APPENDIX 3B

SUMMARY OF SCIENTIFIC ACCOMPLISHMENTS

Viral infections: Culture models and detection techniques

Krzysztof Pyrć, Ph.D.
Faculty of Biochemistry, Biophysics and Biotechnology
Jagiellonian University

Pyrć

1. Name and Surname

Krzysztof Pyrć

2. Diplomas and scientific degrees – including title, date and title

2.1. Master of Science

- Date: 16th of June 2003.
- Title: Oxidative DNA damage in presence of quercetin and etoposide.
- Field: biotechnology, specialization: molecular biology.
- Place: Faculty of Biotechnology, Jagiellonian University in Kraków, Poland
  - promoter: Maria Kapiszewska, PhD, DSc.
- Unit: Jagiellonian University in Kraków, Poland.
- Diploma: WBT-5250/17/02/03 (**Appendix 2**).

2.1. Doctor of philosophy

- Date: 29th of June 2007r.
- Title: Virus discovery and human coronavirus NL63.
- Place: Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), University of Amsterdam, Amsterdam, The Netherlands
  - promoter: prof. dr Ben Berkhout
  - co-promoter: dr Lia van der Hoek
  - Committee members:
    - Prof. R.S. Baric, PhD (UNC, Chapel Hill, USA)
    - Prof. M. Van Ranst, PhD (UL, Leuven, Belgium)
    - Prof. W.J.M Spaan, PhD (LUMC, Leiden, Netherlands)
    - Prof. T. van der Poll, PhD (AMC UVA, Amsterdam, Netherlands)
    - Prof. J. Goudsmit, PhD (AMC UVA, Amsterdam, Netherlands)
    - R. Lutter, PhD (AMC UVA, Amsterdam, Netherlands)
    - H.L. Zaaijer, PhD (AMC UVA, Amsterdam, Netherlands).
- Unit: University of Amsterdam, Amsterdam, the Netherlands.
- Diploma: 14031 (**Appendix 2**).

According to the Act on Science and Fine Arts Degrees and Titles dated as of March 14th, 2003, chapter 2 article 24.1, the degree granted by the University of Amsterdam is equivalent to the academic degree referred to in the aforementioned Act.

Pyrć

3. INFORMATION ON EMPLOYMENT IN SCIENTIFIC UNITS.

Time period	Position	Unit
2012-until now	**Head of the Laboratory of Virology.**	**Malopolska Centre of Biotechnology, Jagiellonian University, Kraków, Poland.**
2009-2012	Scientific coordinator at Molecular Biotechnology for Health project	Faculty of Biochemistry Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland.
2008- until now	**Assistant professor**	**Department of Microbiology, Faculty of Biochemistry Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland.**
2007-2008	Assistant in research	Department of Microbiology, Faculty of Biochemistry Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland.
2003--2007	Assistant in research	Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), University of Amsterdam, Amsterdam, The Netherlands.

4. SCIENTIFIC PUBLICATIONS CONSTITUTING A SCIENTIFIC WORK SUBMITTED AS HABILITATION:

4a) Title of the work.

Viral infections – culture models and detection techniques.

4b) Scientific publications.

1. **Pyrc K#**, Stozek K, Galan W, Potempa J. (2012) HexaPrime: a novel method for detection of coronaviruses.
   Journal of Virological Methods. 188(1–2):29–36.

2. **Pyrc K#**, Stożek K, Wojcik K, Gawron K, Zeglen S, Karolak W, Wojarski J, Ochman M, Hubalewska-Mazgaj M, Bochenek G, Sanak M, Zembala M, Szczeklik A, Potempa J. (2012) Use of Sensitive, Broad-Spectrum Molecular Assays and Human Airway Epithelium Cultures for Detection of Respiratory Pathogens.
   PLoS ONE 7(3): e32582.

3. **Pyrc K#**, Strzyz P, Milewska A, Golda A, Schildgen O, Potempa J. (2011) *Porphyromonos gingivalis* enzymes enhance infection with human metapneumovirus *in vitro*.
   Journal of General Virology. 92(Pt 10):2324-32.

4. **Pyrc K#**, Milewska A, Potempa J. (2011) Development of loop-mediated isothermal amplification assay for detection of human coronavirus-NL63.
   Journal of Virological Methods. 175(1): 133-136.

5. Golda A, Malek N, Dudek B, Zeglen S, Wojarski J, Ochman M, Kucewicz E, Zembala M, Potempa J, **Pyrc K#**. (2011) Infection with human coronavirus NL63 enhances streptococcal adherence to epithelial cells.
   Journal of General Virology. 92(6):1358-68.

6. **Pyrc K**, Sims AC, Dijkman R, Jebbink M, Long C, Deming D, Donaldson E, Vabret A, Baric R, van der Hoek L, Pickles R#. (2010) Culturing the Unculturable: Human Coronavirus HKU1 Infects, Replicates, and Produces Progeny Virions in Human Ciliated Airway Epithelial Cell Cultures.
   Journal of Virology. 84(21):11255-63.

Corresponding author.

Pyrć

4.c. Discussion on the scientific aim of the work and obtained results.

In the current section the work „Viral infections: Culture models and detection techniques" is presented. Complete list of scientific achievements is presented in **Appendix 5** (Information on published scientific works and other achievements in field of didactics, scientific collaborations and science popularization).

4.c.1. Introductory note

Respiratory infections are an important cause of morbidity and mortality, with a worldwide disease burden estimated at almost 90,000,000 disability adjusted life years (DALYs) and more than 4,000,000 deaths per year[1,2]. Respiratory tract diseases are therefore one of the major causes of death both in developing and developed countries. Viral infection of respiratory tract may result in development of upper (e.g., common cold, rhinitis, sinusitis, otitis media) and lower (e.g., pneumonia, bronchopneumonia, bronchitis, bronchiolitis, tracheitis and croup) respiratory illness. The disease course depends on patient age and general health condition, but the pathogen itself is the most important determinant of the disease[3-5].

Despite social and economic importance, the data on respiratory viral pathogens is limited. This may be attributed to several factors, yet lack of appropriate infection models and appropriate detection techniques appear to be the most relevant.

In contrast to microorganisms, viruses present very narrow species and tissue specificity. Even minor differences in protein expression profiles or structure of particular proteins in host cells may directly affect viral replication. As there are numerous, not well defined factors affecting viral replication, it is essential to develop research models that facilitate viral propagation. Unfortunately, in most of the cases, existing culture models utilize cell lines permissive for certain isolates. These systems are of limited usability, due to altered cellular physiology of the model. Moreover, cell lines usually allow replication of a single viral species, what hinders comparison between pathogens[6].

In frames of this work culture models were developed for respiratory viral pathogens. These systems were proven useful for basic research, but they were also included in a complex system for identification of known and novel respiratory pathogens.

In chapter **4.c.2.** development of the first and the only model for human coronavirus HKU1 replication was described *(Human airway epithelium cultures)*. This system was utilized in research presented in chapter **4.c.3.** to study the influence of human coronaviruses on secondary bacterial infections. In chapter **4.c.4.** a model for human metapneumovirus culture in presence of bacterial enzymes is presented. Human metapneumovirus is the only human respiratory pathogen that failed to replicate on HAE cultures (unpublished data).

The second, factor limiting research in virology is lack of appropriate detection methods. As shown in chapter **4.c.5.**, universal culture system for respiratory tract infections – HAE cultures – appears to be ideal for identification and isolation of viral pathogens. Concurrently, in the same section a novel set for detection of human respiratory viruses is described.

Methods described in subsequent sections together with diagnostic system and HAE cultures constitute a complex model for identification of pathogens (**Figure 1**). Briefly, in section **4.c.6.** a new method HexaPrime for detection of coronaviral nucleic acids using isothermal amplification is described, followed by section **4.c.7.**, where the new method for identification of RNA viruses is presented.

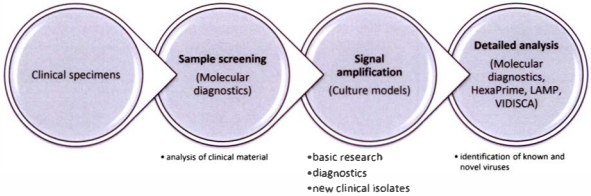

Figure 1. Model for detection, identification and characterization of human respiratory viruses (LAMP: loop-mediated isothermal amplification; VIDISCA: Virus Discovery based on cDNA-AFLP).

4.c.2. Culturing the Unculturable: Human Coronavirus HKU1 Infects, Replicates, and Produces Progeny Virions in Human Ciliated Airway Epithelial Cell Cultures. Journal of Virology. 84(21):11255-63.

Major achievements

- Development of HAE culture system, as the only model for replication of HCoV-HKU1.
- Assessment of full genomic sequence of HCoV-HKU1 Caen1 virus, able to replicate on HAE cultures.
- Evaluation of HCoV-HKU1 cellular specificity.
- Identification and characterization of HCoV-HKU1 TRS regions and subgenomic mRNA molecules.
- Determination that HLA-C does not serve as a cellular receptor for HCoV-HKU1.

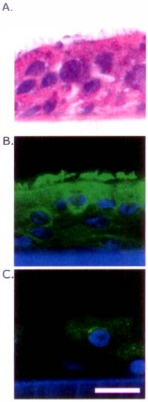

Figure 2. HAE culture architecture. A) Haematoxylin and eosin staining B) β-tubulin and nuclear staining, C) mucin 5AC and nuclear staining. Images were obtained employing fluorescence microscope at 600× magnification; Scale bar: 25mm. Data source and experimental details: *Pyrc et al.*, (2012). *PLoS ONE* 7(3): e32582.

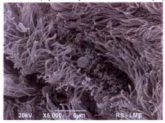

Figure 3. HAE culture architecture. Micrograph of HAE culture (electron microscopy). Source: unpublished data.

Coronaviruses are large, enveloped, single-stranded RNA viruses. In humans, these viruses cause respiratory tract infections; however, some reports suggest their potential to cause also gastrointestinal disorders. On the other hand, animal coronaviruses are described to cause diversified spectrum of diseases, including respiratory and gastrointestinal tract infections, but also other conditions as hepatitis and acute peritonitis.

Until 2003, only two human coronaviruses (HCoV-229E and HCoV-OC43) were recognized and associated with relatively mild common cold symptoms. Emergence of SARS-CoV in 2002/2003 winter season put these pathogens in the limelight and intensified efforts resulted in identification of two novel human coronaviruses – HCoV-NL63 and HCoV-HKU1[7,8]. Subsequent research studies led to identification of surprising number of animal coronaviruses[9-11]. One may question the importance of this fact for humans, though it is worth to mention that in 2012 another animal coronavirus has crossed the species border[12]. This newly emerged pathogen was named HCoV-EMC and is described to cause severe acute respiratory disease. The exact clinical characteristics of this virus are not available thus far due to limited number of cases. Extensive research on human coronaviruses

(e.g., development of anti-SARS-CoV medicinal products) was possible only because *in vitro* models were readily available. Clinical isolates of previously isolated human coronaviruses have been adapted to replicate in standard transformed cell culture; for example, SARS-CoV, ECoV-EMC and HCoV-NL63 replicate efficiently in epithelial monkey kidney cells (VeroE6 and LLC-MK2), HCoV-OC43 in BHK21 (fibroblasts, hamster) cells, and HCoV-229E in MRCS cells (fibroblasts, human)[7,13-15]. Despite the successful amplification of these human coronaviruses in cell lines, all previous attempts to culture a clinical isolate of HCoV-HKU1 have failed. In order to develop a universal system allowing HCoV-HKU1 replication human airway epithelium cultures were used[16]. This culture model is derived from freshly isolated human tracheobronchial airway epithelial cells and mirrors the morphology and physiology of the human cartilaginous airway epithelium. Following 6 to 8 weeks of cell culture growth, a fully differentiated respiratory epithelium is established, with predominant ciliated cells interspersed with goblet cells overlying a basal epithelial cell population. **Figure 2** illustrates the structure of HAE cultures and presence of certain biochemical markers. In **Figure 3** a micrograph obtained with scanning electron microscopy, clearly showing microvilli and cilia is presented.

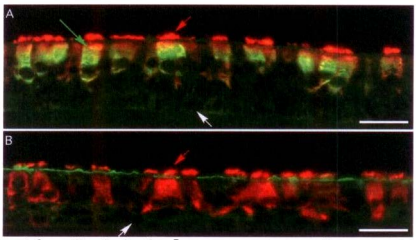

Figure 4. HCoV-HKU1 infects ciliated cells of HAE. Representative images of HCoV-HKU1 immunoreactivity in histological sections of HAE 72h post inoculation with HCoV-HKU1. Histological sections were probed with antisera directed to the virus (green) and β-tubulin (red). (**A**) HCoV-HKU1 immunoreactivity in histological sections, demonstrating that HCoV-HKU1 infects ciliated cells. (B) HCoV-HKU1-inoculated HAE probed with pre immune rabbit serum, demonstrating no HCoV-HKU1 immunoreactivity. Original magnification ×40. Scale bars, 100μm; red arrows, cilial shafts of ciliated cells; green arrow, HKU1-infected cells; white arrows, basal cells. Data source and experimental details: *Pyrc et al.*, (2010) *Journal of Virology*. 84(21):11255-63.

Conducted research showed that HAE cultures may constitute an *in vitro* model for HCoV-HKU1 virus, what enables studies on this pathogen. Subsequent results showed that the virus is able to infect ciliated cells of the respiratory tract epithelium (**Figure 4**).

One of the distinct features of the *Coronaviridae* is the synthesis of a nested set of subgenomic mRNAs (sg mRNAs) *via* a mechanism called discontinuous transcription. During this process TRS (*Transcription Regulating Sequence*) elements located upstream of each viral gene pair with the leader TRS element located at the 5' end of the genome. Due to template switching process, sg mRNAs are formed, which serve as templates for translation

of structural and accessory proteins. Employment of such replication strategy, among others, allows fine-tuned regulation of viral protein expression. On the other hand, detection of these intermediate products of RNA replication provides an ultimate proof that the virus is actively replicating[17-19]. Experiments conducted confirmed that in HAE cultures HCoV-HKU1 sg mRNA molecules are formed, kinetics of sg mRNAs formation was evaluated and its structure was resolved (**Figure 5**). Detailed discussion on obtained results is presented in the manuscript.

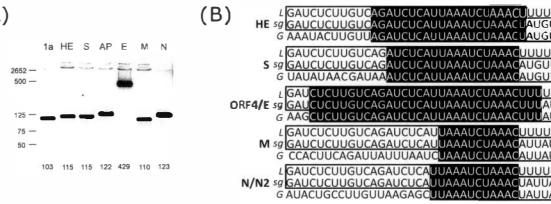

Figure 5. HCoV-HKU1 subgenomic species. (A) Subgenomic mRNA species generated during HCoV-HKU1 replication in HAE. Each lane contains RT-PCR products from total RNA isolated from HCoV-HKU1-inoculated HAE, amplified using a 5' HCoV-HKU1 leader primer and the indicated ORF-specific 3' primer. (B) Leader-body junctions of all HCoV-HKU1 sg mRNAs. Shown on the top row is the leader (L) sequence, and the bottom row shows the specific sequences upstream of the structural genes (G). The sequence in the middle (sg) represents the mature sg mRNA generated during coronavirus replication. Sequence homology between the strands near the junction is highlighted in black. 1a, open reading frame 1a replicase proteins; HE, hemagglutinin; S, spike; AP or ORF4, accessory open reading frame; E, envelope; M, membrane; N, nucleocapsid; N2, internal ORF in N. Data source and experimental details: *Pyrc et al.*, (2010) *Journal of Virology*. 84(21):11255-63.

Available literature data suggest that HCoV-HKU1 employs HLA-C molecule as cellular receptor[20]. These data were obtained using pseudovirus system on A549 cell line, though studies conducted with the virus and HAE cultures showed that this molecule is not essential for cell entry and does not serve as the receptor. At present, studies on identification of actual HCoV-HKU1 receptor are in progress.

Potential application

- Developed model is the only system that allows replication of HCoV-HKU1. Thereby data presented in the manuscript enables studies on this virus.
- Developed culture system may be used for studies on human respiratory tract viruses.
- Developed model is superior to standard in vitro culture systems: (a) primary cells are used, (b) due to the presence of numerous, differentiated cell types the system mimics *in vivo* microenvironment, (c) cultures are polarized and (d) cultures are maintained on air/liquid interphase and can be infected from the apical side (typical for respiratory viruses).
- Assessment of the full genome sequence of HCoV-HKU1 and sg mRNAs enables preparation of the molecular clone model.

Pyri

4.c.3. Infection with human coronavirus NL63 enhances streptococcal adherence to epithelial cells. Journal of General Virology. 92(6):1358-68.

Major achievements

- Assessment of the influence of HCoV-NL63 infection of HAE cultures on adhesion of *Streptococcus pneumoniae*.
- Assessment of surface expression of major receptors of *S. pneumoniae* (PAF-R, E-cadherin, TLR-2 and TLR-4) during HCoV-NL63 infection.
- Evaluation of the role of PAF-R surface expression of *S. pneumoniae* adherence.
- HCoV-NL63 infection does not affect adhesion of *Staphylococcus aureus*, *Haemaphilus influenzae*, *Pseudomonas aeruginosa*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tanerella forsythia*, *Fusobacterium nucleatum* to epithelial cells.

The concept of excessive morbidity and mortality of bacterial infection occurring during or shortly after viral infection was first formulated for influenza virus in the early 20th century[21,22]. Analysis of influenza pandemics showed that the incidence of bacterial pneumonia was increased and contributed substantially to mortality rates. Comparison of bacteriological and virological data from children hospitalized for respiratory disease shows a high degree of occurrence of viral and bacterial infections positively correlating with the severity of illness. Recent research confirmed these observations[23-27]. To illustrate the magnitude of the problem, it is worth mentioning that preceding viral infection could be detected in up to 90% of children with acute otitis media, which is the most common bacterial infectious sequelae of initial viral infection. Moreover, clinical and epidemiological data showed that *Streptococcus pneumoniae* is a leading pathogen, involved in primary or secondary community-acquired pneumonia in hospitalized children, and in more than half of all cases a secondary viral agent could be detected[28]. Although the role of a preceding viral infection in development and severity of bacterial respiratory diseases is a clinically well-documented phenomenon, the exact mechanism has not been elucidated fully.

Initially, it was proposed that respiratory viruses facilitate bacterial colonization through physical damage of the respiratory tract epithelium, with exposed basement membrane components being responsible for increased bacterial adherence[22]. Such a mechanism undoubtedly occurs for highly pathogenic viral species, but it does not explain the occurrence of increased severity of bacterial infection during and shortly after relatively mild viral infections. It was also suggested that numerous other factors, as overexpression of highly glycosylated viral proteins or alteration of a bacterial receptor expression pattern (e.g., PAF-R), may modulate bacterial adhesion and facilitate bacterial colonization. Other theories include hypersensitization of infected cells leading to enhanced immune-mediated lung damage and modulation of innate immune responses resulting in delayed clearance of bacteria[29-32].

The main goal of conducted research was to determine the influence of HCoV-NL63 infection on bacterial adhesion to airway epithelium. In order to obtain reliable data, HAE

model described in section **4.c.2**. was employed. This is of most importance, as it has been previously shown that such effects heavily depend on the culture system used[33]. Analysis of HCoV-NL63 mediated modulation of adhesion of *S. aureus*, *P. aeruginosa*, *H. influenza*, *S. pneumoniae*, *P. gingivalis*, *T. forsythia*, *F. nucleatum* and *P. intermedia* to LLC-MK2 cells revealed that only for *S. pneumoniae* the marked increase in number of adherent bacteria is increased. Similar results were obtained using fluorescent microscopy and flow cytometry on both LLC-MK2 cells and HAE cultures (**Figure 6**).

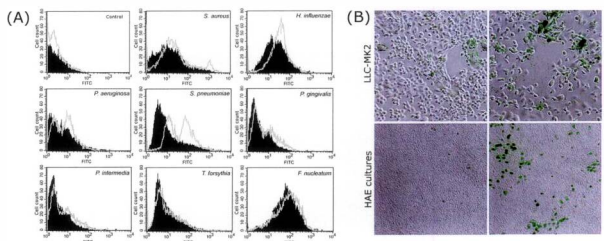

Figure 6. HCoV-NL63-mediated modulation of bacterial adherence to infected cells. (**A**) Adhesion of various bacterial species to HCoV-NL63 infected LLC-MK2 cells (grey lines) or mock-infected LLC-MK2 cells (black shapes). Data was acquired with flow cytometry and presented histograms illustrate the fluorescence intensities and corresponding numbers of FITC-labeled bacteria attached to mock-treated or virus-infected cells. (**B**) Adhesion of *S. pneumoniae* to LLC-MK2 cells and HAE cultures is increased following infection with HCoV-NL63. Fluorescence images from FITC-labeled bacteria and bright-field images were obtained using a fluorescence microscope. Magnification: ×200 (LLC-MK2); ×100 (HAE). Data source and experimental details: *Goldo et al.*, (2011) *Journal of General Virology*. 92(6):1358-68.

In order to assess the molecular mechanism of the phenomenon, surface expression of known *S. pneumoniae* receptors was evaluated. These included PAF-R (platelet activating factor receptor), E-cadherin (epithelial cadherin), Toll-like receptors type 2 (TLR-2) and type 4 (TLR-4). Conducted experiments revealed that viral infection has no effect on TLR-2, TLR-4 and E-cadherin expression, though there is a marked increase in the surface level of the PAF-R protein. PAF-R is a cell-surface G protein-coupled receptor, facilitating adherence of *S. pneumoniae* to epithelial cells by binding to phosphorylcholine, a component of the bacterial cell wall. It has been suggested previously that PAF-R is an essential factor for the development of secondary bacterial pneumonia after viral infection[34]. During rhinovirus and RSV infection, upregulation of PAF-R surface levels has been documented[33,35,36]. In an *in vivo* experiment, it was also shown that influenza virus infection in mice caused enhanced PAF-R expression in the lungs[33,37].

Identification of PAF-R as one of the markers of HCoV-NL63 infection process is therefore not surprising and one may assume that observed increase in bacterial adherence is a direct result of this alteration. However, the presence of PAF, PAF-R antagonist (ABT-491) or PAF-R-specific antibody did not affect the adherence of *S. pneumoniae* to cells pre-

infected with HCoV-NL63. This strongly suggests that increased adherence of pneumococci following HCoV-NL63 infection is not solely mediated by PAF-R, and that some other cellular receptors are involved in this interaction. Surprisingly, obtained data are also consistent with the most recent reports, as it was shown that increased adhesion of pneumococcus to influenza virus infected cells is not PAF-R dependent. Further, evaluation of PAF-R knock-out mice revealed that the *S. pneumoniae* infection process is not severely affected by absence of this receptor[38,39].

Potential application

- Obtained results may be used during development of treatment strategies for patients suffering from secondary infections.
- Employment of HAE cultures allows simulating the in vivo microenvironment and obtaining relevant results.
- Obtained data allow bettering understanding the mechanism of aggravated secondary bacterial infections. Obtained results question the widely accepted theory on PAF-R importance during and after viral infections.

4.c.4. *Porphyromonas gingivalis* enzymes enhance infection with human metapneumovirus in vitro. Journal of General Virology. 92(Pt 10):2324-32.

Major achievements

- Enzymes produced by *P. gingivalis* can activate hMPV virions.
- Activation of hMPV virions results from proteolytic processing of the F protein.
- Proteolytic enzymes produced by *S. aureus*, previously described as potent activators of the influenza virus are not active towards hMPV.

Human metapneumovirus (hMPV) is a pathogen that was first recognized in 2001 in the Netherlands[40]. HMPV infection has been associated with upper and lower respiratory illness, with 2–15 % prevalence among patients suffering from respiratory tract infections. Clinical manifestations include bronchitis, croup, pneumonia and otitis media, as well as asthma exacerbations[41-46]. Successful infection with HMPV depends on two major determinants – receptor recognition and fusion of cellular and viral membranes. These actions are carried out by the F protein, which is a trimeric, transmembrane, type I viral fusion protein. The F protein is expressed in the host cell as an inactive F_0 form, which is subsequently activated by proteolytic cleavage, probably carried out by eukaryotic endogenous proteases, such as transmembrane protease, serine 2 (TMPRSS2) and human airway trypsin-like protease (HAT). Such an event enables the virus to enter new target cells and initiate a new replication cycle[47-50].

As demonstrated previously for influenza virus, extracellular proteases may efficiently promote viral infection. For example, it has been shown that *Staphylococcus aureus* extracellular proteases may promote influenza disease progression in a mouse model and that inhibition of their proteolytic activity prevents the development of fatal pneumonia in mice[51-53]. Surprisingly, current study revealed that *S. aureus* proteases are not able to activate the hMPV virus. Howether, detailed study has shown that proteases secreted by *P. gingivalis* can activate the virus.

Periodontitis is one of the most common infectious diseases inflicting humans (estimated prevalence of 4.2 % in the human population). *P. gingivalisis* is considered to be one of the major players in the development of periodontitis, and proteolytic enzymes (gingipains) have been identified as major contributors to its pathogenicity. Arginine-specific (RgpA and RgpB) and lysine-specific (Kgp) gingipains process numerous protein targets and are responsible – directly or indirectly – for the destruction of periodontal tissues and for interference with several host processes[54].

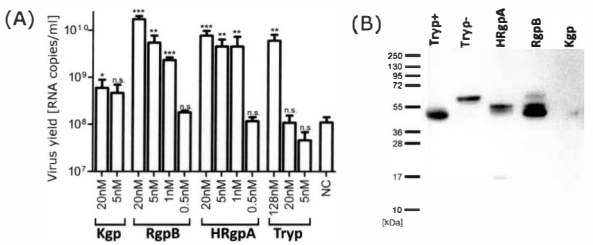

Figure 7. Gingipains, the major extracellular proteases of *P. gingivalis*, facilitate HMPV replication by proteolytic activation of the F protein. (**A**) The influence of gingipains at various concentrations on the replication of HMPV was assessed. HMPV replication in the presence of HRgpA or RgpB was similar to that observed in the presence of trypsin, whilst the lysine-specific Kgp had a significantly lower effect. Data are presented as HMPV RNA copies/ml (means±SD). (**B**) Proteolytic processing of the HMPV F protein visualized by Western blotting. Kgp, virus cultured in the presence of lysine-specific gingipain; RgpA and HRgpA, virus cultured in the presence of arginine-specific gingipains;+Tryp, virus cultured in the presence of trypsin; negative control (-Tryp), virus cultured in the absence of trypsin; NC, Negative control (protease-free medium). Data source and experimental details: *Pyrc et al.*, (2011) *Journal of General Virology*. 92(Pt 10):2324-32.

In the current work authors showed that proteases secreted by *P. gingivalis* are able to activate the F protein by proteolytic processing. Most prominent activity was observed for HRgpA and RgpB enzymes. Activation of HMPV virions by gingipains HRgpA and RgpB is consistent with the substrate specificity of these enzymes. As with trypsin, HRgpA and RgpB cleave substrates after arginine at the P1 position, whilst the canonical cleavage site for hMPV is RQSR↓FVLG. Surprisingly, incubation with Kgp also resulted in a slight increase in virus yield. Kgp recognizes and hydrolyses peptide bonds after lysine at the P1 position, which is inconsistent with the canonical activation cleavage site for HMPV. Thus, it can be assumed that Kgp targets an alternative cleavage site, resulting in the generation of partially defective virus particles. These observations are consistent with the results obtained by Western blotting (partial degradation) and low activity of viruses treated with Kgp (**Figure 7**).

The clinical relevance of our findings is highlighted by the fact that *P. gingivalis* and increased levels of trypsin-like proteases are found in the saliva of patients with periodontal disease[55-59]. One may assume that high prevalence of periodontitis combined with clinical correlation between dental hygiene and the prevalence of influenza and influenza-like illnesses, and increased levels of proteases may influence the hMPV-associated disease[60-63].

Potential application

- An alternative system for hMPV replication has been developed. Concentrations of HRgpA and RgpB required for activation of hMPV are significantly lower compared to trypsin.
- For assessment of the role of bacterial proteases for respiratory tract infections a model pathogen was used (hMPV). Conducted research showed a possible mechanism for increased prevalence and severity of viral infections in patients with periodontitis. This observation may be useful for development of novel treatment strategies.

4.c.5. Use of Sensitive, Broad-Spectrum Molecular Assays and Human Airway Epithelium Cultures for Detection of Respiratory Pathogens. PLoS ONE 7(3): e32582.

Major achievements

- A set of diagnostic tools was developed for use in patients with respiratory disease.
- A model for pre-amplification of infectious respiratory viruses using HAE cultures was developed.
- Combination of molecular detection methods with tissue cultures enables identification of viral pathogens in otherwise negative samples.

Virus detection and typing is a tedious task because the presence of multiple viral species and strains blurs the image, and infection with a wide range of pathogens may result in a similar clinical outcome. Numerous clinical virology laboratories continue to employ diagnostic algorithms, which incorporate antigen or culture-based methods. Indisputably, the most sensitive and specific methods for detection of respiratory viruses are molecular techniques. Molecular methods may be roughly divided into (a) sensitive and specific assays such as real-time PCR or loop-mediated isothermal amplification (LAMP), and (b) techniques with broad specificity that are capable of detecting a wide variety of pathogens. The latter group includes sequence-independent methods (e.g., VIDISCA, SISPA, differential display). Some previously developed methods (e.g., universal primers) are designed to detect a broad range of targets and combine some advantages of these two approaches.

In the current work development of new universal primer set for detection of respiratory viruses was conducted. Developed assays allow identification of alpha and beta coronaviruses, RSV, influenza A and B, parainfluenza types 1, 2 and 3, human bocavirus, human adenoviruses, human metapneumovirus and picornaviruses in clinical samples (**Figure 8**).

Panel of assays was designed based on complete GenBank information, and developed primers are complementary to 95–100% of the sequences of archival and contemporary viral strains. Such an approach limits the influence of intra–species sequence variability and facilitates the detection of contemporary and emerging strains. The developed diagnostic set is characterized by high sensitivity (1–10 copies per reaction) and can be used in a routine diagnostic laboratory. The obtained results clearly show that the evaluated tests are highly specific and sensitive. Thorough testing showed that these primer sets do not cross react with other respiratory pathogens and are able to detect viruses in a wide range of clinical samples.

Pyr

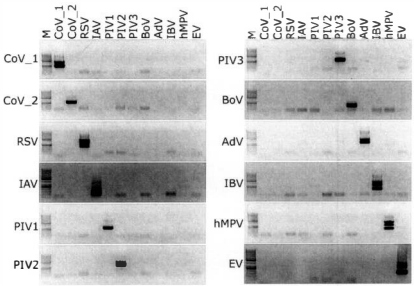

Figure 8. Specificity of the developed assays. All primer pairs (names on top of the figure) were used to amplify all virus stocks included in the study (names on the left side of each panel). M: size marker; CoV_1: HCoV-NL63, CoV_2: HCoV-HKU1; RSV: respiratory syncytial virus; IAV: influenza A virus; PIV: parainfluenza virus type 1, 2 or 3; BoV: bocavirus; AdV: adenovirus type 4; IBV: influenza B virus; hMPV: human metapneumovirus; EV: echovirus 9. Analysis was performed on 1.5% agarose gel. Data source and experimental details: *Pyrć et al.*, (2012). *PLoS ONE* 7(3): e32582.

In the current study, detection of viral pathogens was possible in ~76% of clinical samples, leaving ~24% of cases undiagnosed. Such a detection rate is consistent with that in previously published reports, as it is estimated that no etiological agent can be identified in ~30% of patients suffering from respiratory tract diseases[64-66]. Previous studies hypothesized that this might be due to the presence of as yet unknown viruses or imperfections of existing diagnostic tools. To test whether it was possible to type the infectious agents in the remaining samples, developed molecular assays were coupled fully differentiated human airway epithelium cultures. Pre-amplification of the infectious material in cell culture may result in enhanced detection of viruses in clinical samples, even those containing PCR inhibitors or with low virus load. Conducted analysis resulted in the detection of viral agent(s) in 100% of cases (**Figure 9**). This observation is compliant with the fact that the HAE model is superior to any other culture system in terms of amplifying respiratory viruses.

Developed model allows two-stage analysis of infected samples. In the first stage samples are screened for the presence of known pathogens using molecular assays, while in stage 2 selected samples are used as an inoculum for HAE cultures and further analyzed using more sophisticated methods as HexaPrime or VIDISCA.

Pyrć

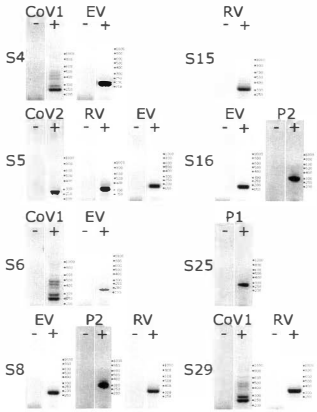

Figure 9. Pre-amplification on HAE cultures improves detection of viral pathogens. RT-PCR analysis of clinical specimens tested negatively for all known pathogens (denoted as "-") and pre-amplified on HAE cultures (denoted as "+"). "S" denotes the sample number. CoV_1: alphacoronaviruses; P1: parainfluenza virus type 1; P2: parainfluenza virus type 2; EV: enteroviruses; RV: rhinoviruses. Analysis was performed on 1.5% agarose gel. Data source and experimental details: *Pyrc et al.*, (2012). *PLoS ONE 7*(3): e32582.

Potential application

- Developed assays may be used for detection of respiratory viruses in clinical material.
- Combination of HAE cultures and molecular detection methods potentially allow identification of novel pathogens.
- Combination of HAE cultures and molecular detection methods allow identification of pathogens in samples containing residual amounts of infectious material.
- Obtained results confirm that HAE model may be used as universal system for respiratory viruses.

4.c.6. Development of loop-mediated isothermal amplification assay for detection of human coronavirus NL63. Journal of Virological Methods. 175(1): 133-136.

Major achievements

- A novel detection system for HCoV-NL63 based on isothermal amplification of nucleic acids was developed.
- Developed assay was validated in laboratory conditions.

The loop-mediated isothermal amplification (LAMP) is a relatively new method for amplification of nucleic acids. It is based on the principle of the strand displacement reaction, which occurs under isothermal conditions. Such amplification is possible due to employment of polymerase that except for normal activity is also able to carry on the strand displacement process; therefore no thermal DNA helix denaturation is required. Due to such approach no thermal cycler is needed and the whole procedure may be carried over using a

water bath or a laboratory incubator. Furthermore, the reverse transcription reaction may be conducted in the same experimental vessel.

For LAMP amplification a set of 6 primers is being used. In contrast to a standard PCR reaction, specific primers anneal to the viral template only in the beginning of the reaction and in subsequent stages 5' and 3' hairpin elements are mostly used for amplification (these structures are introduced on synthetic primers). Such amplification results in generation of complex, cauliflower-like DNA structures forming typical DNA ladder visible during gel analysis of reaction products (see also **Figure 10**).

In current work development and optimization of single-step LAMP reaction for detection of HCoV-NL63 is described. In the study the Bsm polymerase was used. This enzyme is encoded in the genome of *Bacillus smithii*, and catalyze 5' → 3' DNA synthesis. It lacks the 5' → 3' and 3' → 5' exonuclease activity, though it is able to carry on the strand displacement reaction. Developed assay (described in detail in the manuscript) appear to be highly sensitive (~1 copy of viral RNA per reaction) (**Figure 10**). Furthermore, the complete reaction is carried over in a single tube (reverse transcription and amplification) and is time and cost effective (60 minutes, 7 times lower cost compared to PCR detection). Method optimization showed that it is specific towards HCoV-NL63 (no cross reactivity detected). It also allows amplification of viral genetic material from different clinical materials (**Figure 10**).

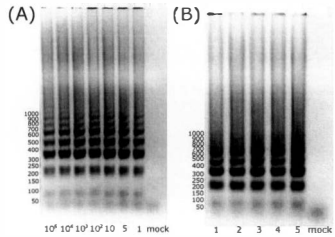

Figure 10. LAMP amplification. (**A**) Sensitivity of the HCoV-NL63 LAMP assay. The assay was performed with serial dilutions of viral RNA (10^5, 10^4, 10^3, 10^2, 10, 5, and 1 copy per reaction). Mock: negative control from mock-infected LLC-MK2 cells. (**B**) Detection of HCoV-NL63 in clinical specimens with the LAMP assay. 1: Cell culture supernatant; 2: bronchoalveolar lavage; 3: nose wash; 4: sputum; 5: human sera; Mock: negative control from mock-infected LLC-MK2 cells. Data source and experimental details: *Pyrc et al.*, (2011) *Journal of Virological Methods*. 175(1): 133-136.

Potential application

- Developed assay may be used for virus detection in clinical material and for screening of clinical samples.
- Developed assay can be carried on in field conditions, where no infrastructure is available.

Pyrć

4.c.7. HexaPrime: a novel method for detection of coronaviruses. Journal of Virological Methods. 188(1–2):29–36.

Major achievements

- A novel method for identification of RNA viruses was developed.
- The method is characterized by broad specificity due to employment of short conserved regions as primer attachment sites within the viral genome.
- HexaPrime software was developed to support identification of conserved sites in viral genomes, which may be used for amplification.
- Method was optimized on members of *Coronoviridoe* family.

As described in section **4.c.5.**, identification of known and novel viruses is a tedious task due to high variability. In section **4.c.5.** a new set of molecular assays for detection of human respiratory viruses is described. Further, a system for universal pre-amplification of the infectious material that allows analysis of otherwise negative samples is presented. In the current work a novel method for identification of RNA viruses in infectious material, which may be combined with HAE cultures pre-amplification system.

The HexaPrime method combines to some extent the selectivity of the universal primer approach (e.g., selectivity, simplicity) with the broad specificity of sequence-independent methods. The method uses short 6–8 nt conserved regions in viral genomes as primer attachment sites. This is the main advantage of the method, compared to standard PCR, where primers are 18 – 25 nt long and therefore longer conserved sites are required. Identification of such long, conserved sites, in some cases may not be possible. The method therefore facilitates the design and development of broad-spectrum assays, and increases the chance of identification of the novel pathogen. Development of the HexaPrime method was possible as primer attachment is carried on at lower temperatures during first and second strand synthesis.

The method relies on the design of synthetic oligonucleotides that comprise short (6-8 nt) elements that recognize conserved regions of viral nucleic acids and longer (16–18 nt) anchor elements. These oligonucleotides are then added to RNA fragments termini during first- and second-strand DNA synthesis. The anchoring regions are subsequently used for the first and nested PCR amplification of viral nucleic acids. In the nested PCR, an additional nucleotide is added on the 3' terminus of each primer, which increases selectivity of amplification. Agarose gel analysis and sequencing of the product allow identification of the virus contained in the original sample. Flow chart for the method is presented in **Figure 11**.

The use of short (6–8 nt) conserved regions to recognize target sequences facilitates the design and development of broad-spectrum assays, and therefore increases the applicability of the method. Although the sensitivity of the method is limited, and thus is mostly suitable for the detection of cultivable pathogens, its potential for recognizing unknown viral pathogens remain unquestionable.

Pyrć

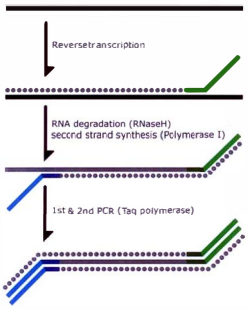

Figure 11. The HexaPrime assay. Reverse transcription (RT) and second-strand (SS) synthesis reactions were conducted on total RNA (black) in the presence of primers comprising short (6–8 nt) elements that recognize conserved regions of viral nucleic acids (dark green and dark blue for RT and SS primers, respectively) and longer (16–18 nt) anchors that serve as a template for subsequent PCR amplification (light green and light blue for RT and SS primers, respectively). Products of the PCR amplification of coronaviral RNA may be analyzed further by gel electrophoresis and sequencing. Data source and experimental details: *Pyrc et al.*, (2012) *Journal of Virological Methods*. 188(1–2):29–36.

The method was optimized for members of the *Coronaviridae* family. Genetic analysis was carried on using the complete set of sequence data available. Inspection of viral genomes for the presence of short, conserved sites was performed with three independent approaches: (a) Bioedit. Conserved sequences were identified based on the automatic conserved site finder feature of this software; (b) HexaPrime software developed for the current study; (c) Manual analysis. All sequences were further inspected by eye and conserved sequences were marked.

The HexaPrime software for the identification of conserved sites in multiple viral genomes was prepared in the Java environment and it is aimed to identify conserved regions regardless the flanking sequences (i.e., it is possible to identify short, highly conserved RNA stretch in the region forming secondary RNA structures). Before the HexaPrime analysis, the collection of all sequences of interest in the FASTA format is prepared. During analysis each sequence is divided into overlapping words of desired length. Subsequently, these words are placed into the matrix. Matrices are formed for each sequence and elements that are not present in every sequence are discarded. Resulting common elements are further sorted and each word is assigned with parameters describing e.g., its location in each original sequence. Further, words pairs (separated by a given number of nucleotides) are identified. To make an example, if the distance was set to 100-250 by the user, words that do not have a partner in such neighborhood are discarded. In such a manner a list of words is being formed, which represent sites within the genome that may serve as primer attachment sites. Using aforementioned methods it was possible to prepare a list of primers, which should be able to amplify RNA of each member of the *Coronaviridae* family. All stages of the process were carefully optimized and a single set was selected. Results of HexaPrime amplification are presented in **Figure 12**.

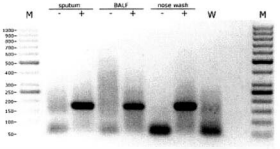

Figure 12. Detection of HCoV-NL63 in different types of clinical samples. Different sample types (sputum, bronchoalveolar fluid (BALF) and nose wash) were spiked with HCoV-NL63. No inhibition of HexaPrime reactions and no additional bands were observed. W: water; – and + signs: samples not inoculated or inoculated with HCoV-NL63. Data source and experimental details: *Pyrc et al.*, (2012) *Journal of Virological Methods*. 188(1–2):29–36.

The developed method was evaluated for its specificity and sensitivity, also in different types of clinical material (**Figure 12**).

Potential application

- HexaPrime method may be used for identification of novel RNA viruses.
- Application of the HexaPrime method in combination with a diagnostic set and HAE culture pre-amplification system allows identification of (potentially) novel viruses in clinical material.
- Developed method may be adjusted for detection of virtually all RNA viruses.

5. OTHER SCIENTIFIC ACHIEVEMENTS.

In this part scientific achievements not included in the habilitation theses are presented. Conducted research focused on respiratory pathogens and different aspects of conducted studies are presented in separate chapters.

5.1. Development of the VIDISCA method, identification and characterization of the novel human coronavirus NL63.

To date, there are still a variety of human infections with unknown etiology. Identification of previously unrecognized viral agents in patient samples is of great medical interest, but remains a major technical challenge. Virus discovery based on cDNA-AFLP (VIDISCA) is a novel approach that provides a fast and effective tool for amplification of unknown nucleic acid species, e.g. of human viruses. The VIDISCA method is based on double restriction enzyme processing of a target sequence and ligation of oligonucleotide adaptors that subsequently serve as priming sites for amplification. As the method is based on the presence of restriction sites, it results in the generation of reproducible, specific amplification patterns. The method allows amplification and identification of viral RNA / DNA, with a lower cutoff value of 10^5 copies/ml for DNA viruses and 10^6 copies/ml for the RNA viruses. With this technique human coronavirus NL63 was discovered, as well as several known viruses that were not detectable using available diagnostic assays

Relevant publications

1. van der Hoek L, **Pyrć K**, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B. (**2004**) Identification of a new human coronavirus. Nature Medicine. 10(4):368-73.
2. **Pyrć K**, Jebbink MF, Berkhout B, van der Hoek L. (**2008**) Detection of new viruses by VIDISCA: virus discovery based on cDNA-amplified fragment length polymorphism. SARS-and Other Coronaviruses, Laboratory Protocols; Methods in Molecular Biology, Vol. 454, Ed. Cavanagh, Dave, ISBN: 978-1-58829-867-6.
3. de Vries M, **Pyrć K**, Berkhout R, Vermeulen-Oost W, Dijkman R, Jebbink MF, Bruisten S, Berkhout B, van der Hoek L. (**2008**) Human parechovirus type 1, 3, 4, 5 and 6 detection in picornavirus cultures. Journal of Clinical Microbiology. 46(2):759-62.
4. **Pyrć K**, Berkhout B, van der Hoek L. (**2007**) Identification of new human coronaviruses. Expert Review of Anti-infective Therapy. 5(2):245-253.
5. **Pyrć K**, Berkhout B, and van der Hoek L. (**2007**) The novel human coronaviruses NL63 and HKU1. Journal of Virology. 81(7):3051-7.
6. van der Hoek L, **Pyrć K**, Berkhout B. (**2006**) Human coronavirus NL63, a new respiratory virus. FEMS Microbiology Reviews. 30(5): 760-773.
7. van der Hoek L, **Pyrć K**, Berkhout B. (**2006**) Coronavirus NL63, the new Dutch. Tijdschrift voor Infectieziekten. 1(3):108-114.

5.2. Molecular characterization of human coronavirus NL63

Genomic analysis of HCoV-NL63 revealed presence of following genes: 1ab-S-ORF3-E-M-N, which are transcribed into sub-genomic mRNAs. Inspection of the genome sequence indicates that all open reading frames have functional transcription regulating sequence (TRS) elements, with the exception of the E gene. The TRS of the E gene possesses a defective core sequence that may result in decreased production of E mRNA. Furthermore, *in silico* analysis revealed some unique features, as unique substrate specificity of the main proteinase.

Genomic analysis of HCoV-NL63 revealed that the RNA genome composition is very low in the C nucleotide and high in U, which is also reflected in the codon usage. Inspection of the nucleotide composition along the genome indicates that this bias in nucleotide count is not random and may suggest importance of this phenomenon in virus evolution. Investigation of the HCoV-NL63 genome variability led to the identification of two genetically distinct lineages that apparently have recombined, resulting in a mosaic genome structure in many HCoV-NL63 isolates. Molecular clock analysis revealed also that this virus have appeared a separate species already in the 11th century.

Relevant publications

1. **Pyrć K**, Dijkman R, Deng L, Jebbink MF, Ross HA, Berkhout B van der Hoek L. (**2006**) Mosaic structure of human coronavirus NL63, one thousand years of evolution. Journal of Molecular Biology. 364(5):964-73.
2. **Pyrć K**, Berkhout B, van der Hoek L. (**2005**) Molecular characterization of human coronavirus NL63. Recent Research in Infection and Immunity, Transworld Research Network. 3:25-48 ISBN: 81-7895-182-7.
3. **Pyrć K**, Jebbink MF, Berkhout B, van der Hoek L. (**2004**) Genome structure and transcriptional regulation of human coronavirus NL63. Virology Journal. 1(1):7.

5.3. Clinical characterization of human coronavirus NL63

Clinical studies on HCoV-NL63 provided important information on this pathogen. This virus is observed mostly in young children, elderly persons and immunocompromised patients with upper and lower acute respiratory tract disease (1-10% of all respiratory infections). Further, it has been shown that the virus is the major cause of croup in children. Additionally, virtually all sera from persons of 8 years and older are seropositive for HCoV-NL63, suggesting that infection of humans is very common and usually acquired during childhood.

Relevant publications

1. Dijkman R, Jebbink MF, El Idrissi NB, **Pyrć K**, Muller MA, Kuijpers TW, Zaaijer HL, van der Hoek L. (**2008**) Human coronavirus NL63 and 229E seroconversion in children. Journal of Clinical Microbiology. 46(7):2368-73.
2. van der Hoek L, Sure K, Ihorst G, Stang A, **Pyrć K**, Jebbink MF, Petersen G, Forster J, Berkhout B, Uberla K. (**2006**) Human coronavirus NL63 infection is associated with croup. Advances in experimental medicine and biology. 581:485-91.
3. **Pyrć K.** (**2008**) Ludzki koronawirus NL63 - Obraz kliniczny i charakterystyka molekularna. Postępy Mikrobiologii. 47, 3, 257-262.
4. **Pyrć K**, van der Hoek L. (**2007**) Human Coronavirus NL63, a Long Lost Brother, Coronaviruses:

Molecular and Cellular Biology. ISBN: 978-1-904455-16-5.

5. van der Hoek L, Sure K, Ihorst G, Stang A, **Pyrć K**, Jebbink MF, Petersen G, Forster J, Berkhout B, Uberla K. (**2005**) Croup Is Associated with the Novel Coronavirus NL63. PLoS Medicine. 2(8):e240.
6. Moes E, Vijgen L, Keyaerts E, Zlateva K, Li S, Maes P, **Pyrć K**, Berkhout B, van der Hoek L, Van Ranst M. (**2005**) A novel pancoronavirus RT-PCR assay: frequent detection of human coronavirus NL63 in children hospitalized with respiratory tract infections in Belgium. BMC infectious diseases. 5(1):6.

5.4. Virus – receptor interaction

The presence of cellular receptors is one of the determinants of cell, tissue and species specificity of the certain virus. It was generally thought that all alpha coronaviruses use CD13 (also known as aminopeptidase N) as receptor because all representatives (HCoV-229E, porcine, feline and canine coronaviruses) engage this molecule for cell entry. However, HCoV-NL63 can infect cells that are not susceptible for the closely related HCoV-229E, suggesting that HCoV-NL63 binds to a different surface molecule. An analysis of receptor engagement revealed that HCoV-NL63 uses angiotensin converting enzyme 2 (ACE2) for cell entry, the receptor that is used by SARS-CoV. ACE2 plays a protective role during lung damage and it has been suggested that the high pathogenicity of SARS-CoV is actually caused by down regulation of ACE2 during infection. However, HCoV-NL63 infection also results in a decrease of ACE2 protein. This result illustrates that engagement of the ACE2 molecule as receptor, as well as its down regulation during infection, do not necessarily lead to the development of severe lung damage as observed during SARS-CoV infection.

Relevant publications

1. Dijkman R, Jebbink MF, Deijs M, Milewska A, **Pyrć K**, Buelow E, van der Bijl A, van der Hoek L. (**2012**) Replication dependent downregulation of cellular ACE2 protein expression by Human Coronavirus NL63. Journal of General Virology. 93(Pt 9):1924-9.
2. Pohlmann S, Gramberg T, Wegele A, **Pyrć K**, van der Hoek L, Berkhout B, Hofmann H. (**2006**) Interaction between the spike protein of human coronavirus NL63 and its cellular receptor ACE2. Advances in experimental medicine and biology. 581:281-4.
3. Hofmann H, Marzi A, Gramberg T, Geier M, **Pyrć K**, van der Hoek L, Berkhout B, Pohlmann S. (**2006**) Attachment factor and receptor engagement of SARS coronavirus and human coronavirus NL63. Advances in experimental medicine and biology.
4. Schildgen O, Jebbink MF, de Vries M, **Pyrć K**, Dijkman R, Simon A, Muller A, Kupfer B, van der Hoek L. (**2006**) Identification of cell lines permissive for human coronavirus NL63. Journal of Virological Methods. 138(1-2):207-10.
5. Hofmann H, **Pyrć K**, van der Hoek L, Geier M, Berkhout B, Pohlmann S. (**2005**) Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. PNAS Proceedings of the National Academy of Sciences, USA. 102(22):7988-93.

Pyrć

5.5. Interaction between viral and cellular proteins.

Studies on the interaction between viral and cellular proteins were aimed to identify and describe mechanisms important during infection.

Relevant publications

1. Milewska A, **Pyrć K**. (**2012**) Human RIG-1/MdaS and TLR3 signaling pathways and their role in antiviral response during mucosal infections, Recent Research Developments in Virology, Transworld Research Network. 8:71-96, ISBN: 978-81-7895-563-6.
2. Dijkman R, Jebbink MF, Wilbrink B, **Pyrć K**, Zaaijer HL, Minor PD, Franklin S, Berkhout B, Thiel V, van der Hoek L. (**2006**) Human coronavirus 229E encodes a single ORF4 protein between the spike and the envelope genes. Virology Journal. 3(1):106.
3. Piotrowski Y, van der Hoek L, **Pyrć K**, Berkhout B, Moll R, Hiigenfeld R. (**2006**) Nonstructural proteins of human coronavirus NL63. Advances in experimental medicine and biology. 581:97-100.

5.6. Identification of novel inhibitors of viruses infecting human respiratory tract.

HCoV-NL63 infections are frequently involved in hospitalizations of patients with respiratory tract illness. Therefore, there is a need for an antiviral therapy to prevent/cure the disease or a vaccine to prevent new infections. Several existing antiviral drugs and small molecules were evaluated as inhibitors of HCoV-NL63 and some potent inhibitors were identified. These include intravenous immunoglobulins, heptad repeat 2 peptide, small interfering RNAs, β-d-N4-hydroxycytidine, 6-azauridine. Further, synthesis and evaluation of novel polymeric inhibitors was described.

Relevant publications

1. Milewska A, Ciejka J, Kamiński K, Karewicz A, Bielska D, Żegleri S, Karolak W, Nowakowska M, Potempa J, Bosch BJ, **Pyrć K**, Szczubiałka K. (**2013**) Novel polymeric inhibitors of HCoV-NL63. Antiviral Research. 97(2):112-21.
2. Golda A, **Pyrć K**. (**2008**) Recent antiviral strategies against human coronavirus– related respiratory illness. Current Opinion in Pulmonary Medicine. 14(3):248-53.
3. Pyrć K, Berkhout B, van der Hoek L. (**2007**) Antiviral Strategies Against Human Coronaviruses, Infectious Disorders - Drug Targets. 7(1): 59-66.
4. **Pyrć K**, Bosch BJ, Berkhout B, Jebbink MF, Dijkman R, Rottier P, and van der Hoek L. (**2006**) Inhibition of Human Coronavirus NL63 Infection at Early Stages of the Replication Cycle. Antimicrobial Agents and Chemotherapy. 50(6): 2000-2008.

REFERENCE LIST

1. in *Global Burden of Disease and Risk Factors* (eds. Lopez, A.D., Mathers, C.D., Ezzati, M., Jamison, D.T. & Murray, C.J.L.) (Washington (DC), 2006).
2. Mathers, C., Fat, D.M., Boerma, J.T. & World Health Organization. *The global burden of disease : 2004 update*, (World Health Organization, Geneva, Switzerland, 2008).
3. Wald, E.R., Guerra, N. & Byers, C. Upper respiratory tract infections in young children: duration of and frequency of complications. *Pediatrics* 87, 129-133 (1991).
4. Fox, J.P., Cooney, M.K., Hall, C.E. & Foy, H.M. Rhinoviruses in Seattle families, 1975-1979. *American journal of epidemiology* 122, 830-846 (1985).
5. Zeng, D.L., Zhang, Z.J., Wang, Z.L., Zhang, J.N. & Yang, M.L. Surveillance of acute respiratory infections in three kindergartens in Beijing. *Chinese medical journal* 101, 787-792 (1988).
6. Fields, B.N., Knipe, D.M. & Howley, P.M. *Fields virology*, (Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, 2007).
7. van der Hoek, L., *et al*. Identification of a new human coronavirus. *Nature medicine* 10, 368-373 (2004).
8. Woo, P.C., *et al*. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *Journal of virology* 79, 884-895 (2005).
9. Rihtaric, D., Hostnik, P., Steyer, A., Grom, J. & Toplak, I. Identification of SARS-like coronaviruses in horseshoe bats (Rhinolophus hipposideros) in Slovenia. *Archives of virology* 155, 507-514 (2010).
10. Poon, L.L., *et al*. Identification of a novel coronavirus in bats. *Journal of virology* 79, 2001-2009 (2005).
11. Jonassen, C.M., *et al*. Molecular identification and characterization of novel coronaviruses infecting graylag geese (Anser anser), feral pigeons (Columbia livia) and mallards (Anas platyrhynchos). *The Journal of general virology* 86, 1597-1607 (2005).
12. Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D. & Fouchier, R.A. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *The New England journal of medicine* 367, 1814-1820 (2012).
13. Drosten, C., *et al*. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *The New England journal of medicine* 348, 1967-1976 (2003).
14. St-Jean, J.R., *et al*. Recovery of a neurovirulent human coronavirus OC43 from an infectious cDNA clone. *Journal of virology* 80, 3670-3674 (2006).
15. Yeager, C.L., *et al*. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* 357, 420-422 (1992).
16. Fulcher, M.L., Gabriel, S., Burns, K.A., Yankaskas, J.R. & Randell, S.H. Well-differentiated human airway epithelial cell cultures. *Methods in molecular medicine* 107, 183-206 (2005).
17. Baric, R.S. & Yount, B. Subgenomic negative-strand RNA function during mouse hepatitis virus infection. *Journal of virology* 74, 4039-4046 (2000).
18. Sawicki, S.G., Sawicki, D.L. & Siddell, S.G. A contemporary view of coronavirus transcription. *Journal of virology* 81, 20-29 (2007).
19. Schaad, M.C., Chen, W., Peel, S.A. & Baric, R.S. Studies into the mechanism for MHV transcription. *Advances in experimental medicine and biology* 342, 85-90 (1993).
20. Chan, C.M., *et al*. Identification of major histocompatibility complex class I C molecule as an attachment factor that facilitates coronavirus HKU1 spike-mediated infection. *Journal of virology* 83, 1026-1035 (2009).
21. Muir, R. & Wilson, G.H. Observations ON INFLUENZA AND ITS COMPLICATIONS. *British medical journal* 1, 3-5 (1919).
22. Wilson, C.B.P.S. & Steer, P. Bacteriological and pathological observations on influenza as seen in France during 1918. *British medical journal* 1, 634-635 (1919).

Pyrć

23. Duttweiler, L., Nadal, D. & Frey, B. Pulmonary and systemic bacterial co-infections in severe RSV bronchiolitis. *Archives of disease in childhood* 89, 1155-1157 (2004).
24. Kneyber, M.C., *et al.* Concurrent bacterial infection and prolonged mechanical ventilation in infants with respiratory syncytial virus lower respiratory tract disease. *Intensive care medicine* 31, 680-685 (2005).
25. Randolph, A.G., Reder, L. & Englund, J.A. Risk of bacterial infection in previously healthy respiratory syncytial virus-infected young children admitted to the intensive care unit. *The Pediatric infectious disease journal* 23, 990-994 (2004).
26. Thorburn, K., Harigopal, S., Reddy, V., Taylor, N. & van Saene, H.K. High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis. *Thorax* 61, 611-615 (2006).
27. Grijalva, C.G. & Griffin, M.R. Unveiling the burden of influenza-associated pneumococcal pneumonia. *The Journal of infectious diseases* 205, 355-357 (2012).
28. McCullers, J.A. Insights into the interaction between influenza virus and pneumococcus. *Clinical microbiology reviews* 19, 571-582 (2006).
29. Okamoto, T., Akuta, T., Tamura, F., van Der Vliet, A. & Akaike, T. Molecular mechanism for activation and regulation of matrix metalloproteinases during bacterial infections and respiratory inflammation. *Biological chemistry* 385, 997-1006 (2004).
30. McCullers, J.A. & Bartmess, K.C. Role of neuraminidase in lethal synergism between influenza virus and Streptococcus pneumoniae. *The Journal of infectious diseases* 187, 1000-1009 (2003).
31. Patel, J.A., *et al.* Interleukin-1 alpha mediates the enhanced expression of intercellular adhesion molecule-1 in pulmonary epithelial cells infected with respiratory syncytial virus. *American journal of respiratory cell and molecular biology* 13, 602-609 (1995).
32. Terajima, M., *et al.* Rhinovirus infection of primary cultures of human tracheal epithelium: role of ICAM-1 and IL-1beta. *The American journal of physiology* 273, L749-759 (1997).
33. Avadhanula, V., *et al.* Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner. *Journal of virology* 80, 1629-1636 (2006).
34. Cundell, D.R., Gerard, N.P., Gerard, C., Idanpaan-Heikkila, I. & Tuomanen, E.I. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. *Nature* 377, 435-438 (1995).
35. Ishizuka, S., *et al.* Effects of rhinovirus infection on the adherence of Streptococcus pneumoniae to cultured human airway epithelial cells. *The Journal of infectious diseases* 188, 1928-1939 (2003).
36. Hament, J.M., Kimpen, J.L., Fleer, A. & Wolfs, T.F. Respiratory viral infection predisposing for bacterial disease: a concise review. *FEMS immunology and medical microbiology* 26, 189-195 (1999).
37. van der Sluijs, K.F., *et al.* Involvement of the platelet-activating factor receptor in host defense against Streptococcus pneumoniae during postinfluenza pneumonia. *American journal of physiology. Lung cellular and molecular physiology* 290, L194-199 (2006).
38. McCullers, J.A. & Rehg, J.E. Lethal synergism between influenza virus and Streptococcus pneumoniae: characterization of a mouse model and the role of platelet-activating factor receptor. *The Journal of infectious diseases* 186, 341-350 (2002).
39. McCullers, J.A., Iverson, A.R., McKeon, R. & Murray, P.J. The platelet activating factor receptor is not required for exacerbation of bacterial pneumonia following influenza. *Scandinavian journal of infectious diseases* 40, 11-17 (2008).
40. van den Hoogen, B.G., *et al.* A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nature medicine* 7, 719-724 (2001).
41. Banerjee, S., Bharaj, P., Sullender, W., Kabra, S.K. & Broor, S. Human metapneumovirus infections among children with acute respiratory infections seen in a large referral hospital in India. *Journal of clinical virology : the official publication of the Pan American*

Society for Clinical Virology 38, 70-72 (2007).
42. Bastien, N., *et al.* Human metapneumovirus infection in the Canadian population. *Journal of clinical microbiology* 41, 4642-4646 (2003).
43. Cilla, G., *et al.* Hospitalization rates for human metapneumovirus infection among 0- to 3-year-olds in Gipuzkoa (Basque Country), Spain. *Epidemiology and infection* 137, 66-72 (2009).
44. Falsey, A.R., Erdman, D., Anderson, L.J. & Walsh, E.E. Human metapneumovirus infections in young and elderly adults. *The Journal of infectious diseases* 187, 785-790 (2003).
45. Oliveira, D.B., *et al.* Epidemiology and genetic variability of human metapneumovirus during a 4-year-long study in Southeastern Brazil. *Journal of medical virology* 81, 915-921 (2009).
46. Williams, J.V., *et al.* Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. *The New England journal of medicine* 350, 443-450 (2004).
47. Boivin, G., *et al.* Global genetic diversity of human metapneumovirus fusion gene. *Emerging infectious diseases* 10, 1154-1157 (2004).
48. Miller, S.A., Tollefson, S., Crowe, J.E., Jr., Williams, J.V. & Wright, D.W. Examination of a fusogenic hexameric core from human metapneumovirus and identification of a potent synthetic peptide inhibitor from the heptad repeat 1 region. *Journal of virology* 81, 141-149 (2007).
49. Shirogane, Y., *et al.* Efficient multiplication of human metapneumovirus in Vero cells expressing the transmembrane serine protease TMPRSS2. *Journal of virology* 82, 8942-8946 (2008).
50. Smith, E.C., Popa, A., Chang, A., Masante, C. & Dutch, R.E. Viral entry mechanisms: the increasing diversity of paramyxovirus entry. *The FEBS journal* 276, 7217-7227 (2009).
51. Scheiblauer, H., Reinacher, M., Tashiro, M. & Rott, R. Interactions between bacteria and influenza A virus in the development of influenza pneumonia. *The Journal of infectious diseases* 166, 783-791 (1992).
52. Tashiro, M., Ciborowski, P., Klenk, H.D., Pulverer, G. & Rott, R. Role of Staphylococcus protease in the development of influenza pneumonia. *Nature* 325, 536-537 (1987).
53. Tashiro, M., *et al.* Synergistic role of staphylococcal proteases in the induction of influenza virus pathogenicity. *Virology* 157, 421-430 (1987).
54. Guo, Y., Nguyen, K.A. & Potempa, J. Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. *Periodontology 2000* 54, 15-44 (2010).
55. Ingman, T., *et al.* Salivary collagenase, elastase- and trypsin-like proteases as biochemical markers of periodontal tissue destruction in adult and localized juvenile periodontitis. *Oral microbiology and immunology* 8, 298-305 (1993).
56. Kononen, E., *et al.* Population-based study of salivary carriage of periodontal pathogens in adults. *Journal of clinical microbiology* 45, 2446-2451 (2007).
57. Leblebicioglu, B., Kulekci, G., Ciftci, S., Keskin, F. & Badur, S. Salivary detection of periodontopathic bacteria and periodontal health status in dental students. *Anaerobe* 15, 82-86 (2009).
58. Matto, J., *et al.* Detection of Porphyromonas gingivalis from saliva by PCR by using a simple sample-processing method. *Journal of clinical microbiology* 36, 157-160 (1998).
59. Miyamoto, E., *et al.* Bacterial profiles of oral streptococcal and periodontal bacterial species in saliva specimens from Japanese subjects. *Archives of oral biology* 54, 374-379 (2009).
60. Abe, S., *et al.* Professional oral care reduces influenza infection in elderly. *Archives of gerontology and geriatrics* 43, 157-164 (2006).
61. Adachi, M., Ishihara, K., Abe, S. & Okuda, K. Professional oral health care by dental hygienists reduced respiratory infections in elderly persons requiring nursing care.

International journal of dental hygiene 5, 69-74 (2007).

62. Mojon, P., Budtz-Jorgensen, E., Michel, J.P. & Limeback, H. Oral health and history of respiratory tract infection in frail institutionalised elders. *Gerodontology* 14, 9-16 (1997).
63. Okuda, K., Kimizuka, R., Abe, S., Kato, T. & Ishihara, K. Involvement of periodontopathic anaerobes in aspiration pneumonia. *Journal of periodontology* 76, 2154-2160 (2005).
64. Regamey, N., *et al.* Viral etiology of acute respiratory infections with cough in infancy: a community-based birth cohort study. *The Pediatric infectious disease journal* 27, 100-105 (2008).
65. Tsolia, M.N., *et al.* Etiology of community-acquired pneumonia in hospitalized school-age children: evidence for high prevalence of viral infections. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 39, 681-686 (2004).
66. van de Pol, A.C., Wolfs, T.F., Jansen, N.J., van Loon, A.M. & Rossen, J.W. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. *Critical care* 10, R61 (2006).