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

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CHAPTER 8

Discussion

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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.

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