)	
Infectious respiratory complaints				
Respiratory complaints past 6 weeks (%)	153/160 (96)	69/71 (97)	84 (94)	0.464
Current respiratory complaints (%)	49/160 (31)	27/71 (38)	22 (25)	0.070
Risk assessment				
PRISM score at admission (median, IQR) (II)	6 (3-9)	5 (3-9)	6 (3-9.5)	0.670
PRISM III (median, IQR)	3 (1.75-4)	3 (2-4)	3 (1-4)	0.825
PIM (mean, SD)	-3.68 (0.623)	-3.71(0.598)	-3.66 (0.64)	0.672
Prematurity	19/160 (12)	8/71 (11)	11 (12)	0.832
Passive smoking	12/160 (8)	7/71 (10)	5 (6)	0.312
Operation				0.138
- Univentricular	14 (9)	9 (12)	5 (6)	
- Biventricular	149 (91)	65 (88)	84 (94)	
- CPB duration (minutes) (median, IQR)	91 (59-125)	86 (59.5-124)	97 (59-125)	0.664
- Cross clamp time (minutes) (median, IQR)	57 (31-92)	49 (25-91.25)	64 (33.5-93)	0.251

RACHS score (%)				0.268
1	30 (18)	18 (24)	12 (14)	
2	101 (62)	43 (58)	58 (65)	
3	24 (15)	9 (12)	15 (17)	
4	2 (1)	1 (1)	1 (1)	
Total blood products (median, IQR)	272.5 (200-347.75)	292 (210-395)	260 (180-330)	0.219
Cumulative Fluid (median, IQR)	965 (582-1293)	897 (500.5-1267.5)	977.5 (630.78-1340.75)	0.219
Steroids during operation (%)	45 (27)	27 (36)	18 (20)	0.026
Number of inotropes (%)				0.555
0	5	3	2	
1	53	23	30	
2	52	23	29	
3	4	3	1	
4	1	1	0	
Delayed sternal closure (%)	11 (7)	6 (8)	5 (6)	0.549

IQR: interquartile range, PRISM: pediatric risk of mortality score, PIM: pediatric index of mortality, CPB: cardiopulmonary bypass, RACHS: risk adjustment for congenital heart surgery.

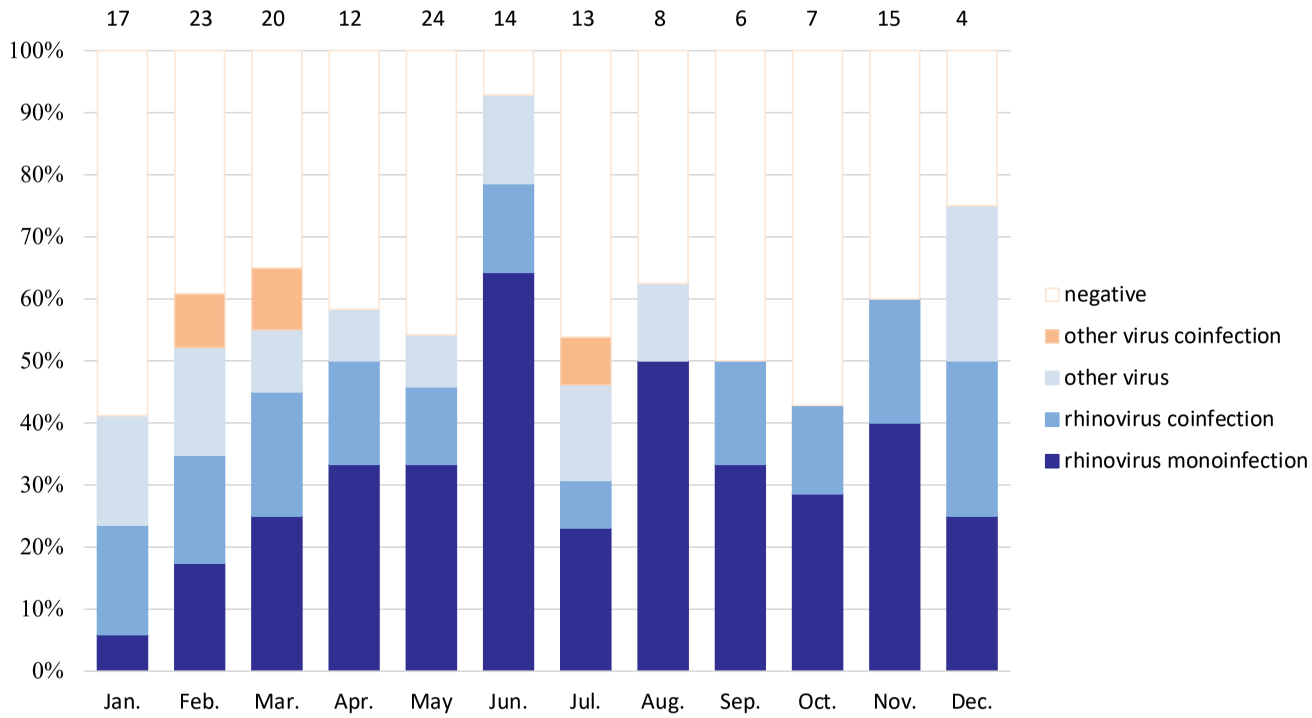


Figure 2. Percentage of rhinovirus mono- and coinfections per month.

Percentages of total number of samples tested per month over the years, total number on top of the bars.

Table 2. Outcome variables of RV-positive versus RV-negative patients.

	all patients n=163	rhinovirus positive n=74	rhinovirus negative n=89	p-value*
PICU LOS (days) (median, IQR)	2 (1-4)	2 (1-3)	2 (1-4)	0.107
Prolonged PICU LOS (=>4 days) (%)	45 (28)	16 (22)	29 (33)	0.094
Hospital LOS (days) (median, IQR)	8 (7-12)	8 (7-10)	9 (7-13.5)	0.002
Duration mechanical ventilation (median, IQR)	0.44 (0.31-1.07)	0.435 (0.2975-0.8350)	0.47 (0.335-1.1)	0.133
Prolonged mechanical ventilation (>48 hours) (%)	22 (13)	11(15)	11 (12)	0.589
Duration of non-invasive respiratory support (hours) (median, IQR)	31 (17-60)	18 (4-52.5)	36 (19.5-61)	0.067
Abnormal laryngoscopy (%)	50(31)	29 (39)	21 (24)	0.064
Oxygenation index on admission to PICU (median, IQR)	2.3 (1.8-3.85)	2.3 (1.8-4.4)	2.4 (1.8-3.7)	0.504
Mean airway pressure (median, IQR)	8.55 (8.0-9.2)	9 (8-9.9)	8.4 (8-9)	0.399
iNO-treatment (%)	2 (1)	0 (0)	1 (2)	0.501
Chest x-ray abnormalities <4 days (%)	54 (33)	25 (34)	29 (33)	0.986
Leucocytes 10x9/L (Median, IQR)	10.1 (7.7-14.2)	11.41 (8.15-14.44)	9.64 (7.3-12.5)	0.050
Extracorporeal life support (%)	3 (2)	1 (1)	2 (2)	0.665
Adverse events (%)	45 (27)	18 (24)	27 (30)	0.531
stridor post extubation (%)	14 (9)	9 (12)	5 (6)	0.138
Suspected of infection postoperatively (%)	26 (16)	7 (10)	19 (21)	0.068
Readmission PICU <48 hours (%)	6 (4)	2 (3)	4 (5)	0.491
Resuscitation (%)	2 (1)	0 (0)	2 (2)	0.501
Rethoracotomy (%)	3 (2)	0 (0)	3 (3)	0.252
JET (%)	4 (3)	3 (4)	1 (1)	0.330
Chylothorax (%)	2 (1)	1 (1)	1 (1)	0.998
AV-block (%)	3 (2)	0 (0)	3 (4)	0.253
Reintubation (%)	2 (1)	0 (0)	2 (2)	0.501
RV Cq value (Mean, SD)		26.8 (4.7)		
Mortality (%)	1(0.6)	0 (0)	1(1)	1.000

PICU: pediatric intensive care unit, LOS: length of stay, IQR: interquartile range, JET: junctional ectopic tachycardia, AV: atrioventricular, RV: rhinovirus, Cq: quantification cycle. * corrected for age and steroid use during operation.

The outcome variables of 'any virus positive' (including RV positive patients, 60%) and 'virus negative' (40%) patients are listed in table 3. The virus negative patients were smaller, younger, had more complicated surgery reflected by their RACHS scores, had more frequent current respiratory complaints, and tended to have more steroids during operation (see supplement 3). After correction for weight, age (per-protocol), and RACHS score, the virus positive patients compared to virus negative patients had significantly shorter PICU LOS (median, 2 versus 3 days, respectively, p=0.048) and hospital LOS (8 versus 9.5 days, respectively, p<0.001). Virus positive patients received shorter ventilatory support (0.41 versus 0.51 days, p=0.042), shorter non-invasive ventilatory support (18 versus 45 hours, p=0.009), and were significantly less often suspected of having a clinical infection postoperatively (10 versus 24%, p=0.017). In virus positive patients, parents reported respiratory complaints on admission significantly more often than in virus negative patients (36 versus 21%, respectively, p=0.03). Similar results, regarding PICU/hospital LOS and duration of mechanical ventilation, were found between virus positive and virus negative patients when we excluded the RV positive patients (data not shown).

Overall, in 50 patients (31%) there was redness or pus during intubation and were suspected of a postoperative clinical infection twice more often than patients without redness or pus (26% versus 12%) (p=0.02). They did, however, not have longer LOS or duration of mechanical ventilation (data not shown).

Table 3. Outcome variables of any virus positive patients.

	any virus positive n=97	virus negative n=66	p-value*
PICU LOS (days) (median, IQR)	2 (1-3)	3 (1-6)	0.048
Prolonged PICU LOS (=>4 days) (%)	20 (24)	25 (38)	0.016
Hospital LOS (days) (median, IQR)	8 (7-10)	9.5 (8-15.25)	<0.001
Duration mechanical ventilation (days) (median, IQR)	0.41 (0.29-0.63)	0.51 (0.35-1.1325)	0.042
Prolonged mechanical ventilation (>48 hours) (%)	11 (11)	11 (17)	0.329
Duration of non-invasive respiratory support (hours) (median, IQR)	18 (5-44.5)	45 (19.88-77.5)	0.009
Abnormal laryngoscopy (%)	35 (36)	15 (23)	0.070
Oxygenation index (Median, IQR)	2.2 (1.8-3.9)	2.6 (1.8-3.7)	0.653
Mean Airway pressure (median, IQR)	8.7 (8-9.5)	8.2 (8-9)	0.292
iNO-treatment (%)	0 (0)	2 (3)	0.162
Chest x-ray abnormalities <4 days (%)	30 (31)	24 (36)	0.606
Leucocytes (median, IQR)	10.6 (8.2-14.3)	9.1 (7.3-13.3)	0.068
Extracorporeal life support (%)	2 (2)	1 (2)	1.000
Adverse events (%)	24 (25)	21 (32)	0.451
stridor post extubation (%)	11 (11)	3 (5)	0.129
Suspected of infection postoperatively (%)	10 (10)	16 (24)	0.017
Readmission PICU <48 hours (%)	4 (4)	2 (3)	0.804
Resuscitation (%)	0 (0)	2 (3)	0.162
Rethoracotomy (%)	0 (0)	3 (5)	0.065
JET (%)	3 (3)	1 (2)	0.648
Chylothorax (%)	1 (1)	1 (2)	1.000
AV-block (%)	1 (1)	2 (3)	0.569
Reintubation (%)	1 (1)	1 (2)	1.000
RV Cq value (mean, SD)	27.9 (5.3)		
Mortality	0 (0)	1 (1.5)	0.405

PICU: pediatric intensive care unit, LOS: Length of Stay, IQR: interquartile range, JET: junctional ectopic tachycardia. AV, atrioventricular, RV: rhinovirus, Cq-value: quantification cycle, *corrected for age, weight and RACHS score

A 'positive' questionnaire, any respiratory symptom in the past six weeks, was found in 96% (153) of the 160 patients (data from 3 children could not be collected). Respiratory symptoms in the past six weeks were not associated with prolonged PICU LOS or any of the secondary outcome measures. Of the 49 (31%) patients whose parents reported mild respiratory complaints on admission, 35 (71%) tested positive for respiratory viruses and 27 (55%) tested RV positive. These 49 patients had similar PICU LOS and hospital LOS compared to patients without respiratory complaints on admission but had significantly longer non-invasive ventilatory support median 17.5 (4.5-38.5) versus 42 (18-73) hours, respectively, p=0.028) and post-extubation stridor significantly more often than patients who did not have respiratory complaints on admission (18 versus 5%, respectively, p=0.012).

As mild respiratory complaints on admission alone were not associated with worse clinical outcome (defined by duration of MV > 2 days or PICU LOS $\geq$ 4 days), we performed multivariate linear regression analysis to determine if a combination of a positive questionnaire, current complaints, abnormal laryngoscopy and RV positive would be predictive of prolonged PICU LOS. However, it was not possible to identify/develop a prediction model based on our results.

Twenty-six patients (15%) developed symptoms suspected for a postoperative respiratory infection. They had significantly longer cardiopulmonary bypass (CPB) times, cross-clamp times, higher PRISM and RACHS scores, more frequent steroid use during operation, and delayed sternal closure more often, indicating more complicated surgeries, as compared to patients without a postoperative infection (data not shown). After correction for all these factors and age, this group still had significantly increased PICU LOS (7 versus 2 days, p =<0.001), and hospital LOS (8 versus 16 days, p=0.002), and prolonged duration of mechanical ventilation (2.8 versus 0.4 days, p =0.003) and compared to patients without clinical infection.

Of all 163 patients, 45 (28%) were still admitted at the PICU at day four, which was similar in RV positive and RV negative patients (16 versus 29, respectively, p=0.119, figure 1). Twenty-two (49%) of these 45 patients were still intubated, and in 17 (77%) patients an follow-up nasopharyngeal swab was obtained for the detection of respiratory viruses. Seven of these 17 patients were RV positive prior to surgery, of which 5/7 (71%) were again RV positive in the follow-up sample, no significant difference in Cq value was found, of which two patients had an infection with another RV type compared to pre-operative (RV-B52 prior to surgery and RV-A41 on day 4, and RV-A71 and RV-A1 respectively). Ten of these 17 patients were RV negative prior to surgery, of which 1/10 (10%) became RV positive in the follow-up sample (RVA9).

The mean Cq value on the day of operation of the 74 rhinovirus positive patients was 27 (range 16.2 to 34.8). Rhinovirus positive patients with a high viral load (Cq<25) and rhinovirus negative patients had comparable hospital length of stay (median 8 vs 9 days respectively, p=0.070, corrected for age

and steroid use). No significant differences were found between rhinovirus Cq<25 and> 25 in hospital length of stay (p=0.812, corrected for age and steroid use). Mixed infections or RV species were not associated with a difference in PICU or hospital LOS.

In 67 (84%) out of 80 RV positive samples RV could be typed, 61 day 1 samples and 6 day 4 samples. Of the 64 unique samples, the majority of the patients had RV species A (56%, 36/64), followed by species C (27%,17/64) and species B (17%,11/64). An overview of the RV genotypes within each species, as detected per month in the study period, is shown in figure 3.

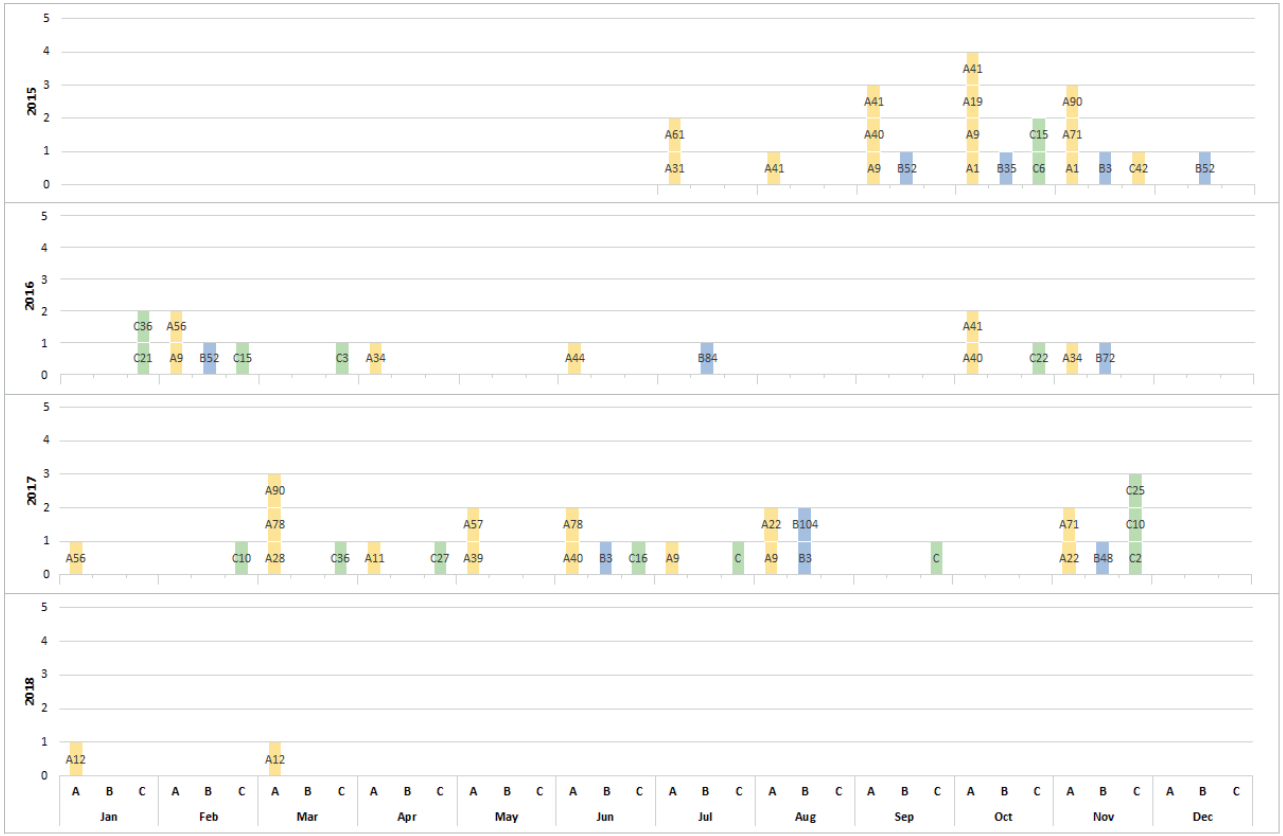


Figure 3. Rhinovirus genotypes per month.

Rhinovirus species per month during the study period are shown in different colors (species A yellow, species B blue and species C green). Genotypes within each species are shown, if available.

DISCUSSION

This prospective study, screening 163 children on the day of elective cardiac surgery during all seasons over several years, showed that RV was detected in 45% of the children and any respiratory virus even in 60%. Contrary to our hypothesis, RV positive patients had a similar duration of PICU LOS but a shorter hospital LOS compared to RV negative patients.

The very high proportion of 45% RV positive patients was unexpected. We predicted to find RV in approximately 20% of children, based on earlier reports of asymptomatic children in the Netherlands (range 14-28%) and infants undergoing cardiac surgery in Utah, USA[14,15]. We hypothesize that this high prevalence might be explained by our geographical location, inclusion throughout the year, young age (15 months), and underlying cardiac disease.

The earliest studies that demonstrated a negative effect of respiratory viruses on postoperative outcomes used ELISA's, in which positivity might have represented a more serious infection[5] compared to the modern highly sensitive PCR assays, which could represent prolonged shedding and asymptomatic carrier status[29-31]. However, we expected a certain number of asymptomatic carriers of RV to develop a symptomatic infection after surgery due to exposure to CPB and subsequent immunoparalysis[32-34]. Our results confirm a very recent and similar, but smaller, study by Delgado et al., who also observed no difference in postoperative outcomes in preoperatively tested (all respiratory viruses) asymptomatic infants[14].

We found a significantly shorter hospital length of stay in the RV positive patients compared to the RV negative patients and they were less often suspected of a postoperative clinical infection. This effect was also present in the 'any virus positive' patients. This might be the effect of an unknown confounder. However, recent studies investigating the relationship between respiratory microbiota and disease suggest that the microbiota acquired during childhood may affect immunological responses and may be related with health[35]. Rhinovirus can also very often be found in healthy children. In a study by Man et al. for instance, RV was significantly less common in children admitted with a lower respiratory infection than in healthy children[36].The precise mechanism as to how the respiratory viral and bacterial microbiota might be associated with health remains to be elucidated.

Almost all patients (96%) in our study had a positive questionnaire indicating respiratory symptoms in the 6 weeks prior to the operation. We deliberately asked parents about this 6-week period as the risk of peri-operative adverse events is increased up to 6 weeks after upper respiratory tract infections (URTI)[20]. Delgado-Corcoran et al. conducted a very similar questionnaire but only focused on two weeks pre-operatively and found a positive questionnaire in 66% of their patients, not related to clinical outcomes[14].

Thirty-one percent of the parents of patients in our study reported rhinorrhea and/or nasal congestion on admission. Parental confirmation of an URTI has been shown to be a better predictor of airway complications than the use of symptom criteria alone[37]. None had signs of active infection (fever, malaise, cough, etc.) and all were medically cleared for surgery. They did have significantly more post-extubation stridor requiring intervention (18 versus 5%; p=0.012). In a study by Malviya et al, children with preoperative signs of an URTI were also found to have more postoperative airway

complications[38]. Our results suggest that it is safe to operate children with rhinorrhea and/or nasal congestion, but the intensive care team should be aware of the higher chance of post-extubation stridor.

In our study, anesthesiologists reported redness and/or secretions on direct laryngoscopy in 31% of all patients, which was significantly associated with the development of a respiratory infection postoperatively but did not influence LOS. As far as we are aware, there is no literature about the relevance of laryngeal redness and/or secretions during elective intubation, although it might be possible that these could represent current mild URTI and might also lead to lower respiratory tract infections.

Patients who developed postoperative clinical signs of infection (16%) had significantly prolonged duration of mechanical ventilation, PICU LOS, and hospital LOS which is consistent with previous studies of children with symptomatic postoperative RV infections[2,4]. We could not identify pre-operative predictors of postoperative clinical infection. We seem to have a similar incidence of postoperative suspected infections compared to the study by Moynihan et al., who performed a PCR based on clinical suspicion of an infection in 18% (318/1737) of their patients following cardiac surgery in Queensland, Australia[4]. Twenty-three percent of their PCR's were virus positive compared to 45% in our cohort. Four percent of their entire cohort had a confirmed post-operative viral infection which is comparable to the 6% in our cohort.

Clinical RV infections tend to be more severe in patients with a higher viral load[39], however we did not find an association between viral load and our primary outcome measures though our study was not powered on comparison of subcategories.

The majority of the patients had a RV type A infections, which is the most prevalent species[40,41]. In this paper, although not powered to detect a difference, the different species were not associated with prolonged PICU LOS. Although previously RV-C was often linked to more serious disease in children, more recent publications do not confirm these findings[42-46]. Future work is needed to determine the optimal rhinovirus genotyping sequencing strategy in the light of recent studies using whole genome sequencing for viral typing[47].

Our study has limitations. First, we had a large number of exclusions, which might have introduced a selection bias. However, the intended sample size was reached and we included children during all months over several years minimizing potential bias. The limited number of patients operated in August and December might be explained by the holidays, in which elective operations are performed less. The second limitation is the lack of standardized pre-operative assessment. To reflect current standard of care we left the decision to clear patients for surgery at the discretion of the medical team. Unfortunately, we do not have data on the number of postponed surgeries. Third, defining an infection in children remains contentious and therefore we based our incidence of postoperative infection on the clinical judgment of the treating intensive care team rather than on set criteria, which does reflect the reality of PICU care. Fourth, we only collected PICU details of the first four days, which may have led to missing data. However, all relevant data regarding the primary

and secondary endpoints were available. Finally, being a single-center study, results might not be applicable to other centers.

We performed the largest, statistically powered, prospective observational study of pre-operative respiratory PCR testing in children undergoing cardiac surgery to date, with as main finding that RV positivity did not negatively impact PICU LOS.

Conclusions

Rhinovirus PCR positivity is highly common in asymptomatic children undergoing cardiac surgery in the Netherlands and is not associated with prolonged PICU LOS, but possibly even with shorter hospital LOS. Our findings do not support the use of routine testing for respiratory viruses in asymptomatic children admitted for elective cardiac surgery.

REFERENCES

1. Spaeder MC, Carson KA, Vricella LA, Alejo DE, Holmes KW. Impact of the viral respiratory season on postoperative outcomes in children undergoing cardiac surgery. *Pediatr Cardiol* 2011; **32**(6): 801-6.
2. Delgado-Corcoran C, Witte MK, Ampofo K, Castillo R, Bodily S, Bratton SL. The impact of human rhinovirus infection in pediatric patients undergoing heart surgery. *Pediatric cardiology* 2014; **35**(8): 1387-94.
3. Simsic J, Phelps C, Yates A, Galantowicz M. Management strategies after cardiac surgery in an infant with human rhinovirus. *Pediatric cardiology* 2013; **34**(8): 1922-4.
4. Moynihan K, Barlow A, Alphonso N, et al. Impact of Viral Respiratory Pathogens on Outcomes After Pediatric Cardiac Surgery. *Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies* 2017; **18**(3): 219-27.
5. Altman CA, Englund JA, Demmler G, et al. Respiratory syncytial virus in patients with congenital heart disease: a contemporary look at epidemiology and success of preoperative screening. *Pediatric cardiology* 2000; **21**(5): 433-8.
6. Moler FW, Khan AS, Meliones JN, Custer JR, Palmisano J, Shope TC. Respiratory syncytial virus morbidity and mortality estimates in congenital heart disease patients: a recent experience. *Critical care medicine* 1992; **20**(10): 1406-13.
7. Navas L, Wang E, de Carvalho V, Robinson J. Improved outcome of respiratory syncytial virus infection in a high-risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. *The Journal of pediatrics* 1992; **121**(3): 348-54.
8. Wang EE, Law BJ, Stephens D. Pediatric Investigators Collaborative Network on Infections in Canada (PICNIC) prospective study of risk factors and outcomes in patients hospitalized with respiratory syncytial viral lower respiratory tract infection. *The Journal of pediatrics* 1995; **126**(2): 212-9.
9. Welliver RC, Sr., Checchia PA, Bauman JH, Fernandes AW, Mahadevia PJ, Hall CB. Fatality rates in published reports of RSV hospitalizations among high-risk and otherwise healthy children. *Current medical research and opinion* 2010; **26**(9): 2175-81.
10. Khongphatthanayothin A, Wong PC, Samara Y, et al. Impact of respiratory syncytial virus infection on surgery for congenital heart disease: postoperative course and outcome. *Critical care medicine* 1999; **27**(9): 1974-81.
11. MacDonald NE, Hall CB, Suffin SC, Alexson C, Harris PJ, Manning JA. Respiratory syncytial viral infection in infants with congenital heart disease. *N Engl J Med* 1982; **307**(7): 397-400.
12. Jain S, Self WH, Wunderink RG, et al. Community-Acquired Pneumonia Requiring Hospitalization among U.S. Adults. *N Engl J Med* 2015; **373**(5): 415-27.
13. Wen S, Lv F, Chen X, et al. Application of a nucleic acid-based multiplex kit to identify viral and atypical bacterial aetiology of lower respiratory tract infection in hospitalized children. *Journal of medical microbiology* 2019.
14. Delgado-Corcoran C, Blaschke AJ, Ou Z, et al. Respiratory Testing and Hospital Outcomes in Asymptomatic Infants Undergoing Heart Surgery. *Pediatr Cardiol* 2019; **40**(2): 339-48.
15. van Benten I, Koopman L, Niesters B, et al. Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants. *Pediatric allergy and immunology* 2003; **14**(5): 363-70.
16. Bonfim CM, Nogueira ML, Simas PV, et al. Frequent respiratory pathogens of respiratory tract infections in children attending daycare centers. *Jornal de pediatria* 2011; **87**(5): 439-44.
17. Vandini S, Biagi C, Fischer M, Lanari M. Impact of Rhinovirus Infections in Children. *Viruses* 2019; **11**(6).
18. Jacobs SE, Lamson DM, St George K, Walsh TJ. Human rhinoviruses. *Clinical microbiology reviews* 2013; **26**(1): 135-62.
19. Choi SH, Huh JW, Hong SB, et al. Clinical characteristics and outcomes of severe rhinovirus-associated pneumonia identified by bronchoscopic bronchoalveolar lavage in adults: comparison with severe influenza virus-associated pneumonia. *Journal of clinical Virology* 2015; **62**: 41-7.
20. Becke K. Anesthesia in children with a cold. *Curr Opin Anaesthesiol* 2012; **25**(3): 333-9.
21. Tait AR, Malviya S. Anesthesia for the child with an upper respiratory tract infection: still a dilemma? *Anesthesia and analgesia* 2005; **100**(1): 59-65.
22. L Van Rijn A, Roeleveld P, Wilde R, et al. The Impact of Rhinovirus Infections in Paediatric Cardiac Surgery (Risk): Study Protocol for a Prospective Cohort Study; 2015.
23. Jacobs JP, Jacobs ML, Lacour-Gayet FG, et al. Stratification of complexity improves the utility and accuracy of outcomes analysis in a Multi-Institutional Congenital Heart Surgery Database: Application of the Risk Adjustment in Congenital Heart Surgery (RACHS-1) and Aristotle Systems in the Society of Thoracic Surgeons (STS) Congenital Heart Surgery Database. *Pediatr Cardiol* 2009; **30**(8): 1117-30.

24. Loens K, van Loon AM, Coenjaerts F, et al. Performance of different mono- and multiplex nucleic acid amplification tests on a multipathogen external quality assessment panel. *Journal of clinical microbiology* 2012; **50**(3): 977-87.
25. Zlateva KT, de Vries JJ, Coenjaerts FE, et al. Prolonged shedding of rhinovirus and re-infection in adults with respiratory tract illness. *The European respiratory journal* 2014; **44**(1): 169-77.
26. Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology* 2012; **19**(5): 455-77.
27. Pollack MM, Patel KM, Ruttimann UE. PRISM III: an updated Pediatric Risk of Mortality score. *Crit Care Med* 1996; **24**(5): 743-52.
28. Shann F, Pearson G, Slater A, Wilkinson K. Paediatric index of mortality (PIM): a mortality prediction model for children in intensive care. *Intensive Care Med* 1997; **23**(2): 201-7.
29. Rhedin S, Lindstrand A, Rotzen-Ostlund M, et al. Clinical utility of PCR for common viruses in acute respiratory illness. *Pediatrics* 2014; **133**(3): e538-45.
30. Jansen RR, Wieringa J, Koekkoek SM, et al. Frequent detection of respiratory viruses without symptoms: toward defining clinically relevant cutoff values. *Journal of clinical microbiology* 2011; **49**(7): 2631-6.
31. Jartti T, Lehtinen P, Vuorinen T, Koskenvuo M, Ruuskanen O. Persistence of rhinovirus and enterovirus RNA after acute respiratory illness in children. *Journal of medical virology* 2004; **72**(4): 695-9.
32. Hall MW, Greathouse KC, Thakkar RK, Sribnick EA, Muszynski JA. Immunoparalysis in Pediatric Critical Care. *Pediatric clinics of North America* 2017; **64**(5): 1089-102.
33. Li YP, Huang J, Huang SG, et al. The compromised inflammatory response to bacterial components after pediatric cardiac surgery is associated with cardiopulmonary bypass-suppressed Toll-like receptor signal transduction pathways. *Journal of critical care* 2014; **29**(2): 312.e7-13.
34. Cornell TT, Sun L, Hall MW, et al. Clinical implications and molecular mechanisms of immunoparalysis after cardiopulmonary bypass. *The Journal of thoracic and cardiovascular surgery* 2012; **143**(5): 1160-6.e1.
35. Koppen IJN, Bosch A, Sanders EAM, van Houten MA, Bogaert D. The respiratory microbiota during health and disease: a paediatric perspective. *Pneumonia (Nathan)* 2015; **6**: 90-100.
36. Man WH, van Houten MA, Merelle ME, et al. Bacterial and viral respiratory tract microbiota and host characteristics in children with lower respiratory tract infections: a matched case-control study. *Lancet Respir Med* 2019; **7**(5): 417-26.
37. Schreiner MS, O'Hara I, Markakis DA, Politis GD. Do children who experience laryngospasm have an increased risk of upper respiratory tract infection? *Anesthesiology* 1996; **85**(3): 475-80.
38. Malviya S, Voepel-Lewis T, Siewert M, Pandit UA, Riegger LQ, Tait AR. Risk factors for adverse postoperative outcomes in children presenting for cardiac surgery with upper respiratory tract infections. *Anesthesiology* 2003; **98**(3): 628-32.
39. Bruning AHL, Thomas XV, van der Linden L, et al. Clinical, virological and epidemiological characteristics of rhinovirus infections in early childhood: A comparison between non-hospitalised and hospitalised children. *Journal of Clinical Virology* 2015; **73**: 120-6.
40. Ng KT, Oong XY, Lim SH, et al. Viral Load and Sequence Analysis Reveal the Symptom Severity, Diversity, and Transmission Clusters of Rhinovirus Infections. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2018; **67**(2): 261-8.
41. van der Linden L, Bruning AH, Thomas XV, et al. A molecular epidemiological perspective of rhinovirus types circulating in Amsterdam from 2007 to 2012. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2016; **22**(12): 1002.e9-.e14.
42. Lau SK, Yip CC, Tsoi HW, et al. Clinical features and complete genome characterization of a distinct human rhinovirus (HRV) genetic cluster, probably representing a previously undetected HRV species, HRV-C, associated with acute respiratory illness in children. *Journal of clinical microbiology* 2007; **45**(11): 3655-64.
43. Piralla A, Rovida F, Campanini G, et al. Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2009; **45**(4): 311-7.
44. Fry AM, Lu X, Olsen SJ, et al. Human rhinovirus infections in rural Thailand: epidemiological evidence for rhinovirus as both pathogen and bystander. *PloS one* 2011; **6**(3): e17780.
45. Henquell C, Mirand A, Deusebis AL, et al. Prospective genotyping of human rhinoviruses in children and adults during the winter of 2009-2010. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2012; **53**(4): 280-4.
46. Ren L, Yang D, Ren X, et al. Genotyping of human rhinovirus in adult patients with acute respiratory infections identified predominant infections of genotype A21. *Scientific reports* 2017; **7**: 41601.

47. Meinel DM, Heinzinger S, Eberle U, Ackermann N, Schonberger K, Sing A. Whole genome sequencing identifies influenza A H3N2 transmission and offers superior resolution to classical typing methods. *Infection* 2018; **46**(1): 69-76.

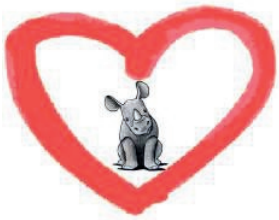
SUPPLEMENTARY FILES

File 1: Questionnaire, which was sent to all patients, asking for signs and symptoms of current and/or recent respiratory infections, see supplementary file study protocol

File 2: Case report form (CRF) for the anesthesiologist during the operation

File 3: Table Baseline characteristics of any virus positive/ negative patients

RISK STUDY



ANESTHESIOLOGY STUDY FORM

Operation date: ……

Weight: ……. (kg)

Induction of anesthesia:

o propofol
o sevoflurane
o esketamine
o other:……..

PATIENT STUDY STICKER

Anesthesia maintenance:

o propofol
o sevoflurane
o sufentanil
o remifentanil
o esketamine
o other: ……

Intubation:

o secretions? □ yes □ no
o redness / swelling? □ yes □ no
o pus? □ yes □ no
o endotracheal tube size: …… mm; cuff inflated: □ yes □ no
o details:……
o ventilation difficulties? □ no □ yes: ……..

nasopharyngeal swab obtained: □ yes □ no

blood products administered?:

o erythrocytes:……..ml
o thrombocytes:……ml
o plasma:……..ml

Bypass

- Dexamethasone? □ no □ yes, dose: ……..
- X-Clamping duration: ……..(min)
- CP-Bypass duration: ……(min)
- antegrade cerebral perfusion? □ no □ yes, duration: …… (min)

Other details:

……

S3 Table Baseline characteristics of any virus positive/negative patients

	Any virus positive n=97	Virus negative n=66	p-value
Demographics			
Age at surgery (months) (median, IQR)	19 (6-47)	10 (3-32)	0.510
Male (%)	45 (46)	36 (55)	0.307
Weight at surgery (kg) (median, IQR)	10.7 (7.1-16.1)	7.8 (5.5-12.1)	0.016
Underlying respiratory conditions (%)	14/94 (15)	8/66 (12)	0.598
Asthma	3 (21)	1 (13)	
Bronchitis	2 (14))	0	
Tracheomalacia	2 (14)	0	
Pleural fluid	0	1 (13)	
Multiple airway infections	3 (21)	0	
Other	4 (29)	6 (75)	
Infectious respiratory complaints			
Respiratory complaints past 6 weeks (%)	90/94 (96)	63 (95)	1.000
Current respiratory complaints (%)	35/94 (37)	14 (21)	0.030
Risk assessment			
PRISM score at admission (median, IQR) (II)	5 (3-9)	6 (3-10)	0.492
PRISM III (median, IQR)	3 (1-3)	3 (3-4)	0.107
PIM (mean, SD)	-3.77(0.60)	-3.56 (0.64)	0.073
Prematurity	11/94 (12)	8 (12)	0.936
Passive smoking	9/94 (10)	3 (5)	0.362
Operation			0.342
- Univentricular	10(10)	4(6)	
- Biventricular	87 (90)	62 (94)	
- CPB duration (minutes) (median, IQR)	87 (58-124.2)	97 (59-126.8)	0.722
- Cross clamp time (minutes) (median, IQR)	49 (25-91)	64.5 (36.2-94.8)	0.191
RACHS score (%)			0.037
1	24 (25)	6 (9)	
2	53 (55)	48 (73)	
3	14 (14)	10 (15)	
4	1 (1)	1 (2)	
Total blood products (median, IQR)	292 (200-375)	257 (195-330)	0.275
Cumulative Fluid (median, IQR)	957 (552.8-1290)	973 (604.5-1365)	0.531
Steroids during operation (%)	31 (32)	14 (21)	0.136
Number of inotropes (%)			0.756
0	3	2	
1	34	19	
2	28	24	
3	3	1	
4	1	0	
Delayed sternal closure (%)	7 (7)	4 (6)	1.000

IQR: interquartile range, PRISM: pediatric risk of mortality score, PIM: pediatric index of mortality, CPB: cardiopulmonary bypass, RACHS: risk adjustment for congenital heart surgery.

CHAPTER 6

Rhinovirus viremia in adult patients with high viral load in bronchoalveolar lavages

A.L. Van Rijn[a], E.C. Claas[a], P.A. von dem Borne[b], A.C.M. Kroes[a], J.J.C. de Vries[a]

[a] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[b] Department of Medical Haematology, Leiden University Medical Center, Leiden, the Netherlands

J Clin Virol 2017 96:105-109

ABSTRACT

Background: In children, rhinovirus viremia has been associated with higher nasopharyngeal loads and increase in severity of clinical signs and symptoms.

Objectives: This study aims to detect rhinovirus viremia in adult patients and to establish potential correlations with the clinical course.

Study design: Adult patients with rhinovirus strongly positive bronchoalveolar lavages (BAL, quantitation cycle, Cq values <25) detected between 2008-2014 were studied retrospectively. Blood sampled between two weeks before and two weeks after BAL sampling was tested for rhinovirus RNA. Underlying conditions, symptoms, radiography, microbiological data, and disease outcome were analysed.

Results: Twenty-seven of 43 patients with rhinovirus positive BAL at Cq values <25 had blood samples available within the prespecified time-frame (mean blood 3-4 samples per patient). Four of these 27 patients (15%) tested rhinovirus RNA positive in their blood (of whom one patient twice). Genotyping demonstrated rhinovirus A01, A24, B52 and B92 in these four immunocompromised patients.

Viremic patients were not significantly different with regard to underlying conditions, respiratory symptoms, radiological findings, co-pathogens nor the number of blood samples tested for RV. However, patients with rhinovirus viremia had significant higher mortality rates compared to patients without viremia, as all four died as a consequence of respiratory problems (100%) versus 22% (5/23), p=0.007 (Fisher's exact).

Conclusions: Rhinovirus viremia can occur in adult patients with a high viral load in BAL fluid. Rhinovirus viremia may be considered a negative prognostic factor, although a causative role with regard to the adverse outcome has yet to be demonstrated.

BACKGROUND

Rhinovirus (RV) is the most common virus found in respiratory tract infections in children and adults[1-3]. Rhinovirus primarily results in mild upper respiratory tract infections, known as the "common cold". However, a role for rhinovirus in lower respiratory tract infections has also been described. Rhinovirus was detected in 2-17% of the community acquired pneumonias[4-8], in children even up to 26%[9]. Severe rhinovirus-associated pneumonias may give similar symptoms and rates of mortality as severe influenza-associated pneumonia, but are more likely to occur in immunocompromised[10]. Generally, the clinical picture of rhinovirus infections appears to be more severe in patients with a higher viral load and in elderly patients, immunocompromised hosts and patients with underlying chronic lung diseases[11-15]. Although rhinovirus C has been implicated in more severe disease[16], other studies failed to show differences in clinical picture between different rhinovirus species[17-19].

In several case reports and prospective case series, rhinovirus RNA has been demonstrated in blood of paediatric patients[20-27]. Approximately 12% of the children with rhinovirus respiratory infections were found to be viremic on admission. This percentage dropped to 7 % on day three after admission[23,25]. Of the viremic paediatric patients, the majority were infected with rhinovirus C, varying from 67-87%[20,23]. Viremia was associated with a history of asthma, higher nasopharyngeal viral loads and more severe clinical signs and symptoms[20,25]. In addition, case reports on rhinovirus viremia have been published in patients with fatal outcome, suggesting a correlation with more severe disease[21,26,27]. Rhinovirus viremia in adult patients has not yet been demonstrated. Rhinovirus RNA was analysed in adult patients, inoculated with rhinovirus-16, by deMore et al.[28] This group found no evidence of viremia. However this were healthy subjects, or with mild asthma, with upper respiratory tract symptoms not requiring hospitalisation.

Objectives

The aim of this study was to determine whether rhinovirus viremia occurs in adults and whether there is a relationship with the clinical course of the infection.

STUDY DESIGN

Patients and samples

A laboratory database (GLIMS, MIPS, Belgium) search was performed for bronchoalveolar lavages (BAL) in adults between January 2008 and June of 2014 in the tertiary care hospital Leiden University Medical Center (LUMC, Leiden, the Netherlands). All BAL samples were included in the initial selection, however this invasive diagnostic procedure is performed primarily in respiratory insufficient and immunocompromised patients. Adult patients with a rhinovirus PCR positive BAL and a quantitation cycle (Cq) value of 25 and below were considered strongly positive, described as risk factor for RV viremia in children[20], and included in the study. Of these patients, stored plasma (-80^{o}C) or serum (-20^{o}C) samples (previously sent to the microbiological lab for routine diagnostics) were collected (serum when plasma was not available). All samples available in the time period from two

weeks before to two weeks after BAL sampling, with a maximum of one per day, were tested for the presence of rhinovirus RNA.

Patient characteristics, underlying diseases, symptoms, laboratory values, chest radiography, and disease outcome were retrospectively obtained from the patient records and laboratory databases. Stem cell transplantation patients were routinely screened for reactivation of cytomegalovirus (CMV), Epstein Barr virus and adenovirus by viral load testing.

Rhinovirus RNA detection

Nucleic acids were extracted from thawed serum or plasma samples with the MagNA Pure LC, using the Total Nucleic Acid Isolation Kit - Large Volume (Roche Diagnostics). The input was 1000 µl serum or plasma with elution of 50 µl extracted nucleic acid. If insufficient clinical sample was available, negative plasma was added up to 1000 µl.

Rhinovirus RNA amplification was performed in duplicate with an in-house real-time polymerase chain reaction (amplifying a 142-bp fragment of the 5'-UTR region), all samples in one run simultaneously, using primers, probes and conditions previously described by Loens *et al*.[29].Cq values, normalized using a fixed fluorescence threshold, were used as an indicative measure of viral load. Rhinovirus typing of respiratory samples from viremic patients was performed by amplification and sequencing of the VP4/VP2 genome region as previously described by Zlateva *et al*.[30].

Statistical analysis

Underlying respiratory illnesses, transplantation status, symptoms, radiographic changes, microbiologic findings, and outcome of rhinovirus viremia positive patients were compared to rhinovirus viremia negative patients. Categorical data were compared using 2x 2 tables with Fisher's exact test and Odds ratios. Numeric variables were compared using the independent t-test for equality of means. All statistical analysis was performed using IBM SPSS Statistics version 20.00 software for Windows.

Ethical approval

The study design was approved by the medical ethics review committee of the LUMC.

RESULTS

Patient selection

Between January 2008 and June of 2014, 791 bronchoalveolar lavages from 638 adult patients were tested for rhinovirus RNA. A total of 114 BAL samples from 84 (13%) patients tested positive for rhinovirus (range Cq values 15-45, randomly distributed, data not shown). Of 43 patients (51%, 48 BAL samples), the RV PCR result had a Cq<25. A total of 84 blood samples were available from 27 of

these 43 patients (63%), on average three- four blood samples per BAL sample (31 BAL samples). In 14 samples from nine patients, the available plasma volume varied from 200 to 950 µl.

Rhinovirus viremia positive patients

Four out of the 27 patients (15%) with BAL RV Cq<25 tested rhinovirus RNA positive in their blood samples (range Cq values 34 – 42) divided over the years 2010, 2011 and 2012. From these viremic patients a total of 22 blood samples were tested of which five tested positive. One patient was found viremic twice, on day -six and day +three relative to BAL sampling. For an overview of tested blood samples relative to the day of the BAL see Figure 1.

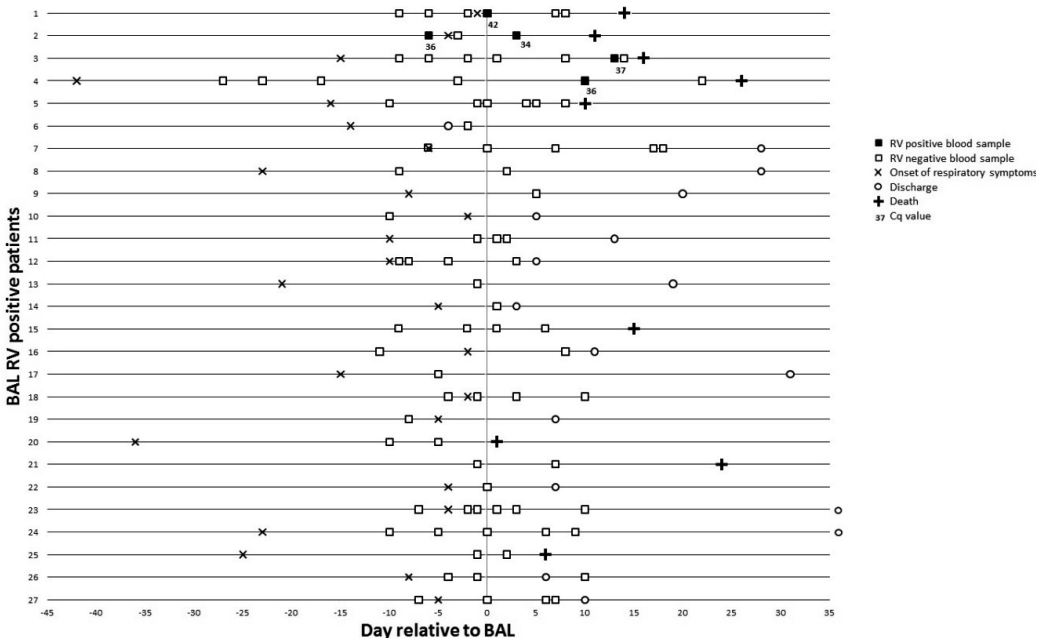


Figure 1. Detection of rhinovirus in blood relative to the bronchoalveolar lavage (day 0).

This figure shows the distribution of the different samples compared to the sample date of the bronchoalveolar lavage, the onset of symptoms, and the day of discharge/ death of the patients. Of patients 3, 4 and 7 multiple BAL samples were included. In this figure the first BAL was shown as day 0 if two BALs were available (patients 3 and 7), or the second BAL of three in total (patient 4).

Horizontal lines represent individual patients; the squares represent individual blood samples, onset of respiratory symptoms, discharge, and death. Cq values are shown for the positive samples.

Abbreviations: RV: rhinovirus, BAL: bronchoalveolar lavage.

Genotyping of RV viremic patients

The rhinovirus load in the blood samples of the viremic patients was considered too low to enable successful genotyping. Therefore, the associated BAL samples were used, which resulted in two patients with RV-A: A24 and A01, and two patients with RV- B: B52 and B92.

Characteristics of rhinovirus viremic patients

Patient 1 (Figure 1), was a 60-year-old female who received allogeneic stem cell transplantation for multiple myeloma. One month after transplantation she was admitted at the haematology department with neutropenic fever with a leukocyte count of 0.1 $x10^9$ g/L. During her stay in the hospital she developed cough and dyspnoea. One day after symptom onset a BAL was performed, which was positive for rhinovirus A01 (Cq 24), parainfluenza 2 (Cq 15), adenovirus (Cq 35) and tested galactomannan positive. Bacterial culture showed *Stenotrophomonas maltophilia*. One of six blood samples tested RV positive, Cq 42. Fourteen days after the BAL was performed the patient died with multiple, respiratory and systemic infections (CMV reactivation- Aspergillus pneumonia-Stenotrophomonas bacteraemia) and unexplained on-going neutropenia. No autopsy was performed. The last two blood samples before her death were RV negative.

Patient 2, was a 68-year-old male with myelodysplastic syndrome for which he received non-myeloablative allogeneic stem cell transplantation four months prior to admittance to the intensive care unit (ICU) due to respiratory failure. At that time leukocyte count was 3.6 $x10^9$ /L and lymphocyte count 0.6 $x10^9$ /L. Four days after symptom onset a BAL was performed, in which a rhinovirus RV-B52 (Cq 23), was detected. No other pathogen (viral, bacterial, parasitic or fungal) could be found in the BAL sample. In total three blood samples were tested. The blood samples drawn six days before and three days after the BAL tested rhinovirus positive, Cq 34 to 36. The blood sample drawn three days before the BAL was rhinovirus negative. The patient died 11 days after the BAL was performed. Autopsy showed extensive pulmonary fibrosis, the lung architecture was completely destroyed without specific characteristics. Reported cause of death was respiratory failure of unknown cause.

Patient 3 was a 65-year-old female with a double cord blood stem cell transplantation for a diffuse large B cell lymphoma. She was admitted to the ICU for neurological deterioration three months after transplantation, leukocyte count was 2.2 $x10^9$ /L and lymphocyte count 0.8 $x10^9$ /L. Two bronchoalveolar lavages were performed, seven days apart, which both contained RV-A24 with Cq values of 19and 18 respectively. Pulmonary symptoms started 15 days prior to the initial BAL. Galactomannan was positive in both BALs, no other viral or bacterial pathogens could be found. In total seven blood samples were tested, one tested positive, Cq 37, taken 13 days after the initial BAL. The patient died 16 days after the first BAL was performed. The last available blood sample before the patient died was rhinovirus negative. Shortly before death, progressive neurological deterioration, kidney insufficiency and Aspergillus pneumonia were observed. Autopsy was not performed.

Patient 4, a 48-year-old male with a kidney transplant was admitted at the ICU with respiratory insufficiency of unknown cause. The patient had 23.3 $x10^9$ /L leukocytes and 0.7 $x10^9$ /L lymphocytes

at the time of the second BAL. Three BALs were performed, with RV Cq values of 24, 22 and 17 respectively. The second BAL, containing RV-B92, is used as index BAL in figure 1. The only pathogen consistently detected in all three BALs was rhinovirus. In total six blood samples were tested for rhinovirus, one tested positive Cq 36, taken 52 days after the first day of illness, being 16 days before the patients died of respiratory failure. Autopsy was not performed, but a lung biopsy, taken before the patient deceased, showed strongly disturbed lung architecture with few inflammatory infiltrate (no PCR performed). This was suggestive for a cryptogenic organizing pneumonia, possibly due to recurrent infections.

Rhinovirus viremia risk factors

In both the viremic and non-viremic groups the majority of patients had received allogeneic stem cell transplantation (Table 1). Almost all patients had clinically and radiologically signs of severe lower respiratory tract disease (cough, dyspnoea, radiological abnormalities).

No significant difference could be found between viremic and non-viremic patients with regard to underlying condition, symptoms, radiological findings or co-pathogens.

All patients with rhinovirus viremia died (4/4, 100%) as a consequence of respiratory problems during their hospital admission. Only five out of 23 (22%) rhinovirus viremia negative patients died during hospital admission, of which three died because of respiratory failure (all three had a co-infection with another virus). This difference in mortality was significant (p=0.007, Fisher's exact).

Table 1. **Characteristics of rhinovirus viremia positive versus rhinovirus viremia negative patients.**

	RV viremia positive (n=4)	RV viremia negative (n=23)	odds ratio	95% confidence interval		P* value
				lower	upper	
General						
Age, years, median (range)	60 (48-68)	56 (21-78)		-10.6	18.9	0.57
Male, no. (%)	2 (50)	15 (65)	0.53	0.06	4.5	0.62
Admission to hospital (%)	4 (100)	22 (96)				0.67
Conditions underlying						
Smoking	2 (2/3)	8 (8/17)	2.25	0.2	29.8	1
Pulmonary condition underlying	1 (1/4)	5 (5/22)	1.13	0.1	13.4	1
STx (%)	3 (75)	12 (52)	2.75	0.2	30.5	0.61
Allogeneic	3 (3/3)	11 (11/12)				1
NMA	2 (2/3)	7 (7/11)				1
Related	0 (0/3)	4 (4/11)				0.51
GVHD	0 (0/3)	6 (6/12)				0.23
Solid organ Tx (%)	1 (25)	5 (22)	1.2	0.1	14.2	1
Prednisone use (%)	1 (25)	12 (52)	0.31	0.03	3.4	0.60
Symptoms						
Fever	2 (2/3)	18 (18/23)	0.56	0.04	7.5	1
Coughing	3 (3/3)	14 (14/15)				1
Dyspnoea	3 (3/3)	21 (21/21)				
Laboratory findings						
CRP mg/L, mean (range)	115 (22-162)	131 (3-417)		-162.4	131.3	0.83
Leukocytes x10^9 /L, mean (range)	7.3 (0.1-23.3)	7.7 (0.1-22.9)		-7.8	6.9	0.91
Lymphocytes x10^9 /L, mean (range)	0.53 (0-0.83)	1.09 (0.01-6.16)		-2.2	1.0	0.47
Saturation %, without oxygen , mean(range)	96 (90-100)	94 (88-100)		-3.4	6.6	0.51
pH, mean (range)	7.45 (7.38-7.54)	7.42 (7.24-7.52)		-0.06	0.1	0.56
Radiography						
Abnormalities (%)	4 (100)	22 (96)				1
Ground glass	2 (2/3)	8 (8/16)	2	0.2	26.7	1
Consolidations (%)	4 (100)	18 (78)				0.56
Tree in bud	1 (1/3)	7 (7/16)	0.64	0.05	8.6	1
Microbiology						
Duration of respiratory disease to BAL, mean (range)	7 (1-15)	12 (2-36)		-14.8	5.5	0.36
Days post-Tx and BAL sampling date, mean (range)	343 (60-1034)	517 (17-4455)		-1300.6	951.8	0.75
Rhinovirus BAL Cq value, mean (range)	22.9 (19.4-24.5)	20.6 (15.5-24.9)		-0.6	5.1	0.12

Respiratory co-pathogens, viral, bacterial or fungal (%)	3 (75)	17 (74)	1.06	0.09	12.2	1
No. of blood samples available for RV testing per BAL, mean (range)	3.6 (2-6)	2.6 (1-6)		-0.8	2.9	0.26
Outcome						
Days admitted, mean (range)	35 (12-53)	24 (2-106)		-16.4	37.26	0.43
Deceased (%)	*4 (100)*	*5 (22)*				*0.01*

Abbreviations: RV: rhinovirus, STx: stem cell transplantation, BAL: bronchoalveolar lavage, Tx: transplantation, GVHD: Graft-versus-host-disease:* Fisher's exact or independent T-test

DISCUSSION

This study demonstrated the presence of rhinovirus viremia in four out of 27 adult patients with high loads in the respiratory tract. In addition, an association with higher mortality in the rhinovirus viremic patients was found. No other correlations could be found.

These results indicate that rhinovirus viremia may be a relevant negative prognostic factor given the significant association with fatal outcome. No other risk factors for viremia could be identified. In previous studies in children, rhinovirus viremia was associated with worse clinical signs and symptoms and was found in several fatal cases, also suggesting that viremia may be a predictor of poor prognosis[20-22,25-27]. Whether rhinovirus viremia would play a causative role in this poor prognosis, in the presence or absence of co-pathogens, or whether it indicates end stage disease needs to be determined. Similarly, while we did not culture our blood samples we cannot deduce whether rhinovirus viremia is the result of leakage of viral RNA from the lungs, as may be suggested by the severity of illness and the high Cq values, or whether active replication takes place outside the respiratory tract. However, others have shown previously that rhinovirus could be cultured from the blood of children with severe rhinovirus infections[21,26]. Furthermore, in vitro, rhinovirus has been cultured on Hela cell suspension[31] suggestive of a broader cell tropism resembling enterovirus.

We were able to test an average of three-four blood samples per BAL sample. However, sufficient volumes of blood were not always available to enable testing with optimal sensitivity. In addition, a potential classification bias may have been introduced if more blood samples were drawn, stored and tested from more severely ill patients with high mortality risk compared to less severely ill patients. However, our comparison of the mean number of blood samples tested for RV per patient shows that this was comparable for the RV viremic patients and controls.

Patients with high viral loads in the BAL were selected for rhinovirus viremia testing. High viral loads in BAL fluid, with an arbitrary threshold of Cq <25, were selected as it was expected these would show stronger correlations with viremia. Despite this potentially high-risk population only a minority of patients were viremic, with low viral loads. This percentage in the selected group of highly immunocompromised adults is comparable to the percentage of viremia described in immunocompetent children, while in immunocompromised children the rate of viremia may be considerably higher. Viremia in immunocompetent adults may occur even less frequently, although

this could not be studied in our population. It must be noted that the low number of viremic patients reduces the power of the risk factor analysis. In children RV viremia has been shown to be associated with asthma[20,25], therefore adults with asthma or COPD may also have a higher risk of RV viremia. The effects of asthma or COPD could not be determined, given the low number of patients with underlying pulmonary conditions.

In children, the percentage of rhinovirus viremia positive cases has been reported to be higher on the day of admission compared to three days later[23,25]. In our adult patients this pattern could not be detected, potentially because blood samples were not drawn on a regular basis and the number of positive blood samples was low.

Genotyping of the rhinovirus viremia positive samples revealed two RV-A, two RV-B and no RV-C genotypes, though RV-C appeared to be the predominant species in viremic children. The percentage RV-C in children with viremia varies from 67% to 87% in studies with six and 30 viremic patients respectively[20,23]. Because the number of viremic patients is low and the local prevalence of the different rhinovirus species over the years is unknown, it cannot yet be concluded whether the outcome of RV-C viremia is different between children and adults.

In conclusion, rhinovirus viremia may occur in adult patients, possibly less frequent compared to children, and appears to be associated with a higher mortality. The role of such viral markers may also be relevant if broader genotypic approaches (sequencing) will increasingly be used in the near future. The pathogenesis and risk factors of rhinovirus viremia need further investigation.

ACKNOWLEDGEMENTS

We thank Lisette Rusman and Margriet Kraakman (Department of Medical Microbiology, LUMC) for assistance with the molecular testing.

REFERENCES

1. Nichol KL, D'Heilly S, Ehlinger E: Colds and influenza-like illnesses in university students: impact on health, academic and work performance, and health care use. *Clinical infectious diseases* 2005, **40**(9):1263-1270.
2. Weigl JA, Puppe W, Meyer CU, Berner R, Forster J, Schmitt HJ, Zepp F: Ten years' experience with year-round active surveillance of up to 19 respiratory pathogens in children. *European journal of pediatrics* 2007, **166**(9):957-966.
3. Makela MJ, Puhakka T, Ruuskanen O, Leinonen M, Saikku P, Kimpimaki M, Blomqvist S, Hyypia T, Arstila P: Viruses and bacteria in the etiology of the common cold. *Journal of clinical microbiology* 1998, **36**(2):539-542.
4. Jennings LC, Anderson TP, Beynon KA, Chua A, Laing RT, Werno AM, Young SA, Chambers ST, Murdoch DR: Incidence and characteristics of viral community-acquired pneumonia in adults. *Thorax* 2008, **63**(1):42-48.
5. Johnstone J, Majumdar SR, Fox JD, Marrie TJ: Viral infection in adults hospitalized with community-acquired pneumonia: prevalence, pathogens, and presentation. *Chest* 2008, **134**(6):1141-1148.
6. Templeton KE, Scheltinga SA, van den Eeden WC, Graffelman AW, van den Broek PJ, Claas EC: Improved diagnosis of the etiology of community-acquired pneumonia with real-time polymerase chain reaction. *Clinical infectious diseases* 2005, **41**(3):345-351.
7. Johansson N, Kalin M, Tiveljung-Lindell A, Giske CG, Hedlund J: Etiology of community-acquired pneumonia: increased microbiological yield with new diagnostic methods. *Clinical infectious diseases* 2010, **50**(2):202-209.
8. Jain S, Self WH, Wunderink RG, Fakhran S, Balk R, Bramley AM, Reed C, Grijalva CG, Anderson EJ, Courtney DM *et al*: Community-Acquired Pneumonia Requiring Hospitalization among U.S. Adults. *N Engl J Med* 2015, **373**(5):415-427.
9. Garcia-Garcia ML, Calvo C, Pozo F, Villadangos PA, Perez-Brena P, Casas I: Spectrum of respiratory viruses in children with community-acquired pneumonia. *The Pediatric infectious disease journal* 2012, **31**(8):808-813.
10. Choi SH, Huh JW, Hong SB, Lee JY, Kim SH, Sung H, Do KH, Lee SO, Kim MN, Jeong JY *et al*: Clinical characteristics and outcomes of severe rhinovirus-associated pneumonia identified by bronchoscopic bronchoalveolar lavage in adults: comparison with severe influenza virus-associated pneumonia. *Journal of clinical virology* 2015, **62**:41-47.
11. Bruning AHL, Thomas XV, van der Linden L, Wildenbeest JG, Minnaar RP, Jansen RR, de Jong MD, Sterk PJ, van der Schee MP, Wolthers KC *et al*: Clinical, virological and epidemiological characteristics of rhinovirus infections in early childhood: A comparison between non-hospitalised and hospitalised children. *Journal of clinical virology* 2015, **73**:120-126.
12. Louie JK, Yagi S, Nelson FA, Kiang D, Glaser CA, Rosenberg J, Cahill CK, Schnurr DP: Rhinovirus outbreak in a long term care facility for elderly persons associated with unusually high mortality. *Clinical infectious diseases* 2005, **41**(2):262-265.
13. Kraft CS, Jacob JT, Sears MH, Burd EM, Caliendo AM, Lyon GM: Severity of human rhinovirus infection in immunocompromised adults is similar to that of 2009 H1N1 influenza. *Journal of clinical microbiology* 2012, **50**(3):1061-1063.
14. Gunawardana N, Finney L, Johnston SL, Mallia P: Experimental rhinovirus infection in COPD: implications for antiviral therapies. *Antiviral research* 2014, **102**:95-105.
15. Piralla A, Zecca M, Comoli P, Girello A, Maccario R, Baldanti F: Persistent rhinovirus infection in pediatric hematopoietic stem cell transplant recipients with impaired cellular immunity. *Journal of clinical virology* 2015, **67**:38-42.
16. Lauinger IL, Bible JM, Halligan EP, Bangalore H, Tosas O, Aarons EJ, MacMahon E, Tong CY: Patient characteristics and severity of human rhinovirus infections in children. *Journal of clinical virology* 2013, **58**(1):216-220.
17. Xiang Z, Gonzalez R, Xie Z, Xiao Y, Liu J, Chen L, Liu C, Zhang J, Ren L, Vernet G *et al*: Human rhinovirus C infections mirror those of human rhinovirus A in children with community-acquired pneumonia. *Journal of clinical virology* 2010, **49**(2):94-99.
18. Jacobs SE, Lamson DM, Soave R, Guzman BH, Shore TB, Ritchie EK, Zappetti D, Satlin MJ, Leonard JP, van Besien K *et al*: Clinical and molecular epidemiology of human rhinovirus infections in patients with hematologic malignancy. *Journal of clinical virology* 2015, **71**:51-58.
19. Choi SH, Hong SB, Kim T, Kim SH, Huh JW, Do KH, Lee SO, Kim MN, Lim CM, Kim YS *et al*: Clinical and molecular characterization of rhinoviruses A, B, and C in adult patients with pneumonia. *Journal of clinical virology* 2015, **63**:70-75.
20. Esposito S, Daleno C, Scala A, Castellazzi L, Terranova L, Sferrazza Papa S, Longo MR, Pelucchi C, Principi N: Impact of rhinovirus nasopharyngeal viral load and viremia on severity of respiratory infections in children. *European journal of clinical microbiology & infectious diseases* 2014, **33**(1):41-48.
21. Urquhart GE, Stott EJ: Rhinoviraemia. *British medical journal* 1970, **4**(5726):28-30.

22. Urquhart GE, Grist NR: Virological studies of sudden, unexplained infant deaths in Glasgow 1967-70. *Journal of clinical pathology* 1972, **25**(5):443-446.
23. Fuji N, Suzuki A, Lupisan S, Sombrero L, Galang H, Kamigaki T, Tamaki R, Saito M, Aniceto R, Olveda R *et al*: Detection of human rhinovirus C viral genome in blood among children with severe respiratory infections in the Philippines. *PloS one* 2011, **6**(11):e27247.
24. Tapparel C, L'Huillier AG, Rougemont AL, Beghetti M, Barazzone-Argiroffo C, Kaiser L: Pneumonia and pericarditis in a child with HRV-C infection: a case report. *Journal of clinical virology* 2009, **45**(2):157-160.
25. Xatzipsalti M, Kyrana S, Tsolia M, Psarras S, Bossios A, Laza-Stanca V, Johnston SL, Papadopoulos NG: Rhinovirus viremia in children with respiratory infections. *American journal of respiratory and critical care medicine* 2005, **172**(8):1037-1040.
26. Lupo J, Schuffenecker I, Morel-Baccard C, Bardet J, Payen V, Kaiser L, Constant S, Lobrinus JA, Lin-Marq N, Lina B *et al*: Disseminated rhinovirus C8 infection with infectious virus in blood and fatal outcome in a child with repeated episodes of bronchiolitis. *Journal of clinical microbiology* 2015, **53**(5):1775-1777.
27. Las Heras J, Swanson VL: Sudden death of an infant with rhinovirus infection complicating bronchial asthma: case report. *Pediatric pathology* 1983, 1(3):319-323.
28. DeMore JP, Weisshaar EH, Vrtis RF, Swenson CA, Evans MD, Morin A, Hazel E, Bork JA, Kakumanu S, Sorkness R *et al*: Similar colds in subjects with allergic asthma and nonatopic subjects after inoculation with rhinovirus-16. *The Journal of allergy and clinical immunology* 2009, **124**(2):245-252, 252.e241-243.
29. Loens K, van Loon AM, Coenjaerts F, van Aarle Y, Goossens H, Wallace P, Claas EJ, Ieven M: Performance of different mono- and multiplex nucleic acid amplification tests on a multipathogen external quality assessment panel. *Journal of clinical microbiology* 2012, **50**(3):977-987.
30. Zlateva KT, de Vries JJ, Coenjaerts FE, van Loon AM, Verheij T, Little P, Butler CC, Goossens H, Ieven M, Claas EC: Prolonged shedding of rhinovirus and re-infection in adults with respiratory tract illness. *The European respiratory journal* 2014, **44**(1):169-177.
31. Lee WM, Chen Y, Wang W, Mosser A: Growth of human rhinovirus in H1-HeLa cell suspension culture and purification of virions. *Methods in molecular biology* 2015, **1221**:49-61.

CHAPTER 7

Molecular epidemiology and clinical impact of rhinovirus infections in adults during three epidemic seasons in 11 European countries (2007-2010)

K.T. Zlateva[a#], A.L. van Rijn[a#], P. Simmonds[b], F.E.J. Coenjaerts[c], A.M. van Loon[c], T.J.M. Verheij[d], J.J.C. de Vries[a], P. Little[e], C.C. Butler[f], E.W. van Zwet[g], H. Goossens[h], M. Ieven[h], E.C.J. Claas[a] on behalf of the GRACE Study Group (www.grace-lrti.org)

[a] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[b] Infection and Immunity Division, Roslin Institute, University of Edinburgh, Edinburgh, United Kingdom
[c] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands
[d] Department of Data management, Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands
[e] University of Southampton Medical School, Primary Care Medical Group, Southampton, United Kingdom
[f] Nuffield Department of Primary Care Sciences, Oxford University, Oxford,
[g] Department of Medical Statistics, Leiden University Medical Center, the Netherlands
[h] Department of Medical Microbiology, Vaccine and Infectious Disease Institute, University Hospital Antwerp, Antwerp, Belgium.
[#] These authors contributed equally to this work

Submitted

ABSTRACT

Background: Differences in clinical impact between rhinovirus (RV) species and types in adults are not well established.

The objective of this study was to determine the epidemiology and clinical impact of the different rhinovirus species.

Methods: We conducted a prospective study of RV infections in adults with acute cough/lower respiratory tract infection (LRTI) and asymptomatic controls. Subjects were recruited from 16 primary care networks located in 11 European countries between 2007-2010. RV detection and genotyping was performed by means of real-time and conventional reverse-transcriptase polymerase-chain-reaction assays, followed by sequence analysis. Clinical data were obtained from medical records and patient symptom diaries.

Results: RV were detected in 566 (19%) of 3016 symptomatic adults, 102 (4%) of their 2539 follow-up samples and 67 (4%) of 1677 asymptomatic controls. Genotyping was successful for 538 (95%) symptomatic subjects, 86 (84%) follow-up infections, and 62 (93%) controls. RV-A was the prevailing species, associated with an increased risk of LRTI as compared to RV-B (relative risk [RR], 4.5; IC 2.5-7.9; P<0.001) and RV-C (RR, 2.2; IC 1.2-3.9; P=0.010). In symptomatic subjects RV-A loads were higher than those of RV-B (P= 0.015). Symptom scores and duration were similar across species. More RV-A infected patients felt generally unwell in comparison to RV-C (P=0.023). Of the 140 RV types identified, 5 were new types; asymptomatic infections were associated with multiple types.

Interpretation: In adults RV-A is significantly more often detected in cases with acute cough/ LRTI than RV-C, while RV-B infection is often found in asymptomatic patients.

INTRODUCTION

Rhinoviruses (RV) are the most prevalent respiratory pathogens in humans, accounting for approximately 20-50% of respiratory tract infections annually[1-4]. Infection with RV can lead to a wide spectrum of clinical manifestations ranging from asymptomatic to severe lower respiratory illness[2,5,6]. RV is also recognized as a major cause of exacerbations of chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis[7,8]. Co-infections with other viral or bacterial pathogens have been frequently reported, potentially increasing clinical severity[5,9,10]. Over 160 RV types have been identified and classified into three genetically distinct species (RV-A, RV-B and RV–C) within the genus *Enterovirus* of the family *Picornaviridae*[11]. RV-A has been the most frequently detected RV species, followed by RV-C and RV-B respectively[3,4]. Although the clinical significance of RV species and types remains poorly defined, RV-C has been predominantly linked to asthma exacerbations and more serious respiratory illness in young children[12-14]. More recently similar clinical presentations across species have been reported[15-18].

Despite the high morbidity and disease burden of RV infections, there are currently no effective and safe antiviral treatments, and efforts to develop an effective vaccine have been hampered by the substantial antigenic diversity between RV types and species. A more practical approach could be the development of vaccines targeting more prevalent or pathogenic rhinovirus types although the feasibility of this depends on whether particular RV species or types are associated with more frequent or severe respiratory illness. Previous studies have been limited by the absence of asymptomatic controls and incomplete microbiological investigation, and were focused mainly on hospitalized young children.

The aim of this study was to determine the epidemiology and clinical impact of the different rhinovirus species through a community-based comparative evaluation of disease severity among adult subjects infected with different RV species and types. Additionally, the multicentre approach provides insight in geographical differences in RV epidemiology.

METHODS

Study design and subjects

Respiratory samples were obtained from subjects aged 18 years and older enrolled as part of the GRACE European Network of Excellence (www.grace-lrti.org) focusing on improving the management of community-acquired LRTIs. Sixteen primary care networks from 11 European countries including: Belgium (Antwerp and Ghent); France (Nice), Germany (Rotenberg), Italy (Milan), the Netherlands (Utrecht), Poland (Bialystok, Lodz and Szczecin), Slovakia (Bratislava), Slovenia (Jesenice), Spain (Barcelona and Mataró), Sweden (Jönköping), and United Kingdom (Cardiff and Southampton) participated in prospective recruitment of patients presenting with acute cough and signs of an acute LRTI over the period October 2007 - June 2010. Cases were patients presenting at the general practitioner (GP) with an acute or worsened cough (≤28 days duration) as the main symptom, or any clinical presentation considered by the GP to be caused by lower respiratory tract infection and consulting for the first time for this illness episode. Exclusion criteria were pregnancy, breast-feeding

and any condition associated with severe impaired immune status. Asymptomatic controls were subjects who consulted their GP for complaints other than respiratory, who had no symptoms and signs of a respiratory tract infection (RTI) and had not used antibiotics or antivirals in the previous 2 weeks. Case and controls were recruited from the same GP center, within 14 days of each other and with a maximum 5-year age difference.

Nasopharyngeal swab (NPS) and sputum (if available) samples were collected from case (V1) and control (V0) patients during their first visit to the GP, for which standardization was obtained by training of staff. Follow-up NPS samples were obtained from case patients at the second visit (V2) to the GP after approximately 28-days. Medical ethics committees in participating countries approved the study and all participants provided written informed consent.

The original study was designed to evaluate the aetiology of lower respiratory tract infections in GP settings, as described by Ieven et. al., this study is a post-hoc analysis[19].

Data collection

Clinical data, including medical history, co-morbidities and their management/treatment, and days the patient felt unwell were recorded by the GP on a case report form (CRF) at the first consultation. The presence or absence of cough, sputum production, shortness of breath, wheeze, coryza, fever, chest pain, muscle aching, headache, disturbed sleep, feeling generally unwell, interference with normal activities, confusion/disorientation and diarrhoea, were documented. If present, the severity of each symptom was rated on a 4-point scale ranging from 'no problem=1' to 'severe problem=4'. Following the first GP consultation, patients were requested to complete a daily symptom diary for the duration of illness (to a maximum of 28 days). The presence and severity of the above symptoms (except diarrhoea), were rated on a 7-point scale ranging from 'normal/not affected =0' to 'as bad as it could be=6'.

Clinical assessment

The disease severity of rhinovirus infections was evaluated by comparing the following clinical characteristics: 1) initial symptom score, 2) duration of illness, 3) maximal symptom score, and 4) duration of higher symptom score. The 'initial symptom score' was estimated as the mean severity score of at least 12 of the 14 symptoms evaluated by the GP during the first visit of the patient. The 'duration of illness', 'maximal symptom score' and 'duration of higher symptom score' were estimated based on the presence and severity of 13 symptoms excluding diarrhoea, recorded in the patient's daily symptom diary as described previously. The duration of illness was defined as the total sum of days a patient felt unwell before the first GP visit and the number of days the patient experienced any of the 13 symptoms over the 28-day follow-up period. The duration of higher symptom score was the number of days a patient had a mean daily symptom score of two or more based on the 7-point scale[19]. The occurrence and severity of each symptom recorded during the first visit to the GP were also compared between RV species.

Laboratory investigation

The overall results of the laboratory diagnostics were previously described by Ieven et al[20]. In short, total nucleic acids were extracted at the University Hospital Antwerp, and examined for RV by real-time PCR at the Leiden University Medical Center. Cycle quantification (Cq) values, which had been normalized using a fixed baseline fluorescence threshold, were used as an indicative measure of viral load. Bacterial and fungal infections were detected in NPS or/and sputum samples by conventional culture and/or molecular methods[21].

RV typing

RV genotyping was conducted by amplification and sequencing of partial VP3/VP1, VP4/VP2 or/and 5'-UTR viral genome fragments as described previously[19]. BLASTn analyses were conducted for initial species identification and then confirmed by phylogenetic analysis. RV sequences were compared to prototype strains and assigned to types based on phylogenetic analysis and pairwise p-distances (see Supplementary Appendix). RV sequences from this study have been deposited in GenBank under the accession numbers KP736530-KP737279 and KR045604.

Statistical analysis

Continuous variables were summarized as medians with interquartile (IQR) ranges or means with 95% confidence interval and for categorical variables, the frequencies in each category were calculated. Demographic and clinical characteristics were compared across RV species within RV-positive symptomatic and asymptomatic adults by using risk-ratio and/or chi-square tests for categorical variables and the non-parametric Kruskal-Wallis test, Mann-Whitney U test or independent t-test or one way ANOVA when appropriate for continuous variables. Correction for possible confounders (age, sex, geographical location, sample season and sample year) was performed using multivariable linear or logistic regression for continuous or binary outcome measures, respectively. The statistical analyses were conducted in the IBM SPSS Statistics software version 25 for Windows (IBM Corp. Released 2011, Armonk, NY: IBM Corp.). A two-sided P-value <0.05 was considered statistically significant.

RESULTS

RV detection and species distribution

In this study 3070 (99%) (median age 50, [IQR, 36-63 years]; male/female ratio 1:1.5) of 3104 recruited adults with acute cough/LRTI and 1677 asymptomatic controls (median age 50, [IQR, 35-62 years]; male/female ratio 1:1.5) were included. Thirty-four patients with no available NPS material or CRF were excluded (Figure 1). NPS samples were obtained from 3016 symptomatic patients during the first visit (V1) to the GP and a follow-up (V2) sample was collected from 2485 (82%) of these patients[19]. For 54 symptomatic patients, only a follow-up NPS was available for testing, resulting in a total of 2539 V2 NPS specimens. Of the asymptomatic controls 1677 samples were tested. Of the

3104 symptomatic patients 1844 (60%) tested positive for a respiratory pathogen, of which 350 (11%) a bacterial pathogen only, 1190 (39%) a respiratory virus and 304 (10%) a double infection. Of the 1677 asymptomatic controls, 205 (12%) had an respiratory virus as previously reported by Ieven et al[19].

RV was detected in 766 out of 7232 samples (11%) and genotyping was successful for 717 (94%) of these samples. Enteroviruses including 13 EV-D68, 10 EV-C104, 5 EV-C105, 2 EV-C117 types, and 1 EV-C (non-typeable), were identified due to cross-reactivity in our diagnostic RV assay. These 31 EV-positive samples were excluded from further analysis. Forty-nine (6%) RV-positive samples with lower viral loads inferred from quantification cycle (Cq) values could not be genotyped due to amplification failure in the genotyping PCR (median Cq values of 29 vs. 36 in the diagnostic RT-PCR assay for genotyped and not-genotyped samples respectively; P<0.001).

RV was detected in 566 (19%) V1 and 102 (4%) V2 samples from case patients (of which 21 were positive in both the V1 and V2 samples) and in 67 (4%) V0 samples from asymptomatic control patients. Of the 735 RV infection episodes, 182 (25%) were co-infections, of which 68 (9%) with another virus, 103 (14%) with a bacterial co-infection, and 11 (2%) had a rhinovirus infection with at least 2 other pathogens. Commensals and *Candida species* were not considered causative pathogens and were excluded from the analysis.

Symptomatic (V1 positive) RV-positive patients tended to be older than asymptomatic patients (median 47 years, [IQR, 33-60 years] vs. median 38 years [IQR, 27-58 years]; P=0.058, respectively), see Table 1. No significant differences were found in sex, location, and sample season and year between RV-positive symptomatic and asymptomatic adults. Of 563 of the 566 V1 patients underlying conditions were stated: 238 (42%) had an underlying condition, including 122 (51%) with respiratory comorbidities or history of hospitalization for respiratory illness. Mixed infections within the RV-positive adults (V1), were associated with symptomatic disease more often (29% vs 7% in the asymptomatic patients, RR 4.9 (95% CI 2.0-11.8) p<0.001, adjusted for age, sex, geographical location, sample season, sample year, and viral load). Mixed infections were not included in further analyses. The RV-viral load was lower in the RV-positive asymptomatic controls (mean Cq value 31 vs 28 in patients, p<0.001, adjusted for age, sex, geographical location, sample season, sample year, and mixed infections).

Virus genotyping to RV species level was successful in 538 (95%) V1, 86 (84%) V2 RV-positive samples from symptomatic patients and in 62 (93%) V0 control subjects. The number and proportion of RV species among symptomatic, follow-up and asymptomatic infections are shown in Figure 1. Prolonged RV shedding in symptomatic patients (V1) was identified in 6 patients as previously described[19]. RV re-infection with another rhinovirus type, was identified in 11 symptomatic (V1) patients.

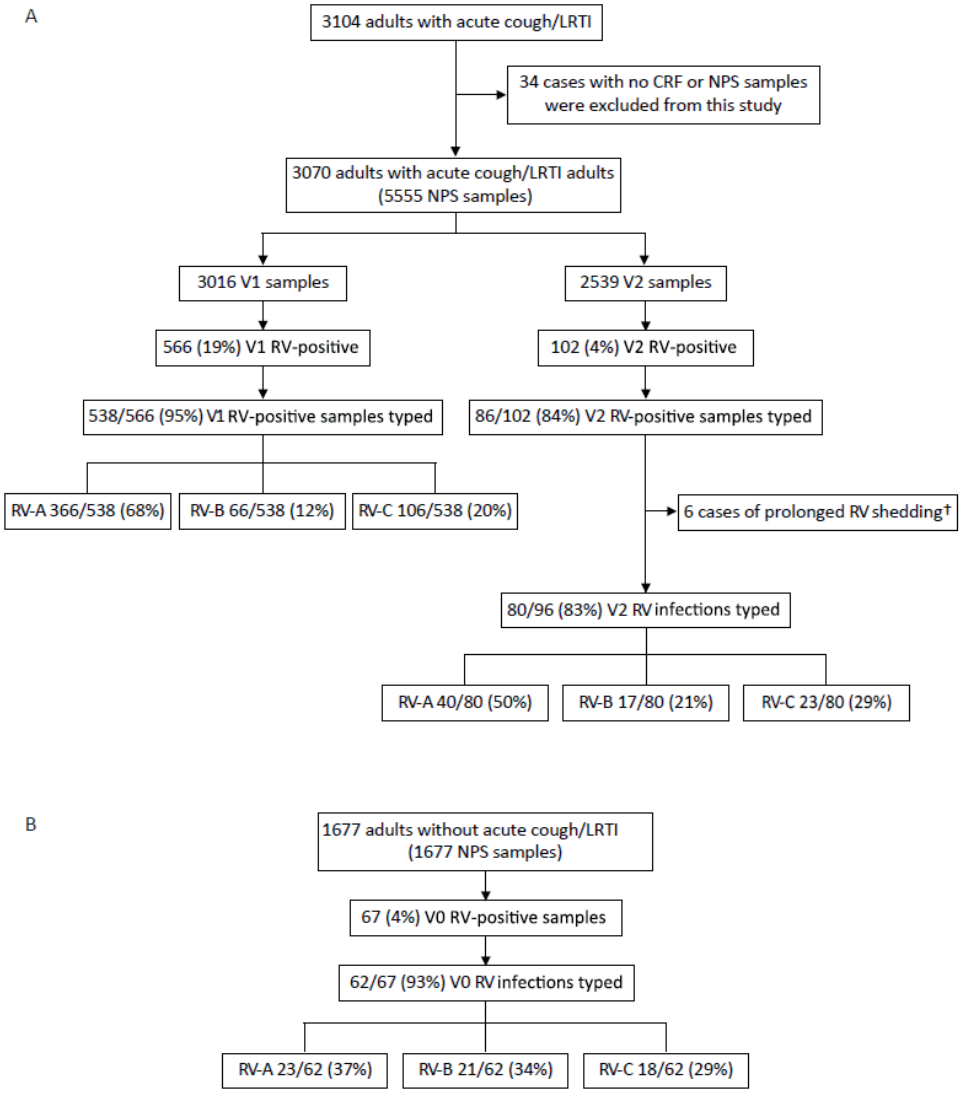


Figure 1. Rhinovirus prevalence and species distribution among (A) adults enrolled with acute cough/LRTI and asymptomatic (B) controls.

Abbreviations: LRTI, lower respiratory tract infection; CRF, case report form, NPS, nasopharyngeal swab.
†Prolonged rhinovirus shedding was identified in 6 patients, described previously[19].

Table 1. **Demographics of symptomatic (V1) vs asymptomatic (V0) RV-positive patients**

		Total n=633	RV+ V1 n=566	RV+ V0 n=67	p-value
Male (%)		263 (42)	229 (40)	34 (51)	0.106
Age (median, IQR)		47 (32-60)	47 (33-60)	38 (27-58)	0.058
Ethical background (%)		563			
	Caucasian		540 (96)		
	African		8 (1)		
	Asian		10 (2)		
	Other		5 (1)		
Study site (%)					0.281
	Antwerp BE	95 (15)	85 (15)	10 (15)	
	Barcelona SP	66 (10)	64 (11)	2 (3)	
	Bialystok PL	28 (4)	24 (4)	4 (6)	
	Bratislava SL	30 (5)	29 (5)	1 (1)	
	Cardiff GB	64 (10)	57 (10)	7 (10)	
	Ghent BE	25 (4)	20 (4)	5 (7)	
	Jesenice SVN	10 (2)	10 (2)	0 (0)	
	Jonkoping SW	8 (1)	7 (1)	1 (1)	
	Lodz PL	52 (8)	47 (8)	5 (7)	
	Mataró SP	79 (12)	68 (12)	11 (16)	
	Milan IT	7 (3)	17 (3)	0 (0)	
	Nice FR	3 (0.5)	3 (1)	0 (0)	
	Rotenberg DU	27 (4)	25 (4)	2 (3)	
	Southampton GB	43 (7)	39 (7)	4 (6)	
	Szczecin PL	27 (4)	22 (4)	5 (7)	
	Utrecht NL	59 (9)	49 (9)	10 (15)	
Sample season &year (%)		632	565	67	0.918*
	autumn 07	37 (6)	34 (6)	3 (4)	
	winter 07/08	49 (8)	43 (8)	6 (9)	
	spring 08	73 (12)	67 (12)	6 (9)	
	summer 08	4 (1)	4 (1)	0 (0)	
	autumn 08	118 (19)	106 (19)	12 (18)	
	winter 08/09	64 (10)	55 (10)	9 (13)	
	spring 09	44 (7)	40 (7)	4 (6)	
	summer 09	4 (1)	4 (1)	0 (0)	
	autumn 09	131 (37)	118 (21)	13 (19)	
	winter 09/10	77 (12)	69 (12)	8 (12)	
	spring 10	31 (5)	25 (4)	6 (9)	

Underlying condition (%)		238/563 (42)
	asthma	67/563 (12)
	COPD	41/563 (7)
	other respiratory comorbidities	7/563 (1)
	history of hospitalization for respiratory illness	24/563 (4)
	cardiac disease	47/563 (8)
	diabetes	35/562 (6)
	allergic diseases	105/563 (23)

*Overall sample season and year, symptomatic (V1) versus asymptomatic (V0)

Clinical manifestations

Complete clinical record forms and symptom diaries were available for 563 (99.5%) and 383 (67.7%) RV-positive symptomatic V1 patients, respectively. Age and sex characteristics of symptomatic and asymptomatic subjects, infection outcome, viral loads inferred from Cq values, and duration and severity of disease were compared across RV species (Table 2). The age and sex distribution was similar across RV-species in the symptomatic and control groups. In 538 RV-infected symptomatic patients the proportion of RV-A infections was 68% versus 12% for RV-B and 20% for RV-C infections. In contrast, in the 62 asymptomatic infections the proportions were similar: RV-A: 37%, RV-B: 34% and RV-C: 29% (Figure 1). For single infections RV-A was 4.5 and 2.2 times more often associated with a LRTI outcome than RV-B ($P<0.001$) and RV-C ($P=0.010$), respectively, and RV-C more often than RV-B (RR 2.1, $P=0.015$). The results were similar for RV co-infected cases. In symptomatic patients with a RV mono-infection RV-A viral loads (mean Cq=27) were significantly higher than RV-B viral loads (mean Cq=30, $P= 0.015$). The duration, overall disease severity, and maximum daily symptom score were similar between RV species. The severity of the individual symptoms was also compared across RV species (Table 3). Significant differences were observed only for “feeling generally unwell”. In particular RV-A infected patients generally felt worse than those with RV-C ($P=0.023$). No other significant differences were observed between RV-A and RV-B, or RV-C and RV-B infected patients.

Table 2. Age and sex distribution, infection outcome and disease severity according to rhinovirus species.

Characteristics†	RV-A	RV-B	RV-C	RR (95% CI)	P value
Age/Sex -V1 infections					
No. of cases	366	66	106		
Median (IQR) years	47 (33-60)	43 (30-61)	50 (33-62)		0.571
No. (%) of male	155 (42)	22 (33)	43 (41)		0.389
Age/Sex -V0 infections					
No. of cases	23	21	18		
Median (IQR) years	37 (25-53)	36 (28-51)	50 (30-61)		0.472
No. (%) of male	13 (57)	9 (43)	10 (56)		0.613
Symptomatic (V1) vs. asymptomatic (V0) infections **No (%) of V1/V0 cases**	366 (94)/23 (6)	66 (76)/21 (24)	106 (85)/18 (15)	RV-A vs. RV-B: RR 4.6 (2.7-8.0) RV-A vs. RV-C: RR 2.4 (1.4-4.3) RV-C vs. RV-B: RR 1.9 (1.1-3.4)	RV-A vs. RV-B (<0.001)* RV-A vs. RV-C (0.003)* RV-C vs. RV-B (0.026)*
Symptomatic (V1) vs. asymptomatic (V0) single infections **No (%) of V1/V0 cases**	261 (92)/22 (8)	43 (70)/18 (32)	80 (82)/17 (18)	RV-A vs. RV-B: RR 4.5 (2.5-7.9) RV-A vs. RV-C: RR 2.2 (1.2-3.9) RV-C vs. RV-B: RR 2.1 (1.1-3.7)	RV-A vs. RV-B (<0.001)* RV-A vs. RV-C (0.010)* RV-C vs. RV-B (0.015)*
Cq values- V1 single infections **Mean (95%CI)**	27 (27-28)	30 (28-31)	27 (26-29)		0.053* RV-A vs. RV-B (0.015)* RV-A vs. RV-C (0.780)* RV-C vs. RV-B (0.068)*
Cq values- V0 single infections					RV-A vs. RV-B (0.922)*

Mean (95% CI)					RV-A vs. RV-C (0.576)*
	31 (29-33)	32 (30-33)	31 (29-32)	0.838*	RV-C vs. RV-B (0.642)*
Initial symptom score[a]				0.697*	RV-A vs. RV-B (0.426)*
No. of cases	260	43	80		RV-A vs. RV-C (0.666)*
Median (IQR)	1.6 (1.1-2.0)	1.4 (0.9-2.1)	1.4 (1.1-1.9)		RV-C vs. RV-B (0.684)*
Duration of illness				0.987*	RV-A vs. RV-B (0.893)*
No. of cases	200	31	62		RV-A vs. RV-C (0.948)*
Median (IQR) days	21 (14-31)	20 (13-31)	19 (13-32)		RV-C vs. RV-B (0.870)*
Maximal daily symptom score[b]					RV-A vs. RV-B (0.216)*
No. of cases	200	31	62	0.426*	RV-A vs. RV-C (0.519)*
Median (IQR)	2.3 (1.6-3.1)	2.0 (1.3-3.1)	2.2 (1.5-3.0)		RV-C vs. RV-B (0.499)*
Duration of higher symptom score					RV-A vs. RV-B (0.985)*
No. of cases	158	23	48	0.942*	RV-A vs. RV-C (0.740)*
Median (IQR) days	4 (3-7)	4 (2-7)	3 (2-6)		RV-C vs. RV-B (0.842)*

[†] Characteristics related to disease severity and duration of illness were evaluated only for patients with rhinovirus single infections who had complete case report forms or/and symptom diaries. Abbreviations: RV rhinovirus; IQR, interquartile range; RR, relative risk; CI, confidence interval; Cq, cycle of quantification.

*Corrected for age, sex, sample season, and sample year

[a] Initial symptom score scale: 1=no problem, 2=mild problem, 3=moderate problem, 4=severe problem.

[b] Maximal daily symptom score scale: 0=no problem/not affected, 1=very little problem, 2=slight problem, 3=moderately bad, 4=bad, 5=very bad, 6=as bad as it could be.

Table 3. **Frequency and severity of symptoms in adults with rhinovirus associated acute cough/LRTI**

Characteristics	No. (%) of events (absent-mild/moderate-severe)†			
	RV-A N=261	RV-B N=43	RV-C N=80	P- value*
Cough	1 (0.4) /260(99.6)	1 (2)/42 (98)	1 (1)/79 (99)	RV-A vs. RV-B (0.053) RV-A vs. RV-C (0.400) RV-B vs. RV-C (0.180)
Phlegm	131 (50)/130 (50)	22 (51)/21 (49)	48 (60)/32 (40)	RV-A vs. RV-B (0.739) RV-A vs. RV-C (0.091) RV-B vs. RV-C (0.397)
Shortness of breath	171 (66)/90 (34)	27 (63)/16 (37)	55 (69)/25 (31)	RV-A vs. RV-B (0.549) RV-A vs. RV-C (0.578) RV-B vs. RV-C (0.371)
Wheeze	199 (76)/62 (24)	32 (74)/11 (26)	64 (80)/16 (20)	RV-A vs. RV-B (0.798) RV-A vs. RV-C (0.606) RV-B vs. RV-C (0.566)
Running nose	109 (42)/152 (58)	21 (49)/22 (51)	37 (46)/43 (54)	RV-A vs. RV-B (0.306) RV-A vs. RV-C (0.667) RV-B vs. RV-C (0.545)
Fever	226 (87)/35 (13)	38 (88)/5 (12)	69 (86)/11 (14)	RV-A vs. RV-B (0.958) RV-A vs. RV-C (0.883) RV-B vs. RV-C (0.962)
Chest pain	205 (79)/56 (21)	32 (74)/11 (26)	60 (75)/20 (25)	RV-A vs. RV-B (0.352) RV-A vs. RV-C (0.534) RV-B vs. RV-C (0.690)
Muscle aching	189 (72)/72 (28)	32 (74)/11 (26)	58 (73)/22 (27)	RV-A vs. RV-B (0.662) RV-A vs. RV-C (0.990) RV-B vs. RV-C (0.708)
Headache	181 (69)/80 (31)	25 (58)/18 (42)	55 (69)/25 (31)	RV-A vs. RV-B (0.287) RV-A vs. RV-C (0.996) RV-B vs. RV-C (0.361)
Disturbed sleep	141 (54)/120 (46)	25 (58)/18 (42)	38 (48)/42 (52)	RV-A vs. RV-B (0.428) RV-A vs. RV-C (0.259) RV-B vs. RV-C (0.150)
Diarrhea	256 (98)/5 (2)	41 (95)/2 (5)	78 (98)/2 (2)	RV-A vs. RV-B (0.087) RV-A vs. RV-C (0.744) RV-B vs. RV-C (0.225)

Confusion/ disorientation	257 (98)/4 (2)	43 (100)/0	79 (99)/1 (1)	RV-A vs. RV-B (0.999) RV-A vs. RV-C (0.999) RV-B vs. RV-C (0.999)
Interference with normal activities	156 (60)/105 (40)	23(53)/20 (47)	54 (68)/26 (33)	RV-A vs. RV-B (0.364) RV-A vs. RV-C (0.281) RV-B vs. RV-C (0.130)
Feeling generally unwell	118 (45)/143 (55)	22 (51)/21(49)	49 (61)/31 (39)	RV-A vs. RV-B (0.442) RV-A vs. RV-C (0.023) RV-B vs. RV-C (0.392)

[†] The presence and severity of symptoms were evaluated only for patients with rhinovirus single infections. Abbreviations: RV, rhinovirus; CI, confidence interval. *Corrected for age, sex, sample season, and sample year

RV prevalence and species distribution according to site and year of detection

The number of tested NPS samples, RV detection rate and species distribution among the 16 sites in Europe over the 3-year study period are presented in Figure 2. The majority of subjects were recruited in the months October to April and years were defined from July until June of the following year. No patients were recruited from Nice, Bialystock, Szczecin, Bratislava and Jesenice during 2007/2008 and only 9 samples from 6 patients were available from Milan for 2009/2010. RV accounted for 1% up to 21% of respiratory infections per site during a particular year, when excluding Milan during 2009/2010, because of the low number of included cases.

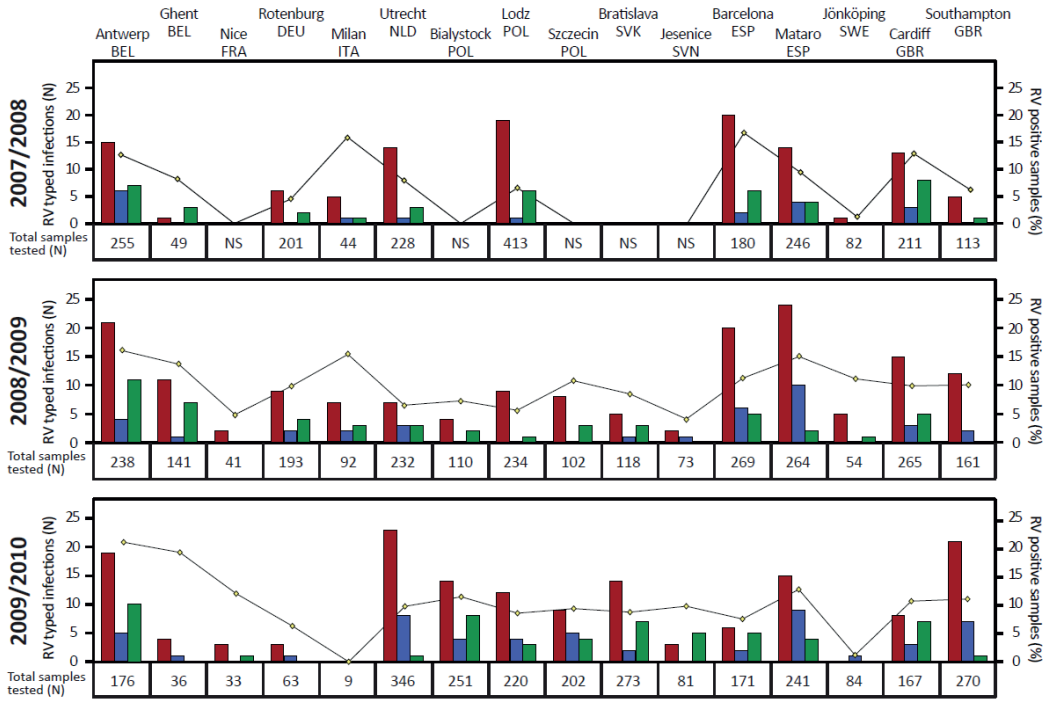


Figure 2. Rhinovirus (RV) detection rate and species distribution according to site and year of detection.

Total numbers of tested samples per location and year of isolation are presented under the corresponding country panel; 'NS' designates that no samples were available. Plot lines represent the proportion (%) of RV infections (right side y-axis) and bar graphs represent the number of RV-A (red), RV-B (blue) and RV-C (green) infections (left side y-axis). Country abbreviations: BEL (Belgium), FRA (France), DEU (Germany), ITA (Italy), NLD (Netherland), POL (Poland), SVK (Slovakia), SVN (Slovenia), ESP (Spain), SWE (Sweden), GBR (United Kingdom).

RV type identification

Of the 686 sequenced rhinoviruses 654 (95%) were characterized by partial VP3/VP1 or/and VP4/VP2 sequencing and 32 (5%) based only on the 5'-UTR (see Supplementary Appendix). The latter were assigned only to species taxa level, because of limited phylogenetic discrimination in the 5'-UTR region. Four VP4/VP2 sequences were classified only to species level based on BLASTn results due to poor sequence quality. A total of 650 RV sequences were assigned to 73 RV-A, 25 RV-B and 42 RV-C types representing 91%, 78% and 76% [11], respectively, of the currently established types within each species (Figure 3). Five new RV types were identified and assigned by the *Picornaviridae* Study Group as RV-A type A109, RV-B types B105 and B106 and RV-C types C52 and C53.

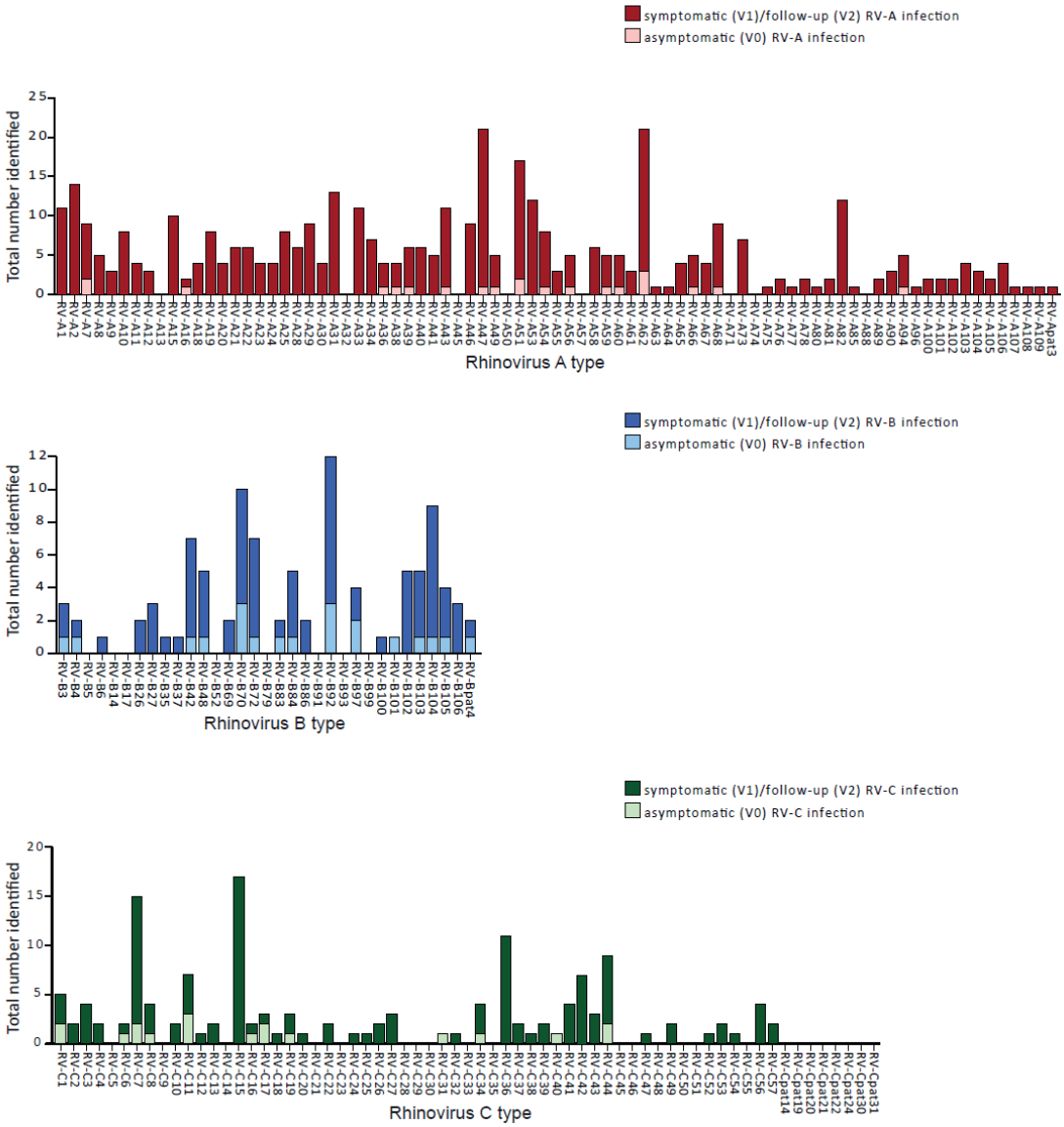


Figure 3. Frequencies of rhinovirus types identified in symptomatic case and asymptomatic control adults.

DISCUSSION

In our study RV was detected in 19% of adults with acute cough/LRTI and in 4% of follow-up samples as well as samples from asymptomatic controls. The overall annual prevalence ranged from 1 to 21% between and within communities. These data indicate that the burden of RV associated illness varies substantially within and between communities in different years. Several other studies have reported rates of RV infection from 7% to 33% in inpatient and outpatient adults with acute LRTI or influenza-like illness[15,22-25], and in 8% or less of asymptomatic adults[3,4,15,26].

RV species were identified with a different frequency among symptomatic and control subjects. With 68% prevalence RV-A strains were significantly more common in RV infected symptomatic patients than RV-B (12%) or RV-C (20%) strains. In contrast, the prevalence of RV-A (37%), RV-B (34%) and RV-C (29%) was more or less similar in asymptomatic infections, suggesting a more pathogenic role of RV-A infections. Also, RV-A was 4.5 and 2.2 times more often associated with LRTI than RV-B and RV-C ($P \leq 0.010$), and RV-C 2.1 times more often than RV-B ($P= 0.015$).

Our findings indicate that in adults, the prevalence of RV-A associated LRTI is considerably higher than that of RV-B and RV-C, while RV-B infections are more often asymptomatic. The latter observation is further supported by the fact that RV-B has been detected infrequently in children and adults hospitalized for severe respiratory illness[15,17,27-31]. Furthermore RV-B symptomatic infections had significantly lower viral loads than RV-A symptomatic infections, suggesting lower pathogenicity of RV-B viruses. Lower viral loads of RV-B relative to RV-A and RV-C in respiratory specimens have previously been reported for adults with pneumonia[32] and viral load has been reported as indicator for severity of RV infections[3]. These findings are in agreement with the *in vitro* study that demonstrated that RV-B types replicate less efficiently and induce lower cytokines and/or chemokine levels than RV-A or RV-C infections[33].

Our results on RV species distribution (RV-A>RV-C>RV-B) among adults with LRTI are consistent with those from a study conducted in Seoul among adults with pneumonia[32]. Other studies in symptomatic patients found a lower prevalence of RV-C than RV-B infections[34,35]. These differences in the relative proportions of RV species in comparison to our findings could be due to differences in the study populations (e.g. URTI instead of LRTI) and annual variations in species distribution. We observed no significant differences between RV species in the duration of illness, maximum symptom scores, overall disease severity and severity of individual common cold symptoms. However, RV-A infected patients subjectively felt in general worse than those infected with RV-C. Our results suggest that RV-A infections have a more debilitating impact on the general health as compared to RV-B and RV-C infections. In children several studies demonstrated an association between RV-C and more serious respiratory illness[13,22,28]. In adult patients RV-C infection has been reported to cause more frequent acute respiratory tract infection and appeared more severe as compared to children[22,36]. Other studies in adults observed no differences in disease severity between rhinovirus species[15,16,26,32,37], though one study found a higher disease severity associated with RV-A in upper respiratory symptoms[35]. However, these studies are limited due to the short surveillance periods, lack of asymptomatic controls and small sample size. It must be noted that the patients included in

this study had per definition acute cough/LRTI, thus differences in disease severity between RV species in patients with milder symptoms cannot be excluded.

RV-A was the most prevalent species in the majority of study locations, followed by RV-C, while RV-B occurrence varied considerably between and within certain communities. Substantial antigenic variation within RV-A could lead to more frequent re-infections in the community and to a higher overall prevalence. Interestingly, not all RV species were identified in the separate locations during the study period. Genotyping of the RV samples lead to the discovery of 5 novel RV types and revealed a remarkably high diversity of RVs represented by 73 RV-A, 25 RV-B and 42 RV-C types. Asymptomatic infections were associated with a variety of RV types, the majority of which were also identified in symptomatic cases.

Although comprehensive, this study has some limitations. Due to the heterogeneity of the RV genomes our RV diagnostic and genotyping assays might have varied in efficacy of amplification and therefore be suboptimal for some genotypes. In particular RV-C infections might be somewhat underrepresented, since a limited number of RV-C complete genome sequences were available at the time our diagnostic assay was developed.

In addition, due to the nature of this study, only associations can be found, and no causality can be proven. With the current study design, an association can be made between RV type and disease severity. However the population studied, is patients visiting their GP that in general will be older and have more co-morbidities as compared to the general population. Nevertheless, these are the patients consulting their GP, and therefore this study is very representative for clinical practice.

Although limited, some CRF's were missing and not all RV positive samples could be typed. Because these numbers are limited a link between the missing data and disease severity is not expected and this probably will not result in a bias.

Finally, the clinical status of cases with RV-positive follow-up samples could not be clearly defined, since symptom manifestations were not recorded by the GP at the time of the second visit.

This is a very comprehensive study, in which RV typing has been performed in symptomatic (LRTI) and asymptomatic adult GP patients in 11 countries. RV typing is not part of standard diagnostic care and our results do not provide an indication for an added value in relation to disease severity. This may change in the future if subtype differences in efficacy are observed following antiviral treatment or vaccination.

In conclusion, LRTI in adults were mainly associated with RV-A, identified in a significantly higher proportion of symptomatic than asymptomatic subjects as compared to RV-B and RV-C. Furthermore RV-A symptomatic infections were associated with significantly higher viral loads than RV-B symptomatic infections, suggesting lower pathogenicity of RV-B.

ACKNOWLEDGEMENTS

This study was funded by the of the European Commision's FP6 Programme under grant number LSHM-CT-2005-518226 and the Department of Medical Microbiology, Leiden University Medical Center, the Netherlands.

We thank all involved in the GRACE Primary Care Network for the opportunity to conduct this study, Stefan van Nieuwkoop, Naim Farzad, Yuvia Guilarte, Iris Hidding, Lisette Rusman, Margriet Kraakman and Mario van Bussel (Department of Medical Microbiology, LUMC, Leiden, The Netherlands) for technical assistance with virus genotyping, and Susan Hemert-Glaubitz (Julius Center of Health Sciences and Primary Care, UMC, Utrecht, The Netherlands) for assistance with the GRACE database.

REFERENCES

1. Heikkinen T, Jarvinen A: The common cold. *Lancet (London, England)* 2003, **361**(9351):51-59.
2. van Gageldonk-Lafeber AB, Heijnen ML, Bartelds AI, Peters MF, van der Plas SM, Wilbrink B: A case-control study of acute respiratory tract infection in general practice patients in The Netherlands. *Clinical infectious diseases* 2005, **41**(4):490-497.
3. Ng KT, Oong XY, Lim SH, Chook JB, Takebe Y, Chan YF, Chan KG, Hanafi NS, Pang YK, Kamarulzaman A *et al*: Viral Load and Sequence Analysis Reveal the Symptom Severity, Diversity, and Transmission Clusters of Rhinovirus Infections. *Clinical infectious diseases* 2018, **67**(2):261-268.
4. van der Linden L, Bruning AH, Thomas XV, Minnaar RP, Rebers SP, Schinkel J, de Jong MD, Pajkrt D, Wolthers KC: A molecular epidemiological perspective of rhinovirus types circulating in Amsterdam from 2007 to 2012. *Clinical microbiology and infection* 2016, **22**(12):1002.e1009-1002.e1014.
5. Ruuskanen O, Waris M, Ramilo O: New aspects on human rhinovirus infections. *The Pediatric infectious disease journal* 2013, **32**(5):553-555.
6. Vandini S, Biagi C, Fischer M, Lanari M: Impact of Rhinovirus Infections in Children. *Viruses* 2019, **11**(6).
7. Goffard A, Lambert V, Salleron J, Herwegh S, Engelmann I, Pinel C, Pin I, Perrez T, Prevotat A, Dewilde A *et al*: Virus and cystic fibrosis: rhinoviruses are associated with exacerbations in adult patients. *Journal of clinical virology* 2014, **60**(2):147-153.
8. Wu X, Chen D, Gu X, Su X, Song Y, Shi Y: Prevalence and risk of viral infection in patients with acute exacerbation of chronic obstructive pulmonary disease: a meta-analysis. *Molecular biology reports* 2014, **41**(7):4743-4751.
9. Goka EA, Vallely PJ, Mutton KJ, Klapper PE: Single, dual and multiple respiratory virus infections and risk of hospitalization and mortality. *Epidemiology and infection* 2014:1-11.
10. Honkinen M, Lahti E, Osterback R, Ruuskanen O, Waris M: Viruses and bacteria in sputum samples of children with community-acquired pneumonia. *Clinical microbiology and infection* 2012, **18**(3):300-307.
11. ICTV. Genus: Enterovirus**.** https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/picornavirales/w/picornaviridae/681/genus-enterovirus Date last update: 02-2019 . Accessed 25-07-2019
12. Lau SK, Yip CC, Tsoi HW, Lee RA, So LY, Lau YL, Chan KH, Woo PC, Yuen KY: Clinical features and complete genome characterization of a distinct human rhinovirus (HRV) genetic cluster, probably representing a previously undetected HRV species, HRV-C, associated with acute respiratory illness in children. *Journal of clinical microbiology* 2007, **45**(11):3655-3664.
13. Piralla A, Rovida F, Campanini G, Rognoni V, Marchi A, Locatelli F, Gerna G: Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients. *Journal of clinical virology* 2009, **45**(4):311-317.
14. Hasegawa K, Mansbach JM, Bochkov YA, Gern JE, Piedra PA, Bauer CS, Teach SJ, Wu S, Sullivan AF, Camargo CA, Jr.: Association of Rhinovirus C Bronchiolitis and Immunoglobulin E Sensitization During Infancy With Development of Recurrent Wheeze. *JAMA pediatrics* 2019.
15. Fry AM, Lu X, Olsen SJ, Chittaganpitch M, Sawatwong P, Chantra S, Baggett HC, Erdman D: Human rhinovirus infections in rural Thailand: epidemiological evidence for rhinovirus as both pathogen and bystander. *PloS one* 2011, **6**(3):e17780.
16. Henquell C, Mirand A, Deusebis AL, Regagnon C, Archimbaud C, Chambon M, Bailly JL, Gourdon F, Hermet E, Dauphin JB *et al*: Prospective genotyping of human rhinoviruses in children and adults during the winter of 2009-2010. *Journal of clinical virology* 2012, **53**(4):280-284.
17. Iwane MK, Prill MM, Lu X, Miller EK, Edwards KM, Hall CB, Griffin MR, Staat MA, Anderson LJ, Williams JV *et al*: Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. *The Journal of infectious diseases* 2011, **204**(11):1702-1710.
18. Ren L, Yang D, Ren X, Li M, Mu X, Wang Q, Cao J, Hu K, Yan C, Fan H *et al*: Genotyping of human rhinovirus in adult patients with acute respiratory infections identified predominant infections of genotype A21. *Scientific reports* 2017, **7**:41601.
19. Zlateva KT, de Vries JJ, Coenjaerts FE, van Loon AM, Verheij T, Little P, Butler CC, Goossens H, Ieven M, Claas EC *et al*: Prolonged shedding of rhinovirus and re-infection in adults with respiratory tract illness. *The European respiratory journal* 2014, **44**(1):169-177.
20. Ieven M, Coenen S, Loens K, Lammens C, Coenjaerts F, Vanderstraeten A, Henriques-Normark B, Crook D, Huygen K, Butler CC *et al*: Aetiology of lower respiratory tract infection in adults in primary care: a prospective study in 11 European countries. *Clinical microbiology and infection* 2018, **24**(11):1158-1163.

21. Loens K, van Loon AM, Coenjaerts F, van Aarle Y, Goossens H, Wallace P, Claas EJ, Ieven M, Group GS: Performance of different mono- and multiplex nucleic acid amplification tests on a multipathogen external quality assessment panel. *Journal of clinical microbiology* 2012, **50**(3):977-987.
22. Lau SK, Yip CC, Lin AW, Lee RA, So LY, Lau YL, Chan KH, Woo PC, Yuen KY: Clinical and molecular epidemiology of human rhinovirus C in children and adults in Hong Kong reveals a possible distinct human rhinovirus C subgroup. *The Journal of infectious diseases* 2009, **200**(7):1096-1103.
23. Templeton KE, Scheltinga SA, van den Eeden WC, Graffelman AW, van den Broek PJ, Claas EC: Improved diagnosis of the etiology of community-acquired pneumonia with real-time polymerase chain reaction. *Clinical infectious diseases* 2005, **41**(3):345-351.
24. Pierangeli A, Scagnolari C, Selvaggi C, Verzaro S, Spina MT, Bresciani E, Antonelli G, Bertazzoni G: Rhinovirus frequently detected in elderly adults attending an emergency department. *Journal of medical virology* 2011, **83**(11):2043-2047.
25. Creer DD, Dilworth JP, Gillespie SH, Johnston AR, Johnston SL, Ling C, Patel S, Sanderson G, Wallace PG, McHugh TD: Aetiological role of viral and bacterial infections in acute adult lower respiratory tract infection (LRTI) in primary care. *Thorax* 2006, **61**(1):75-79.
26. Granados A, Goodall EC, Luinstra K, Smieja M, Mahony J: Comparison of asymptomatic and symptomatic rhinovirus infections in university students: incidence, species diversity, and viral load. *Diagnostic microbiology and infectious disease* 2015.
27. Lee WM, Lemanske RF, Jr., Evans MD, Vang F, Pappas T, Gangnon R, Jackson DJ, Gern JE: Human rhinovirus species and season of infection determine illness severity. *American journal of respiratory and critical care medicine* 2012, **186**(9):886-891.
28. Linsuwanon P, Payungporn S, Samransamruajkit R, Posuwan N, Makkoch J, Theanboonlers A, Poovorawan Y: High prevalence of human rhinovirus C infection in Thai children with acute lower respiratory tract disease. *The Journal of infection* 2009, **59**(2):115-121.
29. Miller EK, Khuri-Bulos N, Williams JV, Shehabi AA, Faouri S, Al Jundi I, Chen Q, Heil L, Mohamed Y, Morin LL *et al*: Human rhinovirus C associated with wheezing in hospitalised children in the Middle East. *Journal of clinical virology* 2009, **46**(1):85-89.
30. Hung HM, Yang SL, Chen CJ, Chiu CH, Kuo CY, Huang KA, Lin TY, Hsieh YC, Gong YN, Tsao KC *et al*: Molecular epidemiology and clinical features of rhinovirus infections among hospitalized patients in a medical center in Taiwan. *Journal of microbiology, immunology, and infection* 2019, **52**(2):233-241.
31. Liggett SB, Bochkov YA, Pappas T, Lemanske RF, Jr., Gern JE, Sengamalay N, Zhao X, Su Q, Fraser CM, Palmenberg AC: Genome sequences of rhinovirus C isolates from wisconsin pediatric respiratory studies. *Genome announcements* 2014, **2**(2).
32. Choi SH, Hong SB, Kim T, Kim SH, Huh JW, Do KH, Lee SO, Kim MN, Lim CM, Kim YS *et al*: Clinical and molecular characterization of rhinoviruses A, B, and C in adult patients with pneumonia. *Journal of Clinical Virology* 2015, **63**:70-75.
33. Nakagome K, Bochkov YA, Ashraf S, Brockman-Schneider RA, Evans MD, Pasic TR, Gern JE: Effects of rhinovirus species on viral replication and cytokine production. *The Journal of allergy and clinical immunology* 2014, **134**(2):332-41
34. Xiang Z, Gonzalez R, Wang Z, Xiao Y, Chen L, Li T, Vernet G, Paranhos-Baccala G, Jin Q, Wang J: Human rhinoviruses in Chinese adults with acute respiratory tract infection. *The Journal of infection* 2010, **61**(4):289-298.
35. Chen WJ, Arnold JC, Fairchok MP, Danaher PJ, McDonough EA, Blair PJ, Garcia J, Halsey ES, Schofield C, Ottolini M *et al*: Epidemiologic, clinical, and virologic characteristics of human rhinovirus infection among otherwise healthy children and adults: Rhinovirus among adults and children. *Journal of clinical virology* 2015, **64**:74-82.
36. Wisdom A, Leitch EC, Gaunt E, Harvala H, Simmonds P: Screening respiratory samples for detection of human rhinoviruses (HRVs) and enteroviruses: comprehensive VP4-VP2 typing reveals high incidence and genetic diversity of HRV species C. *Journal of clinical microbiology* 2009, **47**(12):3958-3967.
37. Pretorius MA, Tempia S, Treurnicht FK, Walaza S, Cohen AL, Moyes J, Hellferscee O, Variava E, Dawood H, Chhagan M *et al*: Genetic diversity and molecular epidemiology of human rhinoviruses in South Africa. *Influenza and other respiratory viruses* 2014.

SUPPLEMENTARY FILE

File 1: Sequencing and phylogenetic analyses

METHODS

Sequence and phylogenetic analyses

RV-positive samples were initially analysed with the VP3/VP1 genotyping assay and samples with negative results were further tested using RT-PCR assays amplifying partial VP4/VP2 or 5'UTR genome fragments[1]. PCR products were purified using the ExoSAP-IT® kit (Isogen, Life Science) and Sanger sequencing was performed at the Leiden Genome Technology Center (LGTC, Leiden, The Netherlands). Sequences were assembled and edited using Geneious version 7.1.5 software package (Biomatters, New Zealand) and BLASTn searches (http://www.ncbi.nlm.gov/BLAST) were conducted to establish virus species. RV data sets included, 212 RV-A (Table S1), 77 RV-B (Table S2), and 185 RV-C (Table S3) reference sequences obtained from the website of the *Picornaviridae* Study Group (http://www.picornastudygroup.com) or through literature search and downloaded from Genbank[2-5]. Multiple sequence alignments were generated with the MUSCLE alignment tool[6]. Neighbor-joining phylogenetic trees were constructed in the MEGA 7.0.26 software package[7] from 1000 bootstrap resampled sequence alignments using the maximum composite likelihood[8] model with pairwise deletions of missing data. RV sequences from this study were assigned to types based on phylogenetic clustering to reference sequences and intra- and inter-clade pairwise nucleotide distances p-distances estimated in MEGA 7.0.26 software package (Figure S1, S2, S3 and Tables S1, S2 and S3). Additional sequence analysis of VP1, VP4/VP2 or 5'UTR genome regions were performed for novel RV types or RV strains with incomplete coverage of the VP3/VP1 analysed fragment, due to poor sequence quality. A primer walking approach with universal primers selected from known or closely related sequences were used to obtain VP1 sequences from novel RV types.

RESULTS

A total of 686 rhinovirus positive samples were successfully amplified and sequenced in the VP3/VP1, VP4/VP2 or/and 5'UTR genome regions.

Rhinovirus typing based on partial VP3/VP1 sequence analysis (Figure S1)

Successful amplification and sequencing of the VP3/VP1 genome region was achieved for 292 RV-positive samples, including 196 RV-A, 49 RV-B and 49 RV-C sequences. Phylogenetic analyses were based on approximately 850 nt and 880 nt VP3-VP1 fragments of respectively RV-A and RV-B sequences (nucleotide positions 2144-2978 according to RV-A2 strain, GenBank accession no. X02316) and 628 nt fragments of the RV-C VP1 gene (nucleotide positions 2174-2801 according to RV-C10 strain, GenBank accession no. EF077278). Twenty-four RV-A, 9 RV-B and 10 RV-C sequences were <90% complete across the analyzed region (sequence lengths ≥354 nt). The majority of VP3/VP1 or VP1 sequences obtained from this study clustered closely with sequences of established RV types (Figure S1 and Table S1, S2, S3). Seven sequences had ≥15% nucleotide divergence from their nearest neighbor type. These sequences were confirmed as new RV types based on complete VP1 sequence analysis and assigned by the *Picornaviridae* Study Group as RV-A109 (F0062-V1), RV-B105 (H4923-V0 and H5048), RV-B106 (D2622-V1 and E2217-V1), RV-C52 (H1380-V1) and RV-C53 (D1880-V1).

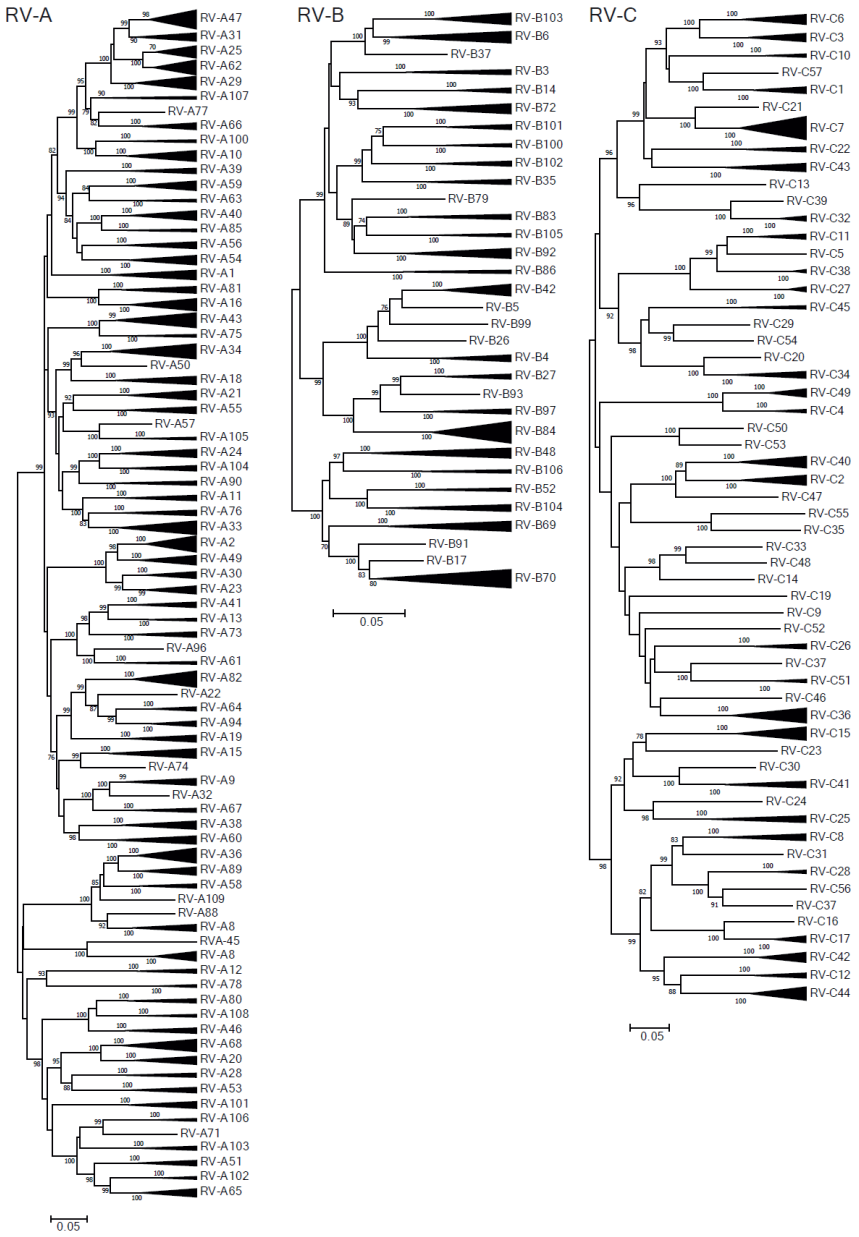


Figure S1. Phylogenetic RV-A, RV-B and RV-C trees of the VP3/VP1 region

Unrooted neighbor-joining phylogenetic trees of RV-A and RV-B partial VP3/VP1 sequences and RV-C partial VP1 sequences were generated using the maximum composite likelihood model. Numerical values (presented when >70%) represent the statistical support for the tree topology as determined by 1000 bootstrap replicates. Multiple sequences from the same type are shown as triangles with heights proportional to their numbers and depths corresponding to their earlier diverging branch.

Table S1. **Rhinovirus A type assignment of partial VP3/VP1, VP4/VP2 and 5'UTR sequences.**

RV-A type	GenBank sequences	VP3/VP1: 196 sequences			VP4/VP2: 253 sequences			VP3/VP1 and VP2/VP4	5'-UTR: 24 sequences		
	Accession no.[a]	Sample Identifier[b]	Intra-clade upper limit divergence[c]	Inter-clade lower limit divergence[d]	Sample Identifier[b]	Intra-clade upper limit divergence[c]	Inter-clade lower limit divergence[d]	Number of typed samples	Sample Identifier[b]	Divergence from nearest RV type[e]	Number of typed samples
RV-A1	D00239† FJ445111† JN798558† JN815255† JN837694† JQ837724†	A3613-V1 F1896-V1 J0892-V1	0.1425	0.2276	A1205-V1 A2843-V1 A3613 V1 F2134-V2 J1088-V1 H3480-V1 I2599-V1 N4548-V1 N3848-V2	0.1284	0.1405	11	B3334-V1 B3152-V1 F0272-V1 J1158-V1 H6700-V1	0.0421 0.0153 0 0 0.003	5
RV-A2	X02316†	A2689-V1 D0256-V1 D2440-V1 D2482-V1 E1615-V2 F1952-V1 F1994-V1 H2346-V1 H2402-V1 E0607-V1 E1867-V2	0.0956	0.1497	E2119-V2 E2931-V2 O1189-V1	0.1121	0.1442	14	O2156-V0	0.0506	1
RV-A7	DQ473503†	B0339-V0	0.0985	0.1482	D0774-V1	0.0892	0.1281	8			

	FJ445176†	I0541-V1 I1186-V0			D1194-V1 E0019-V1 E1615-V1 F0034-V1 I1186-V0 I4699-V1						
RV-A8	FJ445113† FJ445170†	D0298-V1 D1824-V1 E2931-V1 I0471-V1 I0779-V1	0.0835	0.2366	E2931-V1 I0779-V1	0.0984	0.1899	5			
RV-A9	FJ445114† FJ445115† FJ445177†	H2696-V1 I4783-V1	0.1109	0.1417	I3593-V1 I4783-V1	0.0963	0.1281	2			
RV-A10	DQ473498† FJ445178† JN541269 JN798575† JN798582† JN815247†	R2018-V1 R2648-V2	0.0968	0.1452	A3305-V1 B2326-V1 H2024-V1 K0515-V1 K0921-V1 K0977-V1	0.0801	0.1236	8			
RV-A11	EF173414†	E0761-V1 E4485-V1 S1977-V1	0.0844	0.157	E0761-V1 S1711-V1	0.0984	0.1259	4			
RV-A12	EF173415† JF781511 HQ123441		0.0953	0.2529	A0295-V1 H0162-V2 S0535-V1	0.087	0.1922	3			
RV-A13	FJ445116† FJ445117†		0.0729	0.16		0.0778	0.1259				
RV-A15	DQ473493† JN541268†	C5813-V1 D2202-V1	0.1054	0.1588	B3292-V1 C4665-V1	0.0984	0.1236	10	S1809-V1	0.0124	1

		E2329-V1 E1223-V1 I2277-V1			C5813-V1 E3337-V1 H7036-V1 N1790-V1						
RV-A16	JN562722† JN614992† JN798564† JN798574† JN815253† JN990704† JX074057† L24917†	C2859-V2	0.0972	0.1592	H6519-V0	0.0961	0.1602	2			
RV-A18	FJ445118† JF781496† JF781508	A1485-V1 D0032-V1 E3407-V1	0.0882	0.1667	A1485-V1 C6989-V1	0.1053	0.1465	3			
RV-A19	FJ445119† JQ747746† JQ747750†	C1319-V1 C5799-V1	0.0918	0.2097	C4763-V1 C5799-V1 H3200-V1 H5426-V1 H7274-V1 I0177-V2 N4366-V2	0.1009	0.1808	8			
RV-A20	FJ445120† JN541270† JN614993† JN798571 JQ994494	D3420-V1	0.0964	0.144	A0239-V1 B3180-V1 D3420-V1 E4345-V1	0.119	0.1281	4			
RV-A21	FJ445121† JN837693† JQ747747†	B2200-V1 E1783-V1 O2659-V1	0.0882	0.1871	E3379-V1 J1956-V1	0.0915	0.151	6	B3964-V1	0.0062	1

		O2799-V1									
RV-A22	FJ445122†			0.1855	D2146-V1 C1613-V1 C4875-V1 N3624-V1 O2113-V1 P0630-V2	0.1193	0.1514	6	H0582-V1	0.0245	1
RV-A23	DQ473497† JN621244† JN815254† JN837696†	B0898-V1 I2543-V1	0.1028	0.1425	E3911-V1 H4922-V1 I2543-V1	0.0732	0.1098	4			
RV-A24	EF173416† FJ445190† JN798563†	A1107-V1 E1069-V1 E1811-V1	0.0891	0.1235	A3193-V1	0.0915	0.1121	4			
RV-A25	FJ445123† AY450476 AF343617	A0491-V1 A1457-V1 B2550-V1 D1782-V1 I4391-V1 H1478-V1 O1903-V1	0.0865	0.0895	A0491-V1 I1661-V1	0.0801	0.0643	8			
RV-A28	DQ473508† JN798577 JN798580 JQ747751†		0.0953	0.2019	B4146-V1 D1292-V1 D1530-V1 D5170-V1 O3555-V2 S2509-V2	0.1076	0.167	6	N2154-V1	0.0122	1
RV-A29	DQ473499† FJ445125†	A1415-V1 A1527-V1	0.1229	0.1551	H5216-V1 R1864-V1	0.1396	0.1076	9	A2857-V1	0.0247	1

	JN815252†	C3321-V1 F2022-V1 F2092-V1 H3312-V1 I1745-V2						
RV-A30	DQ473512† FJ445179† JN798557	H2892-V1 B2942-V1	0.115	0.1425	D5030-V1 I2781-V1	0.0915	0.1098	4
RV-A31	FJ445126†	E0929-V1 J0248-V1 H1072-V1 H3354-V1 H2374-V1	0.1146	0.1174	A0981-V1 A0155-V1 C2607-V2 C6233-V1 D5044-V1 E0103-V1 E0929-V1 I2333-V1 N1370-V1	0.0984	0.087	13
RV-A32	FJ445127†			0.1417			0.1281	
RV-A33	FJ445128† JN815250 JN990707†	B1178-V1 C5617-V1 D3042-V2 E3477-V1 J1634-V1 N1174-V1 O2183-V1	0.1074	0.1535	A0715-V1 C2033-V1 C5617-V1 D2314-V1 F2652-V1	0.0892	0.1121	11
RV-A34	DQ473501† FJ445189† JF781510 JF781512	B4706-V1 E1363-V2 E1363-V1 F1798-V1	0.0918	0.1559	D0284-V1	0.1007	0.1465	8

	JN562720	J1032-V1 I1955-V1 N0026-V1										
RV-A36	DQ473505† JF781497† JN621243† JN614994† JN798583† JN798584† JN815241† JN815242† JN815246† JN837697† JX074050†	A1206-V0	0.0853	0.1148	C2453-V1 R2116-V1 R2382-V1	0.1232	0.1141	4				
RV-A38	DQ473495† FJ445180† JQ994496† JN541272†	C0367-V2 E3618-V0 E1027-V1	0.1001	0.175	B3208-V1	0.0664	0.1762	4				
RV-A39	AY751783†	B1304-V1 N0236-V1 E1321-V1	0.085	0.1979	C4078-V0 E2749-V1 R1598-V1	0.0847	0.1625	6	E2735-V1	0.0274	1	
RV-A40	FJ445129† JQ245967 JX074051† JN798579	A3333-V1 B2620-V2 O2519-V2	0.0791	0.1381	B2620-V2 E4961-V1 H6630-V1 P0602-V2	0.0801	0.1213	6				
RV-A41	DQ473491†	B0870-V1 E1139-V1 O2155-V1	0.1047	0.16	B0884-V1 E1139-V1 P1330-V1	0.1053	0.1259	5				

RV-A43	FJ445131† JN815237†	B0828-V1 E0369-V1 H1268-V1 H1310-V1 H2738-V1 H3158-V1 K1356-V0 N1020-V1 P0546-V1	0.1188	0.1501	A1667-V1 B0828-V1 B0828-V2 J1270-V1 K1356-V0	0.1102	0.1304	12	F3101-V0	0.003	1
RV-A45	FJ445132†			0.2366			0.1899				
RV-A46	DQ473506†	D2006-V1 E3841-V1 H3228-V1	0.0821	0.1665	D2006-V1 E3771-V2 H1702-V1 H3228-V1 K1271-V1 O2449-V1 O3765-V2 S1389-V1	0.0872	0.131	9			
RV-A47	FJ445133† GQ223229† JN837692†	A3235-V1 A4033-V1 C2776-V0 E2623-V1 J1200-V1 J1144-V1 H2500-V1 I3117-V1 R1626-V1 R2340-V1 E1489-V1	0.1158	0.1174	A3235-V1 B3390-V1 C2776-V0 C6275-V1 C6373-V1 D0130-V1 F2848-V1 I3089-V1 I3117-V1 I3635-V1 I4153-V1 N2896-V1	0.0984	0.087	21	H6420-V1 S2537-V1	0.0095 0.0061	2

					S1487-V1							
RV-A49	DQ473496† FJ445134† JN621241† JN798561† JN798589†	A3515-V1 D1894-V1	0.1006	0.1497	A3068-V0 D5352-V1 S0773-V1	0.0915	0.1442	5				
RV-A50	FJ445135†			0.1559			0.1716					
RV-A51	FJ445136† JN562725	F0972-V1 S2733-V2	0.0948	0.1711	B3558-V1 C0073-V2 D1026-V1 D5968-V1 E4079-V2 F0972-V1 H0442-V1 H0106-V1 H1114-V2 H1814-V1 H3565-V0 I0878-V0 I1465-V1 I1493-V1 I1577-V1 R2060-V1 S2733-V2	0.0847	0.1396	17	I2403-V1	0.0183	1	
RV-A53	DQ473507† JN798587†	B0800-V2 D3182-V2	0.0909	0.2019	B0954-V1 C2705-V1 C5113-V1 D3098-V1 E2273-V1 E2791-V1	0.1144	0.167	12				

					R1822-V1 R1962-V1 R2522-V1 Q0701-V1							
RV-A54	FJ445138† FJ445139† FJ445173†	A0533-V1 E0552-V0 H3242-V1 K1075-V1	0.1334	0.1905	B0688-V1 C3769-V1 D2160-V2 F3212-V1	0.119	0.1405	8				
RV-A55	DQ473511† JQ837718†	C0101-V1 I4069-V1 S2397-V1	0.0718	0.1871		0.0709	0.1595	3				
RV-A56	FJ445140† EU840727†	H1254-V1 F4878-V1 R0479-V0	0.1015	0.1905	C4679-V1 I4979-V1	0.0892	0.1556	5				
RV-A57	FJ445141†			0.1268			0.1327					
RV-A58	FJ445142† JX025558†	B2424 V1	0.0776	0.1459	D6038-V1 E0621-V1 H6462-V1 I3719-V1 O3527-V1	0.094	0.1056	6	C4609-V1	0.0492	1	
RV-A59	DQ473500† JN541266†	A0044-V0 D0200-V1 J0332-V1 H1422-V1 I0093-V2	0.0902	0.1842	A0044-V0	0.0778	0.1556	5				
RV-A60	FJ445143† JN798590†	B2158-V1 B1641-V0 B2788-V1 D1740-V1	0.1071	0.175	F0076-V1	0.0892	0.151	5				
RV-A61	FJ445144†		0.0976	0.1622	B3236-V1	0.0984	0.1533	3				

	JN798560†				E3071-V1 Q0715-V1			
RV-A62	FJ445145† AY458611 AF343618	C4651-V1 C4988-V0 E0887-V1 E3827-V1 I0108-V0 I2725-V2 H2752-V1 H5034-V1 R2004-V1	0.1094	0.0895	B2984-V1 B3404-V1 C5407-V1 E3085-V1 E3183-V1 H5034-V1 H5146-V1 N3484-V1 R0856-V1 S0844-V0 S0955-V1 S1655-V1 S2033-V1	0.0714	0.0643	21
RV-A63	FJ445146†	O2897-V1	0.0803	0.1842			0.1556	1
RV-A64	EF173417† FJ445181†	Q0211-V1	0.0704	0.1449	Q0211-V1	0.0778	0.1556	1
RV-A65	FJ445147† JF781504† JQ245966†	I4811-V1 I4895-V1 I1507-V1	0.0659	0.1295	D1236-V1	0.0641	0.1053	4
RV-A66	FJ445148† JN112340† JN621246 JQ837715†	A1191-V1	0.0767	0.1688	A3347-V2 C0480-V0 C5715-V1 N3428-V1	0.1032	0.1659	5
RV-A67	FJ445149† JN621245†	D2272-V1	0.1024	0.1749	D1362-V1 E3295-V1 N1188-V1	0.0732	0.1442	4
RV-A68	FJ445150† JN798578†	B1080-V1 C5309-V2	0.0903	0.144	C3965-V1 R1836-V2	0.087	0.1281	9

		H6518-V1 R2746-V1 S2243-V1 S2706-V0 S2607-V2										
RV-A71	FJ445152†			0.1631			0.1785					
RV-A73	DQ473492† GU568105	D2650-V1 H2584-V1 E1895-V1	0.102	0.1765	A1877-V1 A0323-V1 C5169-V1 D0872-V1 D2650-V1 E1895-V1 H2584-V1	0.167	0.1533	7				
RV-A74	DQ473494†			0.1588			0.1236					
RV-A75	DQ473510† JF781503† JN837690†		0.0738	0.1501	H0358-V1	0.0778	0.1279	1				
RV-A76	DQ473502† FJ445182† JN815238 JX074049† JX074055		0.0921	0.1535	C6415-V1 F0356-V1	0.0664	0.1259	2				
RV-A77	FJ445154†			0.1688	Q0967-V1	0.0618	0.1287	1				
RV-A78	EF173418† FJ445183†		0.0012	0.2612	A3025-V1 Q0911-V1	0.1121	0.1922	2				
RV-A80	FJ445156† JN798576† JN990705† JN798586†		0.075	0.1647	N4198-V1	0.119	0.1238	1	C3377-V1	0	1	
RV-A81	HQ123442	H1870-V2	0.1263	0.1592	A1121-V1	0.1053	0.1602	2				

	FJ445157† FJ445158† FJ445159†										
RV-A82	DQ473509† FJ445160† JN798556† JN798585† JQ837722†	A4019-V1 B2480-V2 D2062-V1 H2794-V1 H3074-V1 I0891 V1 I3537-V1 O2029-V2	0.1192	0.1926	D0312-V1 H6350-V1 N3862-V1 N0558-V1	0.1281	0.1579	12			
RV-A85	FJ445163†	B1276-V1	0.0883	0.1381	B1276-V1	0.0824	0.1213	1			
RV-A88	DQ473504†			0.1482			0.1412		H5104-V1	0.0336	1
RV-A89	FJ445184† FJ445165† FJ445166† JQ837716† JQ837719† M16248†		0.0818	0.1148	B0408-V1 H6952-V1	0.0984	0.1141	2			
RV-A90	FJ445167†	D2300-V1 C7367-V1	0.0887	0.1623	C7367-V1 P0980-V1	0.087	0.1281	3	C6345-V1	0.0313	1
RV-A94	EF173419† FJ445185†	I3439-V2 S0801-V1	0.0708	0.1449	C1683-V1 D1138-V1 E3926-V0	0.0961	0.1556	5			
RV-A96	FJ445171†			0.1622	B2578-V1	0.0892	0.1442	1	B2578-V1	0.0334	1
RV-A100	FJ445175†	I4293-V1	0.0808	0.1452	I4293 V1	0.0595	0.1236	2			

					J1018 V1						
RV-A101	GQ415051† GQ415052† JQ245965†	A2451-V1 E1951-V1	0.0783	0.2234		0.0778	0.1643	2			
RV-A102	EF155421†	I1115-V1	0.0127	0.1295	D0928-V1	0.0137	0.1053	2			
RV-A103	JF965515† JQ747749† JQ994499		0.0682	0.1961	E3715-V1 E4303-V1 H5314-V1 H6840-V1	0.087	0.1762	4			
RV-A104	JN562727† JX074047† JX193797†	D1446-V1	0.0604	0.1256	H6868-V1 N1384-V1	0.0641	0.1121	3	D1152-V1	0.0162	1
RV-A105	JN614995† JN990699		0.0118	0.1268	A0113-V1 C2467-V1 O3499-V1	0.0405	0.1327	3	A0113-V1 A0113-V2	0.0062	2
RV-A106	JQ245971† JX025555†	D0676-V1	0.0116	0.163	C2873-V1 D0676-V1 D0984-V1 E0033-V1	0.0305	0.1785	4			
RV-A107	KC859319 GQ476621	H0120-V1	0.1557	0.1696	H0120-V1	0.0276	0.1287	1			1
RV-A108	KC859318 EU590059	C3013-V1	0.0284	0.1437	C3013-V1	0.0905	0.1238	1			
RV-A109		F0062-V1		0.1694	F0062-V1		0.1412	1			
RV-Apat3	AB549407				C2411-V1	0.0138	0.1356	1			
Untypable RV-A					B0856-V1 H6420-V1			2			

[a] GenBank rhinovirus (RV) reference sequences included in the phylogenetic and sequence analysis ; (†) indicates RV reference sequences included only in the 5'UTR phylogenetic and sequence analysis.

[b] Sequence identifier including a unique patient code and sampling time; V1 indicates sampling conducted during the first visit to the general practitioner (GP); V2 indicates follow-up sampling conducted 28 days later during the second visit to the GP; V0 indicates sampling from asymptomatic control patients. Complete VP1 sequences are given in bold.

[c] Represents the highest pairwise distance (p-distance) between rhinovirus strains assigned within the same type defined by phylogenetic clustering

[d] Represents the lowest pairwise distance (p-distance) between rhinovirus strains assigned to the nearest neighboring types, defined by phylogenetic clustering.

[e] Represents the pairwise distance (p-distance) to the nearest RV reference strain, defined by phylogenetic clustering.

Table S2. Rhinovirus B type assignment of partial VP3/VP1, VP4/VP2 and 5'UTR sequences.

RV-B type	GenBank sequences	VP3/VP1: 49 sequences			VP4/VP2: 68 sequences			VP3/VP1 and VP4/VP2	5'UTR: 4 sequences		
	Accession number	Sample identifier[a]	Intra-clade upper limit divergece[b]	Inter-clade lower limit divergence[c]	Sample indentifier[a]	Intra-clade upper limit divergence[b]	Inter-clade lower limit divergence[c]	Numberof RV typed samples	Sample identifier[a]	Divergence from nearest neighbor type[e]	Number of RV typed samples
RV-B3	EF173422 DQ473485†	E3030-V0	0.0944	0.2179	C5771-V1 H7078-V1	0.108	0.1671	3			
RV-B4	JN798573 DQ473490†	F3283-V0 H6504-V1	0.0891	0.216	F3283-V0	0.0925	0.162	2			
RV-B5	FJ445112†			0.1609			0.1311				
RV-B6	DQ473486† JQ747748 JX193795 JQ747745† JN562723 JN815243	J1186-V2	0.0936	0.125		0.0951	0.126	1			
RV-B14	X01087 L05355† K02121		0.0162	0.1875			0.1542				
RV-B17	EF173420†			0.1149			0.1054				
RV-B26	FJ445124†			0.1874	E4191-V1 N1006-V1	0.0746	0.1979	2			
RV-B27	EF173421 FJ445186†	F1588-V2	0.0856	0.1586	D1180-V1 E4373-V1	0.0694	0.126	3	D2174-V2	0.0394	1
RV-B35	DQ473487	E0425-V1	0.0734	0.1738	E0425-V1	0.0771	0.1465	1			

	FJ445187†										
RV-B37	EF173423†			0.1528	D2132-V1	0.1003	0.1285	1			
RV-B42	FJ445130† JF781507† JN562724† JF781498	D1684-V1 E0229-V1 E2568-V0	0.1002	0.1609	B3110-V2 D1684-V1 D3602-V2 E0439-V1 E1181-V1	0.0714	0.1311	7			
RV-B48	DQ473488† JN990698†	C0564-V0 F0580-V1 H2612-V1 H3172-V1	0.102	0.1474	E0845-V2	0.1234	0.126	5			
RV-B52	EF173424 FJ445188†			0.124			0.1003				
RV-B69	JN562721 JQ245970† HQ123445 FJ445151†	A1723-V2 B1038-V1	0.0933	0.1854		0.0797	0.0977	2			
RV-B70	DQ473489† JQ245974† JN990706† JX074054†	B1668-V1 B3432-V2 B2803-V0 D1768-V1 E1265-V1 E1783-V2 E3548-V0 H2193-V0 S1221-V1	0.1636	0.1149	E4457-V1	0.0977	0.1054	9	S1221-V1 C0172-V0	0.0031 0.0258	2
RV-B72	FJ445153† JN798562† JN614997†		0.0914	0.1875	A0323-V2 A0351-V1 E0887-V2	0.0959	0.1594	8			

	JQ245969 GU968948 JN562726				E0957-V1 E1924-V0 H0092-V1 H0092-V2 H0652-V1							
RV-B79	FJ445155†			0.1701			0.1311					
RV-B83	FJ445161† JN990701	D1922-V1	0.0775	0.1516	H3845-V0	0.0566	0.108	2				
RV-B84	FJ445162 JQ837723 JN614991 JN541271 JN798588 JF781499 JF781502† JX074048	A2088-V0 C2047-V1 E2385-V1 H0694-V2 H0694-V1 H3396-V1	0.0961	0.2211		0.0771	0.1825	6				
RV-B86	FJ445164†	A3291-V1	0.1242	0.2199	R1948-V1	0.0925	0.1594		2			
RV-B91	FJ445168†			0.1396			0.1054					
RV-B92	FJ445169†	A3893-V1 C2706-V0 C4567-V1 E1182-V0 J0640-V1	0.0905	0.1539	A3627-V1 C2706-V0 C4567-V1 C4637-V1 D2454-V2 E1182-V0 E3911-V2 H5076-V1 I2908-V0 R2032-V1	0.0925	0.1311	12		B3418-V2	0.0377	1
RV-B93	EF173425†			0.1586			0.126					
RV-B97	FJ445172†	B3348-V2	0.0943	0.1863	B3348-V2	0.1285	0.1491	4				

	HM366910	R1837-V0			C6052-V0 R1837-V0 S1963-V1			
RV-B99	FJ445174†			0.1874			0.1311	
RV-B100	HQ123444	H0246-V1	0.0418	0.1518			0.1183	1
RV-B101	JF781500 JX074052 JF781501†		0.0104	0.1518	O2226-V0	0.036	0.1183	1
RV-B102	JX074053†	A1373-V1 C3741-V1	0.0167	0.1533	A1373-V1 C4791-V1 C6247-V1 E4065-V1	0.0308	0.1285	5
RV-B103	JN798572 JQ994497 JN614996 JQ245972† JQ837717 JQ837721	I4727-V2	0.015	0.125	A2507-V1 E1070-V0 J1256-V1 R1934-V1	0.0206	0.126	5
RV-B104	JF781506† FJ445137†	B2662-V1 B2985-V0	0.0641	0.124	C5141 V1 I3103-V1 I3383-V1 K5107 V1 N0054 V1 N2910-V1 R0996-V1	0.0848	0.1003	9
RV-B105	EF077241	H4923-V0 H5048-V1	0.0069	0.1516	E2301-V1 H4923-V0 H5048-V1 I2977-V1	0.1003	0.108	4
RV-B106	HM366914	D2622-V1	0.0011	0.1474	D2622-V1	0.0257	0.126	

	E2217-V1	E2217-V1 S1823-V1 S1823-V2			
RV-Bpat4	AB548901	D5548-V1 O3528-V0	0.0283	0.0977	2
Untypable RV strains		Q0463-V2 H0330-V1			2

[a] GenBank rhinovirus (RV) reference sequences included in the phylogenetic and sequence analysis; (†) indicates RV reference sequences included only in the 5'UTR phylogenetic and sequence analysis.

[b] Sequence identifier including a unique patient code and sampling time; V1 indicates sampling conducted during the first visit to the general practitioner (GP); V2 indicates follow-up sampling conducted 28 days later during the second visit to the GP; V0 indicates sampling from asymptomatic control patients.

[c] Represents the highest pairwise distance (p-distance) between rhinovirus strains assigned within the same type defined by phylogenetic clustering

[d] Represents the lowest pairwise distance (p-distance) between rhinovirus strains assigned to the nearest neighboring types, defined by phylogenetic clustering.

[e] Represents the pairwise distance (p-distance) to the nearest RV reference strain, defined by phylogenetic clustering.

Table S3. Rhinovirus C type assignment of partial VP1, VP4/VP2 and 5'UTR sequences.

RV-C type	GenBank sequences	VP1: 49 sequences			VP4/VP2: 102 sequences			VP4/VP2 and VP1	5'-UTR: 10 sequences		
	Accession number	Sequence identifier[b]	Intra-clade upper limit divergence[c]	Inter-clade lower limit divergence[d]	Sequence identifier[b]	Intra-clade upper limit divergence[c]	Inter-clade lower limit divergence[d]	Number of typed samples	Sequence identifier[b]	Divergence from nearest type[e]	Number of typed samples
RV-C1	EF077279† HQ123443	D3421-V0 I1241-V1	0.0334	0.2192	A2017-V1 H6532-V1 O2142-V0	0.0886	0.1089	5			
RV-C2	EF077280† JN815248 JN837695 JN990703 JQ245968 JX025557		0.0494	0.1576	C3097-V2 I2949-V1	0.0841	0.1316	2			
RV-C3	EF186077† JN798567 JN990700	H0806-V1 H2682-V1	0.035	0.1408	E2245-V2 S1739-V1	0.0386	0.1273	4	A4145-V1	0.0158	1
RV-C4	EF582385† JF781509		0.0158	0.1284	H7134-V1 N2798-V2	0.0337	0.0932	2			
RV-C5	EF582386†			0.1592			0.1401				
RV-C6	EF582387† JF317016 JN990702† JN815245	A1010-V0 S1333-V1	0.0522	0.1322		0.0659	0.1159	2			
RV-C7	DQ875932† JN798559	A0547-V1 A0967-V1	0.0979	0.1369	A0547-V1 D0340-V1	0.0818	0.135	15	A3417-V1	0.0288	1

	JN798570 JN837689 JX025556 JQ994495	A3977-V1 D0662-V1 H2038-V1 H2332-V1 S0858-V0 P0182-V2			D5940-V1 H2038-V1 H5440-V1 N1804-V1 N2168-V1 S1067-V1 E1490-V0						
RV-C8	GQ223227† JQ245964 JQ245973†	Q0897-V1	0.1347	0.2066	E3323-V1 H4363-V0 R1164-V1 Q0897-V1	0.1364	0.1705	4			
RV-C9	GQ223228†			0.2707			0.2391				
RV-C10	GQ323774† EU935600		0	0.2006	E1629-V1 O0755-V1	0.0477	0.1295	2			
RV-C11	EU840952†	H2347-V0 H4978-V1	0.0977	0.1592	A1094-V0 H2347-V0 H7274-V2 E3771-V1 N2924-V1 R2159-V0	0.0942	0.1038	7	D5870-V1 R2159-V0	0.0557 0.0623	2
RV-C12	EF077264 HM236958 JF317017	O2603-V1	0.0264	0.1775	O2603-V1	0.0605	0.1512	1			
RV-C13	EU081795 GU294351 HM236908			0.2739	Q0855-V1 Q1163-V1	0.0304	0.1823	2			
RV-C14	EU081796 GU294362 HM236911			0.2086		0.0101	0.1494				

	EU081800 GU219984† HM236963 JF317014 JN837688†	A1331-V1 I2375-V1 J1074-V1 S2271-V1			A1331-V1 A3165-V1 D1166-V1 H2892-V2 H2920-V1 H3102-V1 J1074-V1							
RV-C15			0.0993	0.2537	J1774-V1 K1355-V1 N1328-V1 N0572-V1 N0222-V1 O2421-V1 O2029-V1 O1861-V2	0.0727	0.1443	17				
RV-C16	EU081808 HM236944			0.1506	D5171-V0	0.0304	0.1114	1				
RV-C17	EU081809 HM236936 JN815244† JN815240† JQ837720		0.0349	0.1506	H4965-V0 N2995-V0 R0646-V1	0.0591	0.1114	3	A0225-V1	0.0252	1	
RV-C18	EU590074 GU294400 HM236918			0.1841	A0309-V1	0.0604	0.108	1				
RV-C19	EU697850 EU840728†			0.288	D2818-V1 O0111-V1 O2016-V0	0.0545	0.2076	3				
RV-C20	EU697851 GQ476677			0.1376	S1305-V2	0.0304	0.0977	1				

	HM236923										
RV-C21	EU752377 GU294433 HM236903			0.1369		0.0475	0.135				
RV-C22	EU752381 JN621242† HM236905	I1017-V1	0.042	0.2294	A3473-V1	0.0583	0.1909	2			
RV-C23	EU752424 GU294358 HM236901			0.2537		0.07	0.2005				
RV-C24	EU752426 HM236939			0.2124	D5884-V2	0.0746	0.2	1			
RV-C25	EU752427 HM236952 HQ123440 JF317013† JN837685†		0.1236	0.2124	H2640-V1	0.1026	0.2	1			
RV-C26	EU752441 HM236904 JX193796	H2472-V1	0.0494	0.2484	R2368-V1	0.0295	0.1628	2			
RV-C27	GQ223122† GU294339 HM236906	I4825-V1 O2001-V1	0.0271	0.2197		0.0531	0.1128	2	H0582-V2	0.0094	1
RV-C28	GQ223134† HM236954 JN798569		0.0396	0.1841		0.029	0.1014				
RV-C29	FJ615699 GU294455 HM236949			0.1752		0.0217	0.1401				
RV-C30	GQ476669			0.1672			0.1342				

	HM236968							
RV-C31	GU294380 GU294429 HM236964			0.2066	C6486-V0	0.0338	0.1787	1
RV-C32	GU294466 HM236897 JN798581† JQ994498†		0.0223	0.1178	H1926-V2	0.0295	0.0942	1
RV-C33	GU294480 GU294428 HM236934			0.1768		0.0402	0.1377	
RV-C34	JF436926 JF519758 JF519759	H3621-V0 D1054-V1	0.0876	0.1376	A0519-V2 D1124-V1	0.0443	0.0977	4
RV-C35	EU081790 JF436925†			0.3041		0.081	0.1418	
RV-C36	EF077256 JF416311 JF416314 JF416316 JF416317 JN541267†	C0129-V2 J1970-V1 D5814-V2 I2851-V1	0.1052	0.2222	C0129-V2 E2693-V2 E4387-V1 H6490-V1 R1766-V1 Q0953-V1 Q1121-V1 S1417-V2	0.0585	0.1888	11
RV-C37	EF077260 GU294476 JF416321			0.1943	H5006-V1 J0108-V2	0.028	0.1436	2
RV-C38	GU294477 EU081791 JF416322		0.0303	0.1799	H0582-V2	0.0304	0.1038	1

	JN837691										
RV-C39	EU081799 JN205461†			0.1178	A1569-V2 F1630-V1	0.0456	0.0942	2			
RV-C40	EU081802 JF416312 JF416313 JF416315 JF781505 JN815251 JQ245963†	R2131-V0	0.0703	0.1576	R2131-V0	0.0582	0.1316	1			
RV-C41	EU081803 JF416305 JF416323 JN798565†	I0135-V1	0.0856	0.1672	A0939-V1 B0534-V2 E0481-V1	0.0864	0.0956	4	A3361-V1	0.1389	1
RV-C42	EU081805 JF416320 JQ994500†	C2299-V1 D1838-V1 F4864-V1 H2878-V1	0.0745	0.2155	I0163-V2 N3890-V1 E1013-V1	0.0818	0.1646	7	N3162-V1 C3293-V2 H2556-V1	0.0342 0.0281 0.0316	3
RV-C43	EU081807 JF416307 JF416309 JN815249 JN837687 JX074056†	H2416-V1	0.1035	0.2294	A1975-V1 C2495-V1	0.0835	0.1646	3			
RV-C44	EU590061 JF416310	A0561-V1 A3277-V1 I4714-V0 F1686-V1 F2806-V1 R1809-V0	0.0412	0.1775	O0741-V1 H5272-V2 H6546-V1	0.0435	0.1512	9			

		O0741-V1							
RV-C45	EU590064 JF416308 JN837686†		0.0334	0.2261		0.0169	0.2536		
RV-C46	GU294446 GU294447 JF416318 JF416319 FJ869950		0	0.2222		0.0145	0.2029		
RV-C47	GQ466482 JF519760 JF519761			0.2006	I2655-V1	0.0483	0.162	1	
RV-C48	JF519762 JF519763			0.1768			0.1494		
RV-C49	EU697839 JF946737 JF946738 JF907574 JN798566† JN798568	S2005-V1	0.0349	0.1284	B1220-V1	0.0557	0.0932	2	
RV-C50	FJ841957 JF316873 JQ739202			0.1458		0.0291	0.1208		
RV-C51	EU743925 JF317015† JX291115†		0.0016	0.1943		0.0371	0.1436		
RV-C52	EU590054	H1380-V1		0.2551	H1380-V1	0.0362	0.2391	1	
RV-C53	FR820909	D1880-V1		0.1458	D1880-V1 F2372-V1	0.0193	0.1208	2	
RV-C54	EU752398			0.1752	D3126-V1	0.0117	0.1401	1	

	KP282614				
RV-C55	KR997885 EU752412		0.0606	0.1472	
RV-C56	LC004772 GU214340	C0633-V1 F0888-V1 S1543-V2 S1641-V1	0.0336	0.108	4
RV-C57	KP890662 EU752358	D0102-V1 E4135-V1	0.0583	0.1013	2
RV-Cpat14	EU697852			0.1013	
RV-Cpat19	FJ598096			0.1472	
RV-Cpat20	FJ615722			0.0956	
RV-Cpat21	FJ615737			0.1888	
RV-Cpat22	FJ615745			0.1646	
RV-Cpat24	FJ869923			0.1014	
RV-Cpat30	AB550405			0.1401	
RV-Cpat31	AB628117			0.1128	

[a] GenBank rhinovirus (RV) reference sequences included in the phylogenetic and sequence analysis; (†) indicates RV reference sequences included only in the 5'UTR phylogenetic and sequence analysis.

[b] Sequence identifier including a unique patient code and sampling time; V1 indicates sampling conducted during the first visit to the general practitioner (GP); V2 indicates follow-up sampling conducted 28 days later during the second visit to the GP; V0 indicates sampling from asymptomatic control patients. Complete VP1 sequences are given in bold.

[c] Represents the highest pairwise distance (p-distance) between rhinovirus strains assigned within the same type defined by phylogenetic clustering

[d] Represents the lowest pairwise distance (p-distance) between rhinovirus strains assigned to the nearest neighboring types, defined by phylogenetic clustering.

[e] Represents the pairwise distance (p-distance) to the nearest RV reference strain, defined by phylogenetic clustering.

Rhinovirus typing based on partial VP4/VP2 sequence analysis (Figure S2)

Successful amplification and sequencing of the VP4/VP2 genome region was achieved for 423 RV-positive samples, including 253 RV-A, 68 RV-B and 102 RV-C viral strains. Phylogenetic analysis were based on approximately 437 nt of the VP4/VP2 genome region (nucleotide positions 611-1047 according to RV-A2 strain, GenBank accession no. X02316). Four sequences were classified to species taxa level based on BLASTn analyses but excluded from the phylogenetic analysis due to bad sequence quality (untypable RV strains). Two RV-A and 2 RV-C sequences were <90% complete across the analyzed VP4/VP2 region (sequence lengths ≥306 nt). The majority of VP4/VP2 sequences clustered closely with reference sequences of established types or potentially assigned types (PAT) described previously by McIntyre and colleagues[2] (Figure S2).

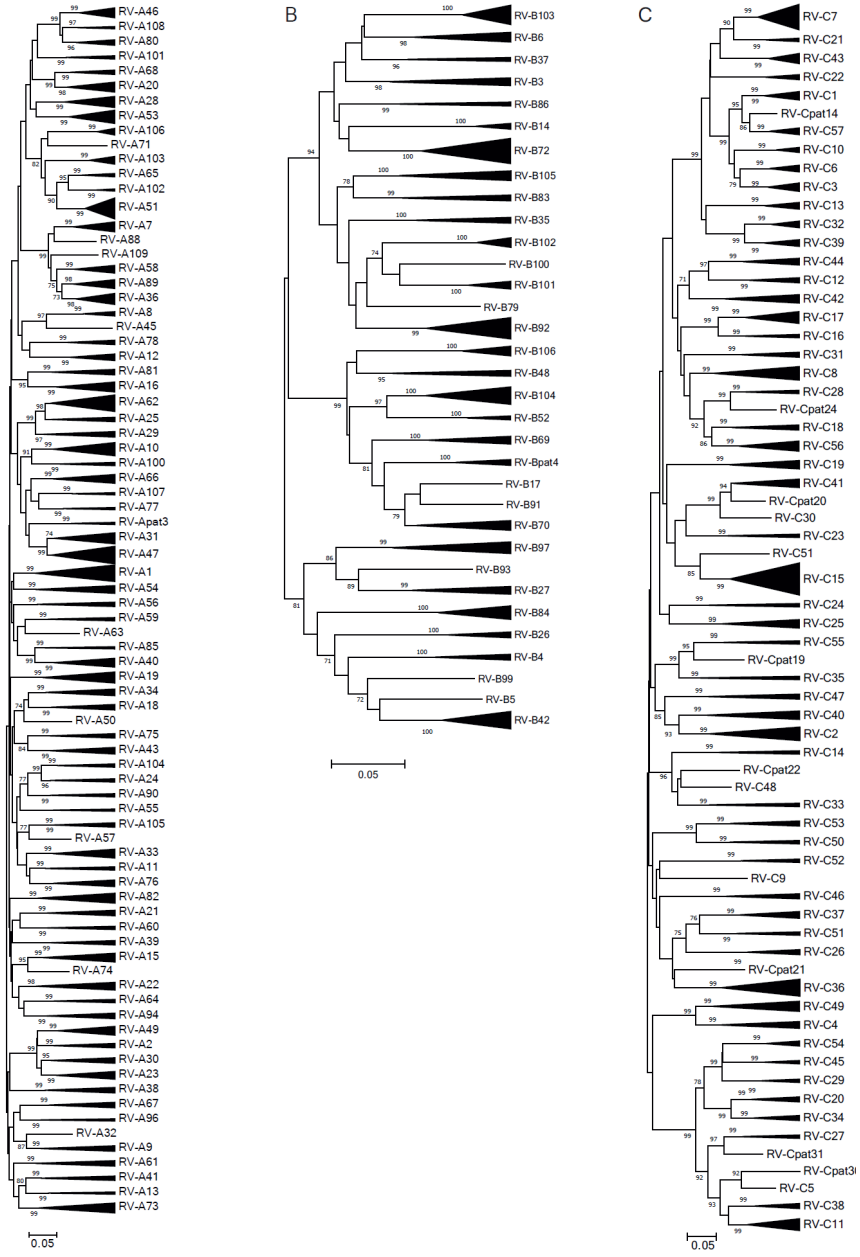


Figure S2. Phylogenetic RV-A, RV-B and RV-C trees of the VP2/VP4 region

Unrooted neighbor-joining phylogenetic trees of RV-A, RV-B and RV-C partial VP2/VP4 sequences were generated with the maximum composite likelihood model. Multiple sequences from the same type are shown as triangles with heights proportional to their numbers and depths corresponding to their earlier diverging branch.

Rhinovirus typing based on partial 5'UTR sequence analysis (Figure S3)

Amplification and sequencing of the 5'UTR was achieved for 38 RV-positive samples, including 6 samples that were typed also in the VP4/VP2 region. Phylogenetic analysis was based on a 326 nt fragment of the 5'UTR (nucleotide positions 218-542 according to RV-A2 strain, GenBank accession no. X02316) including 180 RV-A, 41 RV-B and 36 RV-C reference sequences and using *Enterovirus D* sequence type 68 (GenBank accession no. AB601882) as an outgroup. The phylogenetic dendogram inferred from the 5'UTR revealed that recombination events occur between certain RV-A and RV-C types. Although 5'UTR sequences do not reliably segregate RV-A and RV-C types[9-11], this region can be useful for predicting virus genotype and distinguishing RV from EV types. In our study the 5' UTR genotyping assay was used as a supplemental typing tool. Twenty-four 5' UTR sequences from this study clustered with RV-A, 4 with RV-B and 10 with RV-C types.

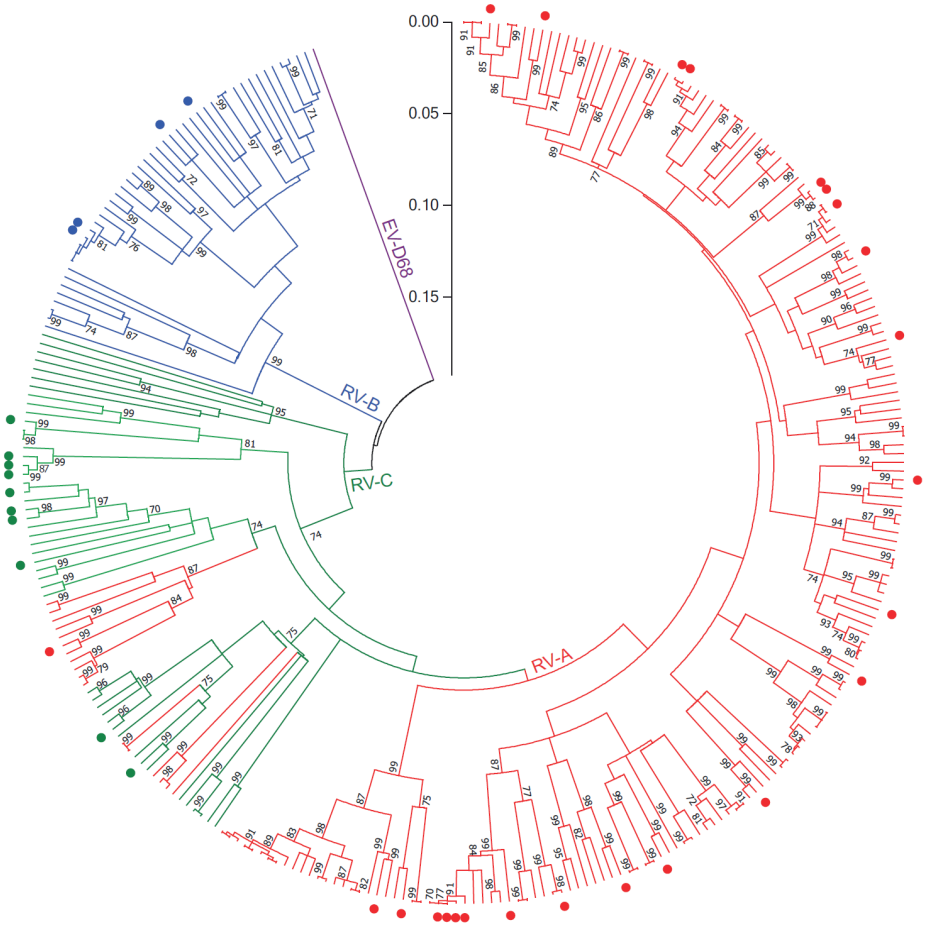


Figure S3. Phylogenetic tree of the 5' UTR region

Neighbor-joining phylogenetic tree of partial 5' UTR of 38 RV clinical isolates and 180 RV-A (red), 41 RV-B (blue) and 36 RV-C (green) GenBank sequences was generated with the maximum composite likelihood model. The tree was rooted by using EV-D68 as an outgroup. 5'-UTR sequences obtained from this study are indicated by solid circles.

REFERENCES

1. Zlateva KT, de Vries JJ, Coenjaerts FE, et al. Prolonged shedding of rhinovirus and re-infection in adults with respiratory tract illness. *The European respiratory journal* 2014; **44**(1): 169-77.
2. McIntyre CL, Knowles NJ, Simmonds P. Proposals for the classification of human rhinovirus species A, B and C into genotypically assigned types. *The Journal of general virology* 2013; **94**(Pt 8): 1791-806.
3. Liggett SB, Bochkov YA, Pappas T, et al. Genome sequences of rhinovirus C isolates from wisconsin pediatric respiratory studies. *Genome announcements* 2014; **2**(2).
4. Liggett SB, Bochkov YA, Pappas T, et al. Genome sequences of rhinovirus B isolates from wisconsin pediatric respiratory studies. *Genome announcements* 2014; **2**(2).
5. Liggett SB, Bochkov YA, Pappas T, et al. Genome sequences of rhinovirus a isolates from wisconsin pediatric respiratory studies. *Genome announcements* 2014; **2**(2).
6. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research* 2004; **32**(5): 1792-7.
7. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* 2011; **28**(10): 2731-9.
8. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic biology* 2003; **52**(5): 696-704.
9. Savolainen-Kopra C, Blomqvist S, Smura T, et al. 5' noncoding region alone does not unequivocally determine genetic type of human rhinovirus strains. *Journal of clinical microbiology* 2009; **47**(4): 1278-80.
10. McIntyre CL, Savolainen-Kopra C, Hovi T, Simmonds P. Recombination in the evolution of human rhinovirus genomes. *Archives of virology* 2013; **158**(7): 1497-515.
11. Thoelen I, Moes E, Lemey P, et al. Analysis of the serotype and genotype correlation of VP1 and the 5' noncoding region in an epidemiological survey of the human enterovirus B species. *Journal of clinical microbiology* 2004; **42**(3): 963-71.

CHAPTER 8

Discussion

Discussion

The research described in this thesis aimed to evaluate several new diagnostic approaches to viral respiratory infections. In addition, relevant patient cohorts were specifically tested for rhinovirus, including subtyping, to increase our insight in the clinical implications of rhinovirus infections, the most prevalent respiratory pathogens.

The first chapters of the thesis present findings regarding new diagnostics. mNGS can result in the detection of all pathogens in a single test and the current protocol already had very high sensitivity and specificity as compared to PCR (chapter 2). Because of the excellent negative predictive value observed in COPD patients, mNGS may be used to exclude any viral infection (chapter 3). In addition, mNGS enabled obtaining detailed information about the pathogens, antiviral resistance, virulence and typing information simultaneously (chapter 2). The advantage of the ePlex® RP assay over in-house respiratory PCR lies in the syndromic testing panel which resulted in the detection of additional pathogens and the reduced time to result, accomplishing a reduction in isolation days and improved antimicrobial and antiviral treatment (chapter 4).

The importance of a short time to result for diagnosing respiratory tract infections is shown in this thesis, and moreover during epidemics when shortage of isolation rooms is reason for concern. Although the mNGS procedure currently takes much more time than rapid syndromic assays like ePlex® RP, it is also capable to detect new pathogens, non-viral pathogens and all relevant properties of pathogens. At a certain moment in future, mNGS may even result in cost-reduction, for example in clinical scenarios where a high number of diagnostic tests are indicated. The potential to analyse expression of immunological human host genes might help to determine severity markers of viral infections, for example the upregulation of ICAM-1, the major receptor of rhinovirus, in COPD patients[1].

The importance of mNGS is also supported by the devastating 2020 SARS-CoV-2 outbreak. This new virus was discovered when a number of patients were admitted with fever and respiratory complaints to a hospital in Wuhan, China, all with an epidemiological link to the Wuhan South China Seafood City. Routine respiratory pathogens, including SARS-CoV and MERS-CoV, all tested negative. Fortunately, by means of mNGS a relevant pathogen could be identified, a novel coronavirus, with around 70% homology to SARS, that was later named SARS-CoV-2. Subsequently, PCR primers and probes became rapidly available for routine diagnostics[2,3]. Although the first known patient with atypical pneumonia of unexplained cause was reported as early as 20 December 2019, of which a bronchoalveolar lavage was collected on December 30, 2019 it was not until 8 January 2020 the first sequence results revealed a novel coronavirus[4,5]. This delay demonstrates that to benefit from the advantages of mNGS, it is essential to obtain results as soon as possible. mNGS testing in this case was only performed when routine diagnostics tested negative. Besides, it is possible other patients with this novel coronavirus, probably solitary cases, before the first recognized patient, were missed at all. Such delay in diagnosis might even have resulted in increased initial spread of the virus. The subsequent development, with spread of the SARS-CoV-2 virus over all continents and a clinical spectrum ranging from asymptomatic to life-threatening pneumonias, still suffered from initial underdiagnosis in some areas[2,5,6]. With the availability of specific primers and probes, PCR is an

adequate diagnostic tool, when applied readily, however when mNGS is used as primary diagnostic tool, all cases will be also diagnosed together with any alternative diagnosis, which is helpful to differentiate an epidemic pathogen reliably from other causes of respiratory infection.

While by NGS analysis this new virus appeared to be genetically related to SARS-CoV it is formally named called SARS-CoV-2[3]. However, the disease it causes is not SARS (severe acute respiratory syndrome) but has been named COVID-19 (corona virus disease). This split in name of the virus and its clinical picture appears confusing, as the virus is often indicated by COVID-19 virus now. It would be advisable to give a new virus a name independent of its clinical picture.

Technically, for viral diagnostics by mNGS, with only a very limited number of viral reads, ultra-deep sequencing, such as Illumina sequencing is necessary. But to be applicable in routine diagnostics the runtime must be as short as possible. In this thesis (chapter 2 and 3) Illumina NextSeq 500 sequencing is used, although several sequencing platforms are available and new platforms are being developed. The advantage of this system is the high output and deep sequencing, the disadvantage is the long runtime, a minimum of 12 hours. Newer machines, like the MiSeq have a minimum runtime of only 4 hours. Other sequencing by synthesis platforms, for example Ion Torrent sequencing, do also have a shorter runtime, however they are not real-time as single molecule sequencing by PacBio or nanopore sequencing. These real-time platforms seem promising, although the limited capacity, high error rates and higher costs limits is usefulness for viral mNGS in routine diagnostics. But with still emerging technologies a combination of deep sequencing, without many errors and with a short runtime might be possible in the near future

Other aspects of the mNGS process have been optimized as well. For example, in this thesis we used Centrifuge software for analysing the sequence data, although several pipelines are available. There are pipelines specifically for virus analyses, for example VirFind, but also total pathogen pipelines like Centrifuge, Surpi, Taxonomer and Kaiju. They can differ in several aspects, such as quality control, database used, speed of processing, output format and user-friendliness. To be applicable in clinical diagnostics a tool suitable to detect all possible pathogens is necessary, as is a tool that is fast and user-friendly. In the context of new viruses, such as SARS-Cov-2, the pipeline must be able to detect these new viruses as well, for example by assigning these reads to the family of *Coronaviridae*. This or a large proportion of unassigned reads should make the clinical microbiologist aware of a possible new virus variant. A good comparison of several pipelines regarding these aspects would be recommendable for implementation in clinical diagnostics.

Another aspect of mNGS to be considered before it will be used in routine diagnostics is the normal background, the virome, and the cut-off number of reads to be considered clinically relevant. In this thesis we have calculated sensitivity as well as specificity with several different cut-off number of reads, but the optimal number must be determined in larger groups with a variety of patients. This might even differ between patient populations, sequencing platforms and bioinformatic pipelines.

When applying mNGS the microbiome is readily available as well. Although the importance of this “bacteriome” is widely demonstrated in many biomedical fields, the virome is studied much less. With bacteriophages being an important part of the virome, it would be plausible that the virome

and bacteriome interact closely, and the virome might be of greater importance than suspected before. It appears mNGS is the ideal tool to study this interaction.

Most likely, mNGS will ultimately determine the future for viral respiratory testing. However, at this moment mNGS is not ready for primary viral respiratory testing, because the advantage of rapid results of current methods still outweighs the additional information gained using mNGS for clinical diagnostics. However, when conventional viral diagnostic tests are negative, mNGS can already be of added value by detection of potential new or unexpected pathogens.

As mentioned before, the other aim of the thesis was to determine several aspects of disease severity of rhinovirus infections. To summarize the new insights obtained, rhinovirus infections in young children cleared for cardiac surgery were not associated with more severe clinical outcome (chapter 5). On the other hand, rhinovirus viremia was associated with fatal outcome in adult stem cell transplant patients (chapter 6). In adult primary care patients, rhinovirus type A was a severity marker, as it was associated, more often than type B and C, with lower respiratory tract infections (chapter 7).

Although the causality of rhinovirus infections with regard to clinical manifestations was already demonstrated in 1978 by inoculation of RV-16 in healthy volunteers, it is still up for debate[7]. The reason is the high percentage of asymptomatic infections, and the high incidence of co-pathogens in symptomatic patients. In this thesis, the high incidence of rhinovirus infections in young children without or with only very mild upper respiratory tract complaints was demonstrated as well. Although several studies have shown an association between rhinovirus and severe disease, it is often still not considered to be a serious pathogen[8,9]. And when rhinovirus is found in patients with a pneumonia admitted to the intensive care unit, it is usually not believed to be the causative agent. mNGS might be the tool solve this long-lasting dubiety, studying not only the virus, but also the virome and the immune response of the host. These factors and their interaction might be the key in understanding the pathophysiology of rhinovirus and the divergence of symptomatology.

Rhinovirus RNA-emia was found in this thesis in adults with high viral loads in their bronchoalveolar lavages. All adults had underlying diseases, and 75% harboured co-pathogens. Another study failed to demonstrate rhinovirus in the blood of adults admitted to the hospital with a community acquired pneumonia[10]. Whether this is because of the difference in patient population (immunocompromised patients were studied in this thesis) or because of differences in the viral load remains to be determined.

In children rhinovirus-C is associated with rhinovirus viremia more often, as well as with the development of asthma and in several studies with more severe disease[10-13]. In this thesis rhinovirus-C was not associated with longer hospital admission in children undergoing cardiac surgery as compared to the other rhinovirus types. While on the contrary in adults rhinovirus-A (instead of C) was associated with respiratory infections more often and only rhinovirus-A and B were found in the viremic patients. The observed differences in pathogenicity between the different rhinovirus species may contribute to the differences in clinical presentation, although host factors will play an important role as well[14,15].

The demonstration of rhinovirus in the lower airways, the blood and even in the stool of patients, and the close relatedness with enterovirus, might suggest the virus is able to cause an ever wider variety of clinical pictures, in which mNGS might provide more insight[10,16].

The shorter duration of hospital length of stay after cardiac surgery in children with rhinovirus was a surprising result. A speculative explanation would be an immunomodulating effect of rhinovirus infections in children. Although strengthening of the immune system has been suggested previously, it is not supported by experimental evidence[17]. The exact role of the different rhinovirus species (as well as types) and the effects of the immune system need further investigation.

Future studies should expand on the performance of mNGS for respiratory infections caused by bacteria, parasites and fungi as well. Using a pan-pathogen protocol, mNGS performance can be compared to standard microbiological testing (PCR and culture) on preferably bronchoalveolar lavages.

To consolidate the findings of this thesis, to investigate the hypothesis of immunomodulation by rhinovirus, and to demonstrate a correlation between disease severity and differences in expression of receptors or inflammatory markers in the host, further investigations on the detection of rhinovirus are required. For such a study, respiratory and plasma samples may be tested with mNGS, involving the host genome as well, in order to study the viral as well as the host factors. Immunocompetent patients, with varying disease severity (from common cold to pneumonia needing hospitalisation), as well as controls without respiratory complaints should be tested. It would be interesting to compare the proportion of rhinovirus positive samples, the rhinovirus species and types, the relative abundancies in the different compartments as well as the expression of host-factors/ inflammatory markers, e.g. rhinovirus receptors.

In conclusion, for the future, when a reduction in time to results is accomplished, mNGS will likely be the primary diagnostic tool for respiratory viral infections. This is mainly due to its abilities to detect all viruses in a single test, to determine precisely all species and types (in for example rhinovirus infections), and to detect resistance and virulence markers, enabling a possible prediction of the disease severity and treatment adjustment in case of antiviral resistant strains (for example, with oseltamivir resistance in influenza cases). For these reasons, the application of this new approach will greatly expand our possibilities to deal effectively with the serious burden respiratory infections impose on mankind.

REFERENCES

1. Shukla SD, Mahmood MQ, Weston S, et al. The main rhinovirus respiratory tract adhesion site (ICAM-1) is upregulated in smokers and patients with chronic airflow limitation (CAL). *Respiratory research* 2017; **18**(1): 6-.
2. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020.
3. Gorbalenya AE, Baker SC, Baric RS, et al. Severe acute respiratory syndrome-related coronavirus: The species and its viruses – a statement of the Coronavirus Study Group. 2020: 2020.02.07.937862.
4. Ralph R, Lew J, Zeng T, et al. 2019-nCoV (Wuhan virus), a novel Coronavirus: human-to-human transmission, travel-related cases, and vaccine readiness. *Journal of infection in developing countries* 2020; **14**(1): 3-17.
5. Zhu N, Zhang D, Wang W, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med* 2020; **382**(8): 727-33.
6. Bai Y, Yao L, Wei T, et al. Presumed Asymptomatic Carrier Transmission of COVID-19. *JAMA* 2020.
7. Bush RK, Busse W, Flaherty D, Warshauer D, Dick EC, Reed CE. Effects of experimental rhinovirus 16 infection on airways and leukocyte function in normal subjects. *The Journal of allergy and clinical immunology* 1978; **61**(2): 80-7.
8. Choi SH, Huh JW, Hong SB, et al. Clinical characteristics and outcomes of severe rhinovirus-associated pneumonia identified by bronchoscopic bronchoalveolar lavage in adults: comparison with severe influenza virus-associated pneumonia. *Journal of clinical Virology* 2015; **62**: 41-7.
9. Choi SH, Hong SB, Ko GB, et al. Viral infection in patients with severe pneumonia requiring intensive care unit admission. *American journal of respiratory and critical care medicine* 2012; **186**(4): 325-32.
10. Lu X, Schneider E, Jain S, et al. Rhinovirus Viremia in Patients Hospitalized With Community-Acquired Pneumonia. *The Journal of infectious diseases* 2017; **216**(9): 1104-11.
11. Baillie VL, Moore DP, Mathunjwa A, Morailane P, Simoes EAF, Madhi SA. A prospective case-control study on the association of Rhinovirus nasopharyngeal viral load and viremia in South African children hospitalized with severe pneumonia. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2020; **125**: 104288.
12. Bergroth E, Aakula M, Elenius V, et al. Rhinovirus Type in Severe Bronchiolitis and the Development of Asthma. *The journal of allergy and clinical immunology In practice* 2020; **8**(2): 588-95.e4.
13. Erkkola R, Turunen R, Raisanen K, et al. Rhinovirus C Is Associated With Severe Wheezing and Febrile Respiratory Illness in Young Children. *The Pediatric infectious disease journal* 2019.
14. Makris S, Johnston S. Recent advances in understanding rhinovirus immunity. *F1000Research* 2018; **7**.
15. Costa LF, Queiroz DA, Lopes da Silveira H, et al. Human rhinovirus and disease severity in children. *Pediatrics* 2014; **133**(2): e312-21.
16. Khoonta P, Linsuwanon P, Posuwan N, Vongpunsawad S, Payungporn S, Poovorawan Y. Prevalence and molecular characterization of human rhinovirus in stool samples of individuals with and without acute gastroenteritis. *Journal of medical virology* 2017; **89**(5): 801-8.
17. Kirchberger S, Majdic O, Stockl J. Modulation of the immune system by human rhinoviruses. *International archives of allergy and immunology* 2007; **142**(1): 1-10.

Nederlandse samenvatting

Ontwikkelingen in de diagnostiek van virale luchtweginfecties en inzicht in de klinische implicaties van rhinovirus infecties

ALGEMENE INTRODUCTIE

Diagnose van virale luchtweginfecties

Respiratoire virussen, virussen die luchtweginfecties veroorzaken, kunnen op verschillende manieren worden gedetecteerd.

Polymerase chain reaction (PCR) is tegenwoordig de meest gebruikte detectiemethode. Hierbij wordt een stukje van het genetische materiaal van het virus vermenigvuldigd, net zo lang tot de hoeveelheid boven de detectielimiet komt. Het nadeel van deze techniek is dat je van te voren moet bepalen naar welke virussen je gaat zoeken. Je kijkt namelijk naar specifieke stukken genetisch materiaal, en het aantal virussen dat je kan detecteren in één test is beperkt. Daarnaast kan het genetisch materiaal binnen een groep virussen variëren of veranderen, waardoor het specifieke stuk genetisch materiaal waar je naar zoekt er niet meer in zit.

Metagenomic next generation sequencing (mNGS) is de nieuwste diagnostische ontwikkeling. Hiermee kan al het genetisch materiaal in één sample gedetecteerd worden. Groot voordeel is dat hierdoor naast de detectie van alle virussen in het sample (het viroom), ook additionele informatie over de virussen, zoals type, virulentie markers en gevoeligheid beschikbaar komt. Helaas zijn de kosten van deze detectiemethode hoog en is de doorlooptijd lang, alhoewel beiden snel afnemen.

Syndromale testen, zoals ePlex® RP panel, zijn andere diagnostische ontwikkelingen, waarbij de meest voorkomende verwekkers van een ziektebeeld, syndroom, tegelijk getest worden. Daarnaast hebben ze als groot voordeel dat ze een hoge doorloopsnelheid hebben.

Rhinovirussen

Rhinovirussen zijn de meest voorkomende respiratoire virussen. Er zijn sinds de ontdekking van dit virus in de jaren 50 meer dan 160 types ontdekt. Deze 160 types worden onderverdeeld in rhinovirus species A, B en C.

Rhinovirussen staan bekend als verwekker van “verkoudheid”. Het klinische spectrum van rhinovirus infecties varieert echter van asymptomatisch tot ernstige onderste luchtweginfecties. Dit verschil in klinische presentatie is waarschijnlijk multifactorieel, maar welke factoren allemaal op welke manier een rol spelen is nog onduidelijk.

DIT PROEFSCHRIFT

Het doel van het onderzoek in dit proefschrift is tweeledig, (I) evalueren van nieuwe virale diagnostiek en (II) meer inzicht krijgen in de klinische implicaties van rhinovirus infecties.

Deel I: Toepassing en toegevoegde waarde van ontwikkelingen in respiratoire virale diagnostiek

In **hoofdstuk 2** wordt een nieuw ontwikkelt mNGS protocol getest, waarbij met één voorbehandeling (in tegenstelling tot wat meestal gedaan wordt; een aparte voorbewerking voor de twee groepen virussen, RNA en DNA virussen) alle virussen in één test gedetecteerd worden. Dit protocol wordt

geëvalueerd met 24 klinische kindersamples en vergeleken met PCR. Het ontwikkelde mNGS protocol had in dit cohort een goede sensitiviteit (bij 83% van de zieke patiënten is de test positief) en specificiteit (bij 94% van de niet zieke patiënten is de test negatief).

In **hoofdstuk 3** wordt met dit nieuw ontwikkelde mNGS protocol luchtwegmateriaal van 88 COPD patiënten met longaanvallen getest en gekeken naar het viroom en naar een mogelijk verband tussen de samenstelling van het viroom en de kliniek. Ook in dit geval wordt een goede sensitiviteit (96%) en specificiteit (98%) van mNGS t.o.v. PCR aangetoond. De negatief voorspellende waarde (dat bij een negatieve testuitslag de patiënt de ziekte ook echt niet heeft) was zelfs 100%, waardoor deze test uitermate geschikt is om in één keer alle respiratoire virussen uit te sluiten. Er kan geen link gevonden worden tussen de kliniek en de samenstelling van het viroom.

In **hoofdstuk 4** wordt het belang van snelle diagnostiek onderzocht. Snelle syndromale diagnostiek (ePlex® RP panel) wordt vergeleken met PCR. Bij 64 patiënten met symptomen passend bij een luchtweginfectie werd tegelijk ePlex® RP panel en PCR diagnostiek aangevraagd. De testen waren qua sensitiviteit en specificiteit vergelijkbaar, maar de ePlex® RP panel gaf significant snellere resultaten (3,4 uur t.o.v. 27,1 uur voor PCR). Dit resulteerde in een reductie van 21 isolatiedagen en een mogelijke reductie van antimicrobiële therapie. Het syndromale testen middels de ePlex® RP panel resulteerde in 2 additionele pathogenen.

Deel II: Klinische implicaties van rhinovirus infecties

In **hoofdstuk 5** worden de gevolgen van een rhinovirus infectie bij kinderen die aan hun hart werden geopereerd beschreven. Van de 163 kinderen testten 74 kinderen (45%) rhinovirus positief ten tijde van hun operatie. In tegenstelling tot onze verwachting hadden de kinderen met een rhinovirus infectie geen verlengde opnameduur op de kinder intensive care. Ze hadden zelfs een kortere opnameduur in het ziekenhuis en hadden minder vaak een klinische verdenking op een postoperatieve luchtweginfectie.

In **hoofdstuk 6** wordt gekeken naar de aanwezigheid van het rhinovirus in het bloed (viremie) van volwassenen en wordt gekeken naar een eventuele associatie met de ziekte ernst. Van de 27 volwassen patiënten met een aangetoonde rhinovirus infectie in de longen, werd bij vier patiënten rhinovirus in het bloed gevonden. Deze rhinovirus viremie wordt geassocieerd met een hogere mortaliteit (100% versus 22% van de patiënten zonder rhinovirus viremie)

Tot slot wordt in **hoofdstuk 7** de associatie tussen de verschillende rhinovirus species en de ziekte ernst beschreven. In 566 (19%) van de 3016 mensen die hun huisarts bezochten vanwege acuut hoesten/ klachten van de onderste luchtwegen werd rhinovirus aangetoond t.o.v. 67 (4%) van de 1677 asymptomatisch controles. Rhinovirus A kwam in verhouding vaker voor bij patiënten met klachten. Daarnaast hadden patiënten met een rhinovirus A infectie over het algemeen meer last van algehele malaise, dan patiënten geïnfecteerd met een ander species.

DISCUSSIE

In de toekomst, wanneer een reductie in doorloopsnelheid van de mNGS detectiemethode is behaald, zal mNGS waarschijnlijk het primaire diagnosticum zijn voor virale respiratoire infecties. Dit komt voornamelijk doordat mNGS in één test (I) alle virussen kan detecteren, (II) alle species en types kan differentiëren (in bv geval van het rhinovirus) en (III) resistentie kan aantonen, waardoor je mogelijk de ziekte ernst van de infectie kan voorspellen en de behandeling kan aanpassen in het geval van resistentie. Hierdoor zullen we effectiever op kunnen treden tegen de hoge morbiditeit en mortaliteit van respiratoire infecties.

List of publications

List of publications

A.L. van Rijn, P.P. Roeleveld, R.B.P. de Wilde, E.W. van Zwet, M.G. Hazekamp, J. Wink. J. Calis, A.C.M. Kroes, J.J.C. de Vries
The impact of rhinovirus infections in paediatric cardiac surgery (RISK): study protocol for a prospective cohort study.
J Clin Trials 2015; 5:246

A.L. van Rijn, J.G.B. Vijverberg, C.F.E. Hoffmann, N.D. van Burgel
infectieziekten in beeld.
Tijdschr Infect 2016; 11(3):102

A.L. van Rijn, E.C. Claas, P.A. von dem Borne, A.C.M. Kroes, J.J.C. de Vries
Rhinovirus viremia in adult patients with high viral load in bronchoalveolar lavages.
J Clin Virol 2017; 96:105-109

A.L. van Rijn, R.H.T. Nijhuis, V. Bekker, G.H. Groeneveld, E. Wessels, M.C.W. Feltkamp, E.C.J. Claas
Clinical implications of rapid ePlex® respiratory pathogen panel testing compared to laboratory developed real-time PCR.
Eur J Clin Microbiol Infect Dis 2018; 37 (3):571-577

A.L. van Rijn, S. van Boheemen, I. Sidorov, E.C. Carbo, N. Pappas, H. Mei, M. Feltkamp, M. Aenerud, P. Bakke, E.C.J. Claas, T.M. Eagan, P.S. Hiemstra, A.C.M. Kroes, J.J.C. de Vries
The respiratory virome and exacerbations in patients with chronic obstructive pulmonary disease.
Plos One 2019;14 (10)

S. van Boheemen*, **A.L. van Rijn***, N.Pappas, E.C. Carbo, R.H.P. Vorderman, I. Sidorov, P.J. van 't Hof, H. Mei, E.C.J. Claas, A.C.M. Kroes, J.J.C. de Vries.
Retrospective validation of a metagenomic sequencing protocol for combined detection of RNA and DNA viruses using respiratory samples from paediatric patients,
Journal of molecular diagnostics 2020 22 (2): 196-207
*These authors contributed equally to this work

A.L. van Rijn, J.J.C. de Vries, E.C.J. Claas
Next generation sequencing in clinical virology
Chapter in: *Application and integration of Omics-Powered diagnostics in clinical and public health microbiology*, Eds. J.Moran-Gilad and Y. Grushka. New York, Springer Publishing, in press

K.T. Zlateva[*], **A.L. van Rijn**[*], P. Simmonds, F.E.J. Coenjaerts, A.M. van Loon, T.J.M. Verheij, J.J.C. de Vries, P. Little, C.C. Butler, E.W. van Zwet, H. Goossens, M. Ieven, E.C.J. Claas on behalf of the GRACE Study Group.
Molecular epidemiology and clinical impact of rhinovirus infections in adults during three epidemic seasons in 11 European countries (2007-2010)
Submitted
[*]These authors contributed equally to this work

P.P. Roeleveld[*], **A.L. van Rijn**[*], R.B.P. de Wilde, E.W. van Zwet, J. Wink, L. Rozendaal, K. Hogenbirk, M.G. Hazekamp, W.H. Man, I. Sidorov, M.E.M. Kraakman, E.C.J. Claas, E. de Jonge, A.C.M. Kroes, J.J.C. de Vries
Rhinovirus detection in the nasopharynx of children undergoing cardiac surgery is not associated with longer PICU length of stay; results of the RISK study
Submitted
[*]These authors contributed equally to this work

Curriculum vitae

Curriculum vitae

Anneloes Liesbeth Klink werd geboren op 7 maart 1984 te Lelystad, als jongste dochter van Tonny en Marianne Klink. Zij groeide met haar 3 zussen, Martine, Annemiek en Marieke op in Bergen, Noord-Holland. In 2002 behaalde zij haar diploma aan het Murmellius Gymnasium te Alkmaar. Hierna verhuisde zij naar Utrecht om daar de studie farmacie te starten. In 2003 begon zij daarnaast met de studie geneeskunde aan de Universiteit Utrecht. In 2009 behaalde ze haar bachelor farmacie en in 2010 haar artsexamen. Na haar artsexamen is zij begonnen aan de opleiding tot arts-microbioloog in het Leids Universitair Medisch Centrum (LUMC) bij opleider prof. dr. A.C.M. Kroes. Tijdens haar opleiding werd in 2014 gestart met het promotieonderzoek onder begeleiding van dr. J.J.C. de Vries, dr. E.C.J. Claas en prof dr. A.C.M. Kroes. In 2015 werd zij geregistreerd als arts-microbioloog en sindsdien is zij werkzaam als zodanig in het LUMC. Hier houdt zij zich voornamelijk bezig met de klinische bacteriologie en parasitologie. In 2015 is zij getrouwd met Lodewijk van Rijn, met wie zij 2 kinderen heeft, Florien (2016) en Pieter (2018).

Dankwoord

Dankwoord

Alhoewel het dankwoord een van de weinige onderdelen van proefschriften is die ik (met plezier) lees, hecht ik meer waarde aan efficiëntie, derhalve;

Eenieder hartelijk dank voor jullie (al dan niet wetenschappelijke) bijdrage aan de totstandkoming van dit proefschrift.

Er zijn echter een aantal mensen die ik toch persoonlijk wil bedanken:

Louis, dank voor de mogelijkheid en de vrijheid om dit proefschrift tot stand te brengen. Je kritische blik en adviezen heb ik enorm gewaardeerd.

Jutte, je vertrouwen, de enorm fijne en betrokken begeleiding en de altijd positieve woorden (ook al kwamen de stukken volledig rood terug) hebben dit proefschrift tot een goed einde gebracht.

Eric, altijd relaxed, praktisch en to the point, ik hou ervan.

En omdat ik er echt niet onderuit kom;

Mijn gezin, Lodewijk, Florien en Pieter, zonder jullie was het vast een stuk sneller gegaan, maar met jullie was het leuker.