Catching the common cold

*Rapid detection and epidemiology of respiratory viruses*

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Acute respiratory tract infections are a leading cause of morbidity and mortality worldwide. Symptoms can be mild, for example those of the ‘common cold’, but severe complications such as pneumonia may develop. Respiratory viruses are thought to be responsible for the vast majority of respiratory tract infections. Rapid identification of these viruses is important for clinical patient management, public health surveillance, and infection prevention. In recent years, the diagnostic possibilities for the detection of respiratory viruses have advanced rapidly. There is a clear trend towards faster diagnostics. Increasing numbers of rapid tests designed for use at the point-of-care have been developed. The aim of this thesis is 1) to evaluate the use and diagnostic accuracy of rapid tests for respiratory viruses in the hospital setting and in primary health care; 2) to increase our insight in the epidemiology and clinical relevance of respiratory viruses.
CATCHING THE COMMON COLD
Rapid detection and epidemiology of respiratory viruses

Andrea Hubertina Lena Bruning
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CATCHING THE COMMON COLD

Rapid detection and epidemiology of respiratory viruses

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Faculteit der Geneeskunde
“I love the doctors—they are dears;
But must they spend such years and years
Investigating such a lot
Of illnesses which no one’s got,
When everybody, young and old,
Is frantic with the common cold?
And I will eat my only hat
If they know anything of that!”

A.P. Herbert, 1936
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Chapter 1

General Introduction
Respiratory tract infections: burden of disease

Acute respiratory tract infections (RTIs) are a leading cause of morbidity and mortality worldwide (1). Every year nearly 3 million children die of acute lower RTIs, such as pneumonia and bronchiolitis (2). Upper RTIs, popularly known as the ‘common cold’, are the most common acute illnesses within the industrialised world. Adults encounter on average two to six colds per year. In young children infection rates are even higher, with an average of five to eight common cold episodes annually. Symptoms can be as mild as just a runny nose, but complications such as asthma exacerbations, otitis media, and pneumonia may develop (3).

Despite the usual self-limiting nature of RTIs, their economic burden on society is enormous. In the United States, the common cold syndrome alone is responsible for about 22 million days of absence from school and 20 million days of absence from work, resulting in lost productivity and medical visits (4). Following this, RTIs are the commonest acute problem dealt with in primary health care (5).

Etiology of respiratory tract infections

Although bacteria are historically considered the main pathogens causing severe RTIs, the importance and contribution of viruses is increasingly recognized. Recent studies suggest more than half of RTIs are caused by viruses, even in severely ill and mechanically ventilated pneumonia patients (6-8). Common respiratory viruses causing RTIs include rhinoviruses (RV), human coronaviruses (HCoV), influenza viruses, respiratory syncytial viruses (RSV), human metapneumovirus (hMPV), parainfluenza viruses (PIV), and adenoviruses (9-11). Several other respiratory viruses have been identified more recently, such as human bocavirus (HBoV), and the respiratory polyomaviruses WU and KI, of which the precise clinical relevance remains under debate, and new viruses continue to emerge (12-14). A brief overview of the characteristics of the most important respiratory viruses is shown in Table 1.

Epidemiology, transmission, and pathogenesis

The epidemiology of respiratory viruses varies considerably, but seasonal patterns can be recognized in temperate climates and less so in (sub)tropical regions. The number of respiratory viruses increases in autumn, is fairly high throughout winter, and decreases in spring (15). RSV and influenza viruses are well-known for their ability to cause seasonal outbreaks, especially in the extremes of the age spectrum and in high-risk individuals, such as immunocompromised patients or patients with chronic underlying cardiac, pulmonary or metabolic comorbidities (4, 16-18).
Respiratory viruses can be transmitted directly from person-to-person through aerosols, respiratory droplets or hand contact, and/or indirectly from contaminated environmental surfaces. Respiratory viruses enter the body through the upper respiratory tract and replicate mainly in respiratory mucosa and respiratory lymphoid tissues. The pathogenesis of viral RTIs likely differs per causative agent and is not yet fully understood for most, but in all depends on the interplay between virus, host and environment. Factors influencing disease manifestations and severity are the virulence of the infecting virus strain, innate and adaptive host immune responses, underlying comorbidities and host genetic factors (19-22).

Treatment, prevention and control

Treatment options for viral RTIs are limited and are still primarily based on supportive care. Symptomatic treatment, e.g. nasal decongestants and paracetamol, is often sufficient for mild infections. In the hospital setting, however, fluid replacement, extra oxygen and eventually mechanical ventilation might be necessary (23). Currently, virus-specific treatment is only available against influenza virus infections. Two classes of antivirals are commercially available for influenza viruses: neuraminidase inhibitors, e.g. oseltamivir and zanamivir, and M2-channel blockers, but due to the high prevalence of resistance for this latter class its use is currently not recommended (24). A number of therapeutic drugs are under development for other respiratory viruses (25). For example for rhinoviruses, the most frequently detected respiratory virus in patients with RTI symptoms and known for its contribution to acute asthma and COPD exacerbations, several antiviral drugs are under evaluation, such as the capsid binder vapendavir (26).

At the moment, influenza is the only vaccine-preventable respiratory virus, although influenza vaccines need to be reformulated periodically due to antigenic drift (27). For RSV, passive immunisation with palivizumab is offered for specific risk groups, such as prematurely born children. New antiviral approaches, including nanobodies, broadly neutralizing monoclonal antibodies, and host-targeted therapeutic approaches, i.e. therapeutics targeted at host cellular pathways required for viral replication, are currently in development, but so far treatment remains largely supportive (28-30).

Infection control of viral RTIs is thus largely based on prevention. Infection control measures, such as respiratory hygiene, cough etiquette and masking and separation of persons with respiratory symptoms, are needed to prevent the spread of respiratory viruses (31, 32).
Diagnostic methods for detecting respiratory viruses

Although certain respiratory viruses are associated with specific syndromes, e.g. RSV bronchiolitis, clinical signs and symptoms related to respiratory viruses infections generally overlap. To confirm the etiological agent responsible for the RTI a laboratory diagnosis is required. Accurate diagnosis of the RTI is important for patient management and infection prevention (33).

At the moment, five general approaches are used for the laboratory detection of viruses: microscopy, culture, detection of viral antigens or nucleic acids, and detection of antibodies against the organism (34). The history of virus detection began in 1898, when Martinus Beijerinck, a Dutch microbiologist, introduced the word 'virus' for an infectious agent causing disease in tobacco plants. The identification of this pathogen is now acknowledged as the foundation of the field of virology (35).

With the introduction of the electron microscope in 1933 it was for the first time possible to visualize virus particles. For clinical purposes the technique is currently outdated because of its limited sensitivity, but it is still in use in research settings to study viral structure. Furthermore, it can have a role in detecting new and unusual virus outbreaks. In 2003, for example, examinations of respiratory samples by electron microscopy resulted in the discovery of Severe Acute Respiratory Syndrome (SARS)-coronavirus (36).

Cell culture has long been considered as the gold standard for detecting respiratory viruses. Viruses are intracellular pathogens requiring host cells for replication. Therefore, in vitro cell culture systems have been developed to facilitate replication. After inoculation with a respiratory sample, the cell culture is incubated for approximately one week and afterwards examined for cytopathogenic effects by light microscopy, suggesting viral replication. The process of cell culture accelerated with the development of so called ‘shell vial spin amplification cultures’. With this technique, the virus is centrifuged on to a thin cell layer, and labelled with fluorescent monoclonal antibodies against viral antigens. Viral growth can then be detected by fluorescence. In mixed cell culture systems several cell lines are combined in one culture and multiple viruses that require different cells for growth can be isolated (34). Cell culture remains a useful approach for virus detection, but has nowadays become mainly a research tool (37).

Several other laboratory techniques make use of viral antigen detection. With Direct Fluorescent Antibody (DFA) staining, cells from nasopharyngeal samples are directly tested for the presence of an antigen with a fluorescent-labelled antibody. These antibodies are incubated with the
sample to allow antigen-specific binding. Excess and unbound antibodies are washed away and areas in which antigens are present can be visualized using a fluorescence microscope (38).

Serology is historically the mainstay of virus diagnostics. It monitors the immune systems’ specific antibody response to viral antigen exposure. Antibodies are produced after the onset of viral illness and can be detected using serological diagnostic techniques such as hemagglutination inhibition tests, enzyme immunoassays, complement fixation, and neutralization tests (38). However, for detection of respiratory viruses these methods are not regarded as clinically relevant diagnostic tools in daily practice as it usually requires acute and convalescent serum samples to detect rises in antibody levels (39).

With the introduction of highly sensitive molecular techniques such as polymerase chain reaction (PCR) a new standard for respiratory virus detection has been set. Nucleic-acid based amplification techniques use primers and probes directed at unique, conserved regions of a viral genome. They are highly specific and bind only to complementary DNA or RNA sequences (38). Advances in technology have allowed for the development of multiplex PCR tests in which several different viruses can be identified in a single test.

All the above-mentioned techniques have their drawbacks. The isolation of respiratory viruses in culture or serological diagnosis requiring paired blood specimens are slow and labour-intensive. DFA is faster, but requires technical expertise as well and is less sensitive than culture. Because of its high sensitivity, high specificity and its ability to also detect viruses that are difficult to culture or simply do not grow at all in cultured cells, PCR has become the reference method for detection of respiratory viruses (40).

However, also PCR techniques require trained laboratory personnel and specialized equipment that is not always directly available resulting in relatively high costs and long turn-around times. Although more rapid and user-friendly PCR devices have been developed (41), rapid antigen detection tests (RADTs) are often used as a simple, cheaper, and time-saving alternative for virus detection.

RADTs are immunoassays that can detect viral antigens in respiratory specimens, such as membrane-based enzyme immunoassays, lateral flow immunochromatography tests, and optical immunoassays. These assays are, like DFA, based on an antigen-antibody reaction, but as the antibody is not fluorescence-labelled but enzyme-labelled, no fluorescence microscopy is required (38, 42). They have the potential to be carried out near the patient at the point-of-care,
such as the emergency department or a general practitioners’ office, and thus have the ability to function as so-called point-of-care tests.

**Point-of-care tests**

Point-of-care tests (POCTs) are defined as rapid, easy-to-use tests carried out near the patient by non-laboratory-trained personnel. PCR-based POCTs are in development, but at the moment RADTs are most often used as POCTs as they are faster (with results typically available within 30 minutes), less expensive, easy-to-use and thus accessible to staff without laboratory training, compared to the other laboratory techniques.

There is an increasing interest in POCTs as according to several cost-effectiveness evaluations the use of these tests could lead to a more effective utilization of healthcare resources (43, 44). With the rapid identification of viral pathogens causing RTI, unnecessary additional testing, such as X-rays, blood count and cultures, and antibiotic prescription may be avoided (43, 45). Besides, prompt viral diagnosis may lead to rapid implementation of infection control measures, early administration of antiviral medication and a decrease in duration of hospital stay resulting in reduced health care costs. POCTs can offer clinicians the ability to manage patients’ expectations for antibiotics and to encourage patients to self-care when suffering from a self-limiting condition (46).

However, the clinical feasibility of POCTs is only sporadically evaluated in clinical settings. Most of the publications regarding POCTs are retrospective studies or studies where the test is used in a laboratory setting. Results are therefore difficult to extrapolate to daily clinical practice. Because of the potentially promising influence of POCTs on patient care, well designed clinical validation studies are needed. An important aim of this thesis is therefore to evaluate the use and diagnostic accuracy of POCTs for respiratory viruses in both the hospital setting and in primary health care.

**Outline of this thesis**

In Part 1 of this thesis, we describe the development and evaluation of POCTs for respiratory viruses. Chapter 2 provides an overview of all commercially available POCTs for the detection of respiratory viruses in patients with RTIs. In chapter 3, we evaluate the use of a POCT for influenza viruses and RSV in a pediatric hospitalized population. Chapters 4 and 5 describe the development of two new viral targets, respectively HBoV and CoV, for a novel POCT. In chapter 6, we determine the diagnostic accuracy and clinical feasibility of a POCT in a primary health care practice.
In Part 2, we focus on the epidemiology of respiratory viruses, in particular rhinoviruses. RVs are frequently detected respiratory viruses that may cause mild common cold symptoms, but can also lead to more severe RTIs. The large number of RV types, classified into species A, B and C, hampers clear insights in the epidemiology and clinical significance of each RV type. Chapter 7 investigates the prevalence of RV types in a hospitalized patient population. In chapter 8, we describe the clinical, virological and epidemiological characteristics of RV infections in young children with mild or no symptoms compared to children with moderate to severe symptoms in order to explore possible associations between different characteristics of RV infections and clinical outcome.

Laboratory testing for acute RTIs is not routinely performed in primary health care. The etiology of RTIs is therefore usually unknown and although RTIs are mainly of viral origin, the contribution of different respiratory viruses is uncertain. The purpose of chapter 9 is to increase our insight into the epidemiology of respiratory viruses in a primary health care setting and to evaluate the accuracy of the local general practitioners in their clinical diagnosis of influenza virus infection compared to most sensitive PCR techniques. The summary and general discussion on the main findings of this thesis and recommendations for future studies is presented in chapters 10 and 11.
Table 1. Virological characteristics of common respiratory viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Genome</th>
<th>Envelope</th>
<th>Characteristics</th>
</tr>
</thead>
</table>
| Rhinovirus            | Picornaviridae   | RNA    | No       | - Rhinovirus species A, B and C  
- >160 types  
- Main pathogen responsible for the 'common cold', but can also elicit more severe disease |
| Influenza virus       | Orthomyxoviridae | RNA    | Yes      | - Influenzavirus A, B and C  
- Influenza A is associated with seasonal epidemics and pandemics, influenza B only with seasonal epidemics |
| Respiratory syncytial virus | Paramyxoviridae | RNA    | Yes      | - RSV subtype A and B  
- Most important cause of acute lower RTIs in children |
| Parainfluenza virus   | Paramyxoviridae  | RNA    | Yes      | - Parainfluenzavirus type 1, 2, 3 and 4  
- Known for its role in croup |
| Human metapneumovirus | Paramyxoviridae  | RNA    | Yes      | - Relatively novel virus (discovered in 2001) with symptoms similar to RSV |
| Human coronavirus     | Coronaviridae     | RNA    | Yes      | - 6 genotypes: 229E, OC43, SARS-CoV, NL63, HKU1, MERS-CoV. |
| Human bocavirus       | Parvoviridae     | DNA    | No       | - Human Bocavirus type 1, 2, 3 and 4  
- Mainly type 1 responsible for respiratory illness |
| Adenovirus            | Adenoviridae     | DNA    | No       | - Besides respiratory symptoms a major cause of keratoconjunctivitis |
REFERENCES


General introduction


PART I

Rapid detection of respiratory viruses
Chapter 2

Rapid tests for respiratory viruses: a systematic review and meta-analysis

Andrea H.L. Bruning, Mariska M.G. Leeflang, Johanna M.B.W. Vos, Rene Spijker, Menno D. de Jong, Katja C. Wolthers, Dasja Pajkrt.

Manuscript submitted for publication
ABSTRACT

**Background:** Rapid diagnosis of respiratory virus infections contributes to patient care. This systematic review evaluates the diagnostic accuracy of rapid tests for the detection of respiratory viruses in patients with respiratory tract infections.

**Methods:** Medline and EMBASE databases were searched for studies evaluating rapid tests for respiratory viruses against PCR as reference standard. We used the bivariate random effects model for quantitative meta-analysis of the results. Quality of included studies was assessed using the Quality Assessment of Diagnostic Accuracy Studies checklist.

**Findings:** 125 articles describing 179 studies were included of which 134 studies evaluated rapid tests for influenza viruses, 32 for respiratory syncytial virus (RSV), and 13 studies for other respiratory viruses. Summary sensitivity and specificity estimates of tests for influenza were 61.1% (95% confidence interval [CI], 53.3-68.3), and 98.9% (95% CI, 98.4-99.3), respectively. For RSV, summary sensitivity was 75.3% (95% CI, 72.6-77.8), and specificity 98.7% (95% CI, 97.3-99.4). Of the studied rapid tests, 26.3% were evaluated at the point-of-care. Because of incomplete reporting of study characteristics, risk of bias was often unclear.

**Interpretation:** Most rapid tests can detect only influenza viruses or RSV. Sensitivity of rapid tests varies considerably, but specificity is high. Despite their intended use at the point-of-care, many are evaluated in a laboratory-setting. Included studies often did not report characteristics that may potentially lead to bias. Although newer tests seem more sensitive, high quality evaluations of these tests are lacking.

**Funding:** This project was funded by European Union’s Seventh Framework People Programme under REA-grant-agreement no 612308.
INTRODUCTION

Acute respiratory tract infections (RTIs) are a leading cause of morbidity and mortality worldwide (1). Although bacteria were historically considered the main etiological agents of severe RTIs, the importance and contribution of viruses is increasingly recognized. Recent studies suggest more than half of RTIs are being caused by viruses, even in severely ill and mechanically ventilated pneumonia patients (2-4). Commonly detected viruses causing RTIs include influenza viruses, respiratory syncytial virus (RSV), adenovirus, human metapneumovirus (hMPV), human parainfluenza viruses, coronaviruses and rhinoviruses (5, 6).

Clinical signs and symptoms of viral RTIs overlap with those of bacterial infections. It is therefore challenging for clinicians to distinguish bacterial from viral infections and between different viral pathogens (7). Because of this diagnostic uncertainty, antibiotics are often prescribed, and rates of extra diagnostic testing such as chest X-rays, blood counts and cultures to rule out bacterial infections are high (8, 9).

If, however, viral pathogens could be rapidly identified, unnecessary additional testing and antibiotic prescription might be avoided. Besides, prompt viral diagnosis may lead to rapid implementation of infection control measures, early administration of antiviral medication and a decrease in duration of hospital stay resulting in reduced health care costs (10-12).

For this reason, rapid diagnostic tests or point-of-care tests (POCTs) have been developed. Compared to other viral diagnostic modalities, i.e. viral culture, polymerase chain reaction (PCR), or immunofluorescence testing, POCTs are faster (with results provided typically in less than 30 minutes), less expensive, easy-to-use and thus accessible to staff without laboratory training. Therefore, they have the potential to be carried out at or near the point-of-care.

There is a clear trend towards point-of-care testing and over the past few years, the number and quality of rapid diagnostic tests for respiratory viruses has rapidly increased (13). For clinicians, it is important to be aware of the diagnostic accuracy of the different rapid viral tests, the factors that affect their accuracies, and how the test performs in daily clinical practice. Previous systematic reviews that addressed the diagnostic accuracy of respiratory tests evaluated either only one respiratory virus, i.e. influenza or RSV, or were conducted in specific populations, e.g. children (14-16).

The aim of our review was therefore to provide a state-of-the-art overview of all commercially available rapid tests for the detection of respiratory viruses in patients of all ages with RTIs.
We systematically summarized the available evidence on their diagnostic accuracy for virus detection compared to polymerase chain reaction (PCR) testing. We assessed the quality of included studies and quantitatively summarized the results in a meta-analysis.

**METHODS**

This systematic review was built on a protocol based on the Preferred Reporting Items for Systematic Review and Meta-Analyses for Protocols 2015 (PRISMA-P 2015). The complete protocol is registered in the Prospero database under CRD42015024581.

**Search strategy**

A systematic literature review was conducted by searching the Medline and EMBASE electronic databases, through the Ovid interface, from inception to January 18, 2016. The reference lists of all articles were hand-searched for additional suitable studies.

The search strategy was developed in collaboration with a medical information specialist (RS) and contained search terms for the most common respiratory viruses (influenza OR respiratory syncytial virus OR metapneumovirus OR parainfluenza virus OR human adenovirus OR human rhinovirus OR human bocavirus OR human coronavirus) combined with search terms for rapid diagnostic tests (diagnostic kit OR antigen (test or detection) OR reagent OR immuno test OR point-of-care systems OR rapid or simple or easy or quick test), including brand names for the most common commercial rapid tests. The complete search strategy is shown in Appendix A. After duplicates were removed the search results were imported into Covidence, a web-based software platform that streamlines the production of systematic reviews (https://www.covidence.org/).

**Selection criteria**

Studies were considered for inclusion if they were written in English or Dutch and reported original data regarding the accuracy (sensitivity and specificity or comparable outcomes with which sensitivity and specificity could be calculated) of a rapid test for the detection of one or more respiratory viruses as compared to PCR. PCR was considered the only acceptable reference standard because of its status as gold standard method for detecting respiratory viruses (17). Rapid tests were defined (partly based on the definition of the World Health Organization simple/rapid tests (18)) as any commercially available quick (up to 2 hours) and easy-to-use test requiring little or no additional equipment or technological skills.
Only original studies were included. Studies evaluating in-house tests and pre-commercial versions of the rapid tests were excluded as well as studies using the result of the rapid test as part of a composite reference standard (to avoid incorporation bias) or if PCR was solely performed on samples in which rapid tests tested negative (to avoid partial verification bias). Case-control studies (use of the rapid test on previously tested known positive or negative samples), case reports, conference abstracts, reviews and veterinary studies were excluded.

Two reviewers (AB and DP) independently assessed inclusion eligibility. Initial selection for inclusion was based on screening of title and abstract. Following this, full-text versions of the selected studies were assessed for eligibility. The reasons for excluding studies were documented.

**Data extraction**
A data extraction form was developed in collaboration with a clinical epidemiologist (ML). Data were extracted independently by two authors (AB and JV). Information collected included author, year of study and publication, country in which the study was performed, study population (children, adults, or both), specimen type, setting (e.g. primary care or hospital), performance of the test in a laboratory or at the point-of-care, brand name of the rapid test, and data required to calculate sensitivity and specificity. With these data we constructed 2x2 contingency tables (true positives, false positives, false negatives, true negatives). If 2x2 tables could not be extracted or calculated from the published data, we contacted authors for additional information. In some articles the same sample sets were tested with different rapid tests. Each test comparison was considered as a separate analysis resulting in a higher total number of included ‘studies’ than the actual number of included articles.

**Quality assessment**
Methodological quality of studies was independently reviewed by two authors (AB and JV) by using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) criteria, a checklist recommended by the Cochrane Collaboration to assess the quality of diagnostic test accuracy studies (19). QUADAS-2 consists of several signalling questions to support the risk of bias for each of four domains: patient recruitment, index test, reference standard and flow and timing of the study. It also contains a judgment about potential concerns regarding the applicability of the included studies for three domains: patient recruitment, index test and reference standard. Disagreement about inclusion, data-extraction and quality assessment was solved by consensus.
Statistical analysis

With the constructed 2x2 contingency tables we estimated sensitivity and specificity of the rapid tests for each study. Each of the different respiratory viruses can be regarded as a separate target condition. Therefore, we analysed the accuracy of the tests for each virus separately. Studies evaluating rapid tests for influenza viruses sometimes reported multiple 2x2 tables for the different virus types (i.e. influenza A and B). Most of the included tests however detect both influenza subtypes and sometimes make no distinction between the subtypes. We therefore used ‘any influenza’ as our main analyses and analysed the accuracy of the tests in subtypes in subgroup analyses. Sensitivity and specificity estimates were pooled into receiver operating characteristic (ROC) plots. As sensitivity and specificity negatively correlated (tests with a higher sensitivity usually have a lower specificity), the meta-analysis should be performed on both outcome measures simultaneously. For this we used a bivariate random effects meta-regression model (20). To investigate the effect of potential sources of heterogeneity on sensitivity or specificity, we added covariates to the model. The following variables were selected as covariates: study population (children versus adults), commercial brand of the rapid test and if the test was performed at the point-of-care. We calculated summary sensitivity and specificity estimates for each covariate. To assess the effect of study quality, we performed sensitivity analyses. All analyses were performed in SAS 9.4.

Role of the funding source

This project has received funding from the European Union’s Seventh Framework People Programme under REA grant agreement no 612308. The funding source had no role in study design, collection, analysis or interpretation of the data, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Search results

Our literature searches identified 3527 articles. After screening of titles and abstracts, 383 articles were eligible for full-text review, of which 258 were subsequently excluded (Figure 1). The main reasons for excluding studies were that the rapid test was evaluated in a case-control study, the test could not be performed rapid (turnaround time was more than two hours), the study did not include original data, another reference test than PCR was used, or because of language. Several reports evaluated more than one rapid test resulting in a total number of 179...
separate studies that were included in the systematic review and meta-analysis. Of these, 134 (74.9%) studies evaluated rapid tests for influenza viruses, 32 (17.9%) studies evaluated rapid tests for RSV, and the remaining 13 (7.3%) studies assessed the accuracy of rapid tests for other respiratory viruses, i.e. hMPV, adenovirus, and parainfluenza viruses (Table 1).

Figure 1. PRISMA Flow-diagram

Characteristics of included studies

Supplemental Table 1 provides the main characteristics of included studies, i.e. author, year of publication, country, study population, if the test was evaluated at the point-of-care, name of rapid test and its estimated sensitivity and specificity. Supplemental Figure 1 displays the accompanying forest plots of the estimated sensitivities and specificities. In Table 1, a summary of included studies characteristics is presented. A total number of 50 different rapid tests have
been evaluated. The most frequently studied tests for influenza were: Alere BinaxNOW® Influenza A&B Test, Quidel QuickVue Influenza A+B, BD Directigen™ EZ Flu A+B, and Quidel Sofia Influenza A+B FIA. For RSV, BD Veritor™ System, Alere BinaxNOW® RSV Card, and Quidel Sofia RSV FIA were evaluated most frequently. Most of the studies were conducted in either children or populations including both adults and children. Of the studied rapid tests, 26.3% were evaluated at the point-of-care.

Table 1. Characteristics of included studies

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Studies (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virus evaluated</strong></td>
<td></td>
</tr>
<tr>
<td>Influenza virus (A, B, H1N1, A and B)</td>
<td>134 (74.9)</td>
</tr>
<tr>
<td>Respiratory Syncytial virus</td>
<td>32 (17.9)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>6 (3.4)</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>5 (2.8)</td>
</tr>
<tr>
<td>Parainfluenza virus type 1-3</td>
<td>2 (1.1)</td>
</tr>
<tr>
<td><strong>Population</strong></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>74 (41.3)</td>
</tr>
<tr>
<td>Adults</td>
<td>14 (7.8)</td>
</tr>
<tr>
<td>Mixed/not reported</td>
<td>91 (50.8)</td>
</tr>
<tr>
<td><strong>Most frequently studied tests</strong></td>
<td></td>
</tr>
<tr>
<td>Influenza virus</td>
<td></td>
</tr>
<tr>
<td>QuickVue Influenza A+B</td>
<td>24 (14.5)</td>
</tr>
<tr>
<td>BinaxNOW Influenza A&amp;B</td>
<td>19 (10.6)</td>
</tr>
<tr>
<td>Directigen EZ FluA+B</td>
<td>16 (8.9)</td>
</tr>
<tr>
<td>Sofia Influenza A+B</td>
<td>9 (5.0)</td>
</tr>
<tr>
<td>Respiratory Syncytial virus</td>
<td></td>
</tr>
<tr>
<td>BinaxNOW RSV</td>
<td>6 (3.3)</td>
</tr>
<tr>
<td>BD Veritor RSV</td>
<td>5 (2.8)</td>
</tr>
<tr>
<td>Sofia RSV</td>
<td>4 (2.2)</td>
</tr>
<tr>
<td>Directigen EZ RSV</td>
<td>3 (1.7)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td></td>
</tr>
<tr>
<td>Immuno Ace Adeno</td>
<td>2 (1.1)</td>
</tr>
<tr>
<td>hMPV</td>
<td></td>
</tr>
<tr>
<td>IC Assay</td>
<td>1 (0.6)</td>
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<tr>
<td>Multiplex test</td>
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<tr>
<td>mariPOC®</td>
<td>15 (8.4)</td>
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<td>Other rapid tests</td>
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<tr>
<td>yes</td>
<td>47 (26.3)</td>
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</table>
Quality of included studies

An overview of the risk of bias and concerns regarding applicability of included articles is presented in Figure 2. Because of the a-priori set exclusion criteria (exclusion of case-control studies and confirmation studies), most studies were free of spectrum bias, incorporation bias, and partial verification bias. Also, because of our inclusion criteria, all included studies used an appropriate reference standard. Nevertheless, several study characteristics might incorporate a risk of bias. In- and exclusion criteria were not or unclearly defined in many studies. It was therefore difficult to assess the risk of bias regarding the selection of patients. Concerns that included patients did not match the review questions were low resulting in a low concern regarding applicability. In at least 37 articles (29.6%), the index test was performed at the point-of-care suggesting that the index test results were interpreted without knowledge of the results of the reference standard. In 8 of these articles some additional information was available on the clinical feasibility or logistical organization of the performance of the rapid test. In conclusion, due to the frequent incomplete description of study characteristics that might result in a risk of bias, e.g. patient selection and conduct or interpretation of the index test, these items were difficult to assess resulting in an ‘unclear’ risk. The concerns regarding the applicability were in general low.

**Figure 2.** Quality assessment of included studies using QUADAS-2
Overall accuracy of rapid tests for influenza and RSV

For influenza, sensitivity ranged from 4.4% to 100.0%. Overall, for all rapid tests that can detect influenza, the summary estimate for sensitivity was 61.1% (95% confidence interval [CI], 53.3 to 68.3), and for specificity 98.9% (95% CI, 98.4 to 99.3). The operating test characteristics of the studies regarding influenza are shown in Figure 3a.

For RSV, variation in sensitivity was greater than variation in specificity, but sensitivity estimates were in general higher than for influenza, ranging from 41.2% to 88.6% (see Figure 3b). Summary sensitivity and specificity for all studies evaluating RSV was 75.3% (95% CI, 72.6 to 77.8), and 98.7% (95% CI, 97.3 to 99.4), respectively.

Due to small number of included studies for adenovirus, hMPV, and parainfluenza virus type 1-3 meta-analysis of the sensitivity and specificity estimates for these viruses had no additional value and was therefore not performed.

**Figure 3.** ROC plot of rapid tests for influenza (3a) and RSV (3b), respectively.
Investigation of heterogeneity

**Virus type**

Overall, sensitivities of rapid tests for influenza A (68.1%, 95% CI 58.9-76.0) and influenza B (71.0%, 95% CI 56.8-82.1) were comparable. Several studies (n=58) only evaluated the diagnostic accuracy of rapid tests for detecting H1N1. Most of these rapid tests were not developed to detect H1N1 specifically. Sensitivity for H1N1 was generally lower (sensitivity: 54.0%, 95% CI 47.6-60.3) compared to the other virus subtypes (Table 2). Specificity was comparable: 99.1% (95% CI 98.5-99.5).

**Population**

Diagnostic test accuracy for influenza significantly decreased when the test was performed in adults (sensitivity 34.1%, 95% CI 14.0-54.1) compared to accuracy in children or a mixed population. For RSV, age did not significantly influence performance characteristics.

**Test type**

To identify the rapid test with the best performance characteristics, we evaluated the pooled summary estimates for each rapid test separately. This was only possible for tests for which we had four or more studies. For influenza, Sofia Influenza A+B FIA had the best over-all performance. For RSV, only for three tests (BD Veritor RSV, Sofia RSV FIA, and BinaxNOW RSV) an estimated sensitivity and specificity per test could be calculated. Of these, Sofia RSV FIA had the best over-all performance.

**Point-of-care testing**

For both influenza and RSV, diagnostic test accuracy was not influenced by setting, i.e. whether the test was performed at the point-of-care or in the laboratory.

**Quality assessment**

Studies evaluating the risk of bias for influenza generally had a low risk of bias for the index test domain, the reference standard domain and flow and timing domain. We assessed the difference between studies with low risk of bias for the patient domain versus those with an unclear risk of bias in this domain. Sensitivity was in general lower in studies with a low risk of bias (53.3%, 95% CI 42.1-64.1). For RSV, the investigated quality criteria did not have a statistically significant effect on diagnostic test accuracy.
Table 2. Subgroup analyses: accuracy estimates

<table>
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<tr>
<th></th>
<th>Pooled sensitivity (95% CI)</th>
<th>p-value</th>
<th>Pooled specificity (95% CI)</th>
<th>p-value</th>
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<td></td>
<td></td>
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<tr>
<td><strong>Virus type</strong></td>
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<td></td>
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<td>Influenza A</td>
<td>68.1 (58.9-76.0)</td>
<td></td>
<td>99.2 (98.5-99.6)</td>
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</tr>
<tr>
<td>H1N1</td>
<td>54.0 (47.6-60.3)</td>
<td></td>
<td>99.1 (98.5-99.5)</td>
<td></td>
</tr>
<tr>
<td>Influenza B</td>
<td>71.0 (56.8-82.1)</td>
<td></td>
<td>99.6 (99.2-99.8)</td>
<td></td>
</tr>
<tr>
<td>Influenza A+B</td>
<td>61.1 (53.3-68.3)</td>
<td></td>
<td>98.9 (98.4-99.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Population</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Children</td>
<td>66.1 (52.9-79.3)</td>
<td>0.76</td>
<td>98.3 (97.2-99.5)</td>
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<td>Adults</td>
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<td>0.01</td>
<td>99.2 (98.2-100.0)</td>
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<td>Yes</td>
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<td>- QuickVue Influenza A+B</td>
<td>44.6 (29.1-60.0)</td>
<td>0.02</td>
<td>99.3 (98.8-99.9)</td>
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<td>- Sofia Influenza A+B</td>
<td>75.3 (59.2-91.5)</td>
<td>0.10</td>
<td>95.3 (91.5-99.2)</td>
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<td>44.1 (23.3-64.9)</td>
<td>0.09</td>
<td>99.4 (98.6-100.0)</td>
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<tr>
<td>- Directigen Flu A+B</td>
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<td>- Children</td>
<td>75.9 (73.1-78.5)</td>
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<td>Yes</td>
<td>76.0 (69.8-81.2)</td>
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<td><strong>Rapid test</strong></td>
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<td>- BD Veritor RSV</td>
<td>76.9 (71.0-82.8)</td>
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<td>98.9 (97.1-100.0)</td>
<td>0.79</td>
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<td>0.24</td>
<td>98.6 (96.5-100.0)</td>
<td>0.94</td>
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<td>- Sofia RSV</td>
<td>80.0 (73.0-86.9)</td>
<td>0.20</td>
<td>97.8 (93.8-100.0)</td>
<td>0.55</td>
</tr>
</tbody>
</table>
DISCUSSION

In this systematic review and meta-analysis we provided an overview of the rapid tests that are available for the detection of respiratory viruses in patients with RTIs. Most rapid tests could detect only influenza viruses or RSV. Overall, the sensitivity of these tests varied considerably, but specificity was high. With the result of the rapid test it is thus possible to rule in a respiratory viral infection, but false-negative results are common. One may therefore consider confirmation of negative test results by more sensitive methods. The performance of rapid tests for RSV was in general superior compared to the performance of rapid tests for influenza. A major advantage of rapid tests is their potential to be performed in a non-laboratory setting, but only 26.3% of them was actually evaluated at the point-of-care.

Although the diagnosis of RTIs requires a syndromic approach as symptoms of respiratory viruses overlap, only few rapid tests capable of simultaneously detecting multiple viruses are currently available. Most rapid tests only detect influenza viruses or RSV. This is historically understandable as these viruses were considered the most important respiratory viruses. Furthermore, availability of antivirals for influenza renders rapid diagnostic testing of influenza of high priority. However, recent studies indicate that other respiratory viruses, such as rhinoviruses and hMPV, can also cause severe respiratory illness and are sometimes detected at higher frequencies than influenza and RSV (21, 22).

The results of our heterogeneity investigation show that rapid test performance is comparable for influenza A and influenza B, which is line with a previous meta-analysis (14). Most of the included studies were performed in children, especially those evaluating rapid tests for RSV, which explains at least in part the lack of influence of age on the diagnostic accuracy estimates for RSV. For influenza, on the contrary, rapid test performance was significantly better in children, which has been confirmed in previous studies (23, 24). Age is inversely associated with viral load, which may explain better results with rapid antigen tests in children.

Testing at the point-of-care did not influence diagnostic test accuracy, although this finding should be interpreted with caution. In many studies, the test was not evaluated at the point-of-care nor was the setting or the personnel who performed the test described. Direct head-to-head comparisons between tests performed at the point-of-care or in the laboratory were limited. Besides, in only 8 of the 37 articles describing tests performed at the point-of-care information was reported on the clinical feasibility of the test, i.e. if the test was easy-to-use, how rapid test results were communicated, or how the personnel performing the rapid tests evaluated its usefulness.
By estimating the pooled sensitivities of the most frequently evaluated rapid tests we aimed to determine which rapid test has the best performance characteristics. However, these conclusions should be interpreted cautiously. Because of our random effects model we could not evaluate all rapid tests separately and the newer molecular testing devices were only sporadically evaluated in prospective cross-sectional studies (25, 26). When analysing only high quality studies (i.e. with a low risk of bias according to our quality assessment) over-all sensitivity decreased, implying an even lower diagnostic accuracy for rapid tests.

**Strengths and limitations of the systematic review design**

Diagnostic accuracy studies are described in different ways and no standard terminology is available (27). To include all available evidence, we constructed our search strategy as broad as possible. Data were extracted and quality of included studies was assessed by two independent reviewers. This decreases the risk of subjectivity to a minimum. In case of methodological shortcomings, e.g. incorporation bias or partial verification bias, diagnostic test performance might be under- or over-estimated. We tried to avoid this bias by applying strict exclusion criteria.

For this systematic review, we deliberately chose to include only studies in which PCR was used as the reference standard because of its status as gold standard method for detecting respiratory viruses (17). This important strength of our systematic review provides a more realistic accuracy estimate for rapid tests. In previous studies where viral culture or immunofluorescence were used as a reference, pooled sensitivities were 9 – 14% higher than in studies that used PCR and test accuracy is thus overestimated (15). We did not assess publication bias as no accurate reporting methods for diagnostic test accuracy studies exist (27).

The accuracy of a systematic review depends on the quality of the studies included. As demonstrated in the two illustrations displaying the outcomes of the quality assessment, many items regarding the risk of bias of included studies were unclear. Sensitivity and specificity calculations are extremely sensitive to the design of a study and are influenced by prevalence of virus activity, time from illness onset to sample collection, type of respiratory specimen tested, quality of the collected sample, age of the patient, and disease severity (11, 28). In many studies that evaluated specific rapid tests these items were lacking or not clearly defined. This is a major limitation in diagnostic accuracy studies in general. Comparisons between tests should be ideally made in the same study, against the same reference standard and in the same patients.
Future perspectives

In the current era of emerging novel respiratory viruses there is a growing need for rapid, sensitive and specific identification of these viral pathogens to allow effective prompt antimicrobial therapy, decrease extra diagnostic testing, and implement pathogen-specific infection control measures. Rapid tests have the potential to fulfil these needs, but one should be aware of their current limitations in diagnostic performance and range of pathogens identified. More sensitive and specific rapid multiplex molecular assays are in development. They have the potential to rapidly and accurately identify not only respiratory viral infections, but also bacterial respiratory pathogens simultaneously. At the moment, fully automated molecular methods, such as the FilmArray and the GenMark DX eSensor, are commercially available and presented as designed to be operated at the point-of-care (29). Although these newer tests seem to have diagnostic performances comparable to laboratory-operated PCRs, comprehensive state-of-the-art clinical evaluations of these tests are currently lacking. Besides, a major drawback of these newer diagnostic devices are the high costs and the low sample throughput (30, 31), which limits their opportunities for direct point-of-care use.

We believe that in the upcoming years also the non-molecular rapid tests will still have a role in practical patient care. It is important for clinicians to be aware of their availability and performance characteristics. Our systematic review provides a helpful tool in this understanding. Although some studies have already been performed evaluating the impact of rapid diagnostics on patient management (32-34), we encourage the planning of randomized controlled trials to assess the clinical relevance of rapid tests in terms of clinically relevant outcomes, such as antibiotic use, length of hospital stay, and cost-efficiency. In addition, point-of-care testing requires novel strategies for logistics and organization (35). For successful implementation of rapid tests in the clinic a laboratory validation of the test is not sufficient and a high-quality evaluation study at the point-of-care is required.

Funding

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REFERENCES

Rapid tests for respiratory viruses


18. WHO. In vitro diagnostics and laboratory technology - simple/rapid tests.


32. Rappo U, Schuetz AN, Jenkins SG, Calfeé DP,


### Supplemental Table 1. Characteristics of included studies

<table>
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<tr>
<th>Author1, year1, (x)</th>
<th>Country</th>
<th>Virus type</th>
<th>Population</th>
<th>Point-of-care</th>
<th>Index test</th>
<th>Sensitivity</th>
<th>Specificity</th>
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<td>Al Johani et al, 2011 (1)</td>
<td>Saudi Arabia</td>
<td>influenza A</td>
<td>not reported</td>
<td>Not reported</td>
<td>Directigen EZ Flu A+B</td>
<td>20.6 (8.7-37.9)</td>
<td>99.1 (95.0-100.0)</td>
</tr>
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<td>Alexander et al, 2005 (2)</td>
<td>Australia</td>
<td>influenza A</td>
<td>children</td>
<td>Not reported</td>
<td>Directigen Flu A+B</td>
<td>83.0 (73.8-90.0)</td>
<td>97.9 (92.8-99.8)</td>
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<td>France</td>
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<td>children</td>
<td>Yes</td>
<td>ClearView Exact Influenza A&amp;B test</td>
<td>59.0 (42.1-74.4)</td>
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<td>RSV</td>
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<td>No</td>
<td>Directigen EZ RSV</td>
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<td>adults</td>
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<td>Directigen Flu A+B</td>
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<td>97.9 (95.7-99.7)</td>
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<td>Not reported</td>
<td>mariPOC</td>
<td>12.5 (0.3-52.7)</td>
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<td>mixed</td>
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<td>BinaxNOW Influenza A&amp;B SD Bioline Influenza</td>
<td>64.5 (56.1-72.4) 69.5 (61.2-77.0)</td>
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<td>Eggers et al, 2015 (26)</td>
<td>Germany</td>
<td>influenza A+B</td>
<td>mixed</td>
<td>No</td>
<td>Directigen EZ FLU A+B  Directigen Flu A+B</td>
<td>47.2 (41.5-53.0) 38.7 (35.5-41.9)</td>
<td>99.5 (97.3-100.0) 99.1 (98.1-99.6)</td>
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<td>Finiti et al, 2009 (27)</td>
<td>USA</td>
<td>H1N1</td>
<td>not reported</td>
<td>Not reported</td>
<td>QuickVue Influenza A+B</td>
<td>51.3 (34.8-67.6)</td>
<td>99.0 (98.0-99.6)</td>
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<td>Fuenzalida et al, 2010 (28)</td>
<td>Spain</td>
<td>H1N1</td>
<td>mixed</td>
<td>Not reported</td>
<td>BinaxNOW Influenza A&amp;B</td>
<td>60.4 (53.7-66.8)</td>
<td>93.7 (90.2-96.2)</td>
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<td>Ganzenmüller, et al 2010 (29)</td>
<td>Germany</td>
<td>H1N1</td>
<td>mixed</td>
<td>No</td>
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<td>18.2 (8.2-32.7)</td>
<td>100.0 (97.2-100.0)</td>
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<td>USA</td>
<td>H1N1</td>
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<td>56.6 (47.3-65.5) 59.1 (46.3-71.1) 48.8 (33.3-64.5)</td>
<td>98.8 (97.7-99.4) 97.6 (93.8-99.3) 98.0 (94.3-99.6)</td>
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<td>Gimeno et al, 2010 (31)</td>
<td>Spain</td>
<td>H1N1</td>
<td>adults</td>
<td>No</td>
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<td>32.9 (22.5-44.6)</td>
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<td>Goodrich et al, 2007 (32)</td>
<td>USA</td>
<td>RSV</td>
<td>mixed</td>
<td>Not reported</td>
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<td>59.5 (47.9-70.4)</td>
<td>98.4 (91.5-100.0)</td>
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<td>NL</td>
<td>influenza A+B</td>
<td>adults</td>
<td>No</td>
<td>Directigen Flu A+B</td>
<td>21.5 (12.3-33.5)</td>
<td>100.0 (83.2-100.0)</td>
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<td>Gordon et al, 2009 (34)</td>
<td>Nicaragua</td>
<td>influenza A+B</td>
<td>children</td>
<td>Yes</td>
<td>QuickVue Influenza A+B</td>
<td>68.5 (63.4-73.3)</td>
<td>98.1 (96.9-98.9)</td>
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<td>Gordon et al, 2010 (35)</td>
<td>Nicaragua</td>
<td>H1N1</td>
<td>children</td>
<td>Yes</td>
<td>QuickVue Influenza A+B</td>
<td>64.1 (53.5-73.9)</td>
<td>98.3 (95.0-99.6)</td>
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<td>USA</td>
<td>influenza A&amp;B</td>
<td>children</td>
<td>Not reported</td>
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<td>63.4 (46.9-77.9)</td>
<td>97.4 (94.4-99.0)</td>
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<td>Germany</td>
<td>RSV</td>
<td>influenza A+B</td>
<td>children</td>
<td>No</td>
<td>Abbott RSV Test pack  Directigen Flu A+B</td>
<td>77.4 (65.0-87.1) 23.0 (13.2-35.5)</td>
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<td>India</td>
<td>RSV</td>
<td>children</td>
<td>Not reported</td>
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<td>60.0 (47.9-74.7)</td>
<td>100.0 (88.1-100.0)</td>
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<td>Hamada et al, 2014 (39)</td>
<td>Japan</td>
<td>hMPV</td>
<td>mixed</td>
<td>Not reported</td>
<td>Check hMPV assay</td>
<td>100.0 (78.2-100)</td>
<td>75.0 (19.4-99.4)</td>
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<td>Hara et al, 2013 (season 1) (40)</td>
<td>Japan</td>
<td>H1N1</td>
<td>children</td>
<td>Not reported</td>
<td>QuickNavi-Flu</td>
<td>87.2 (79.7-92.6)</td>
<td>100.0 (95.1-100.0)</td>
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<td>influenza A+B (including H1N1)</td>
<td>children</td>
<td>Not reported</td>
<td>QuickNavi-Flu</td>
<td>96.4 (90.9-97.1)</td>
<td>100.0 (97.3-100.0)</td>
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<td>Hara et al, 2015 (41)</td>
<td>Japan</td>
<td>adenovirus</td>
<td>children</td>
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<td>Japan</td>
<td>H1N1</td>
<td>mixed</td>
<td>No</td>
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<td>Country</td>
<td>Virus type</td>
<td>Population</td>
<td>Point-of-care</td>
<td>Index test</td>
<td>Sensitivity</td>
<td>Specificity</td>
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<td>Harnden et al, 2003 (43)</td>
<td>UK</td>
<td>influenza A+B</td>
<td>children</td>
<td>No</td>
<td>QuickVue Influenza</td>
<td>44.3 (31.6-57.6)</td>
<td>96.9 (91.1-99.4)</td>
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<td>Hawkes et al, 2010 (44)</td>
<td>Canada</td>
<td>H1N1</td>
<td>children</td>
<td>Yes</td>
<td>BinaxNOW Influenza A&amp;B</td>
<td>61.7 (51.8-70.9)</td>
<td>98.6 (92.4-100.0)</td>
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<tr>
<td>Hazelton et al, 2015 (45)</td>
<td>Australia</td>
<td>influenza A+B</td>
<td>mixed</td>
<td>No</td>
<td>Alere Influenza A&amp;B</td>
<td>77.1 (62.7-88.0)</td>
<td>98.7 (95.4-99.8)</td>
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<td>Hazelton et al, 2015 (46)</td>
<td>Australia</td>
<td>influenza A+B</td>
<td>mixed</td>
<td>No</td>
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<td>Hermann et al, 2001 (47)</td>
<td>Sweden</td>
<td>influenza A+B</td>
<td>mixed</td>
<td>No</td>
<td>Sofia Influenza A+B</td>
<td>61.7 (46.4-75.5)</td>
<td>97.4 (93.5-99.3)</td>
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<tr>
<td>Ivaska et al, 2013 (48)</td>
<td>Finland</td>
<td>influenza A+B</td>
<td>children</td>
<td>Yes</td>
<td>mariPOC</td>
<td>25.0 (5.5-57.2)</td>
<td>97.3 (93.1-99.3)</td>
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<td>Hazelton et al, 2015 (46)</td>
<td>Australia</td>
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<td>mixed</td>
<td>No</td>
<td>Sofia Influenza A+B</td>
<td>61.7 (46.4-75.5)</td>
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<td>Hermann et al, 2001 (47)</td>
<td>Sweden</td>
<td>influenza A+B</td>
<td>mixed</td>
<td>No</td>
<td>FLU OIA</td>
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<td>Ivaska et al, 2013 (48)</td>
<td>Finland</td>
<td>influenza A+B</td>
<td>children</td>
<td>Yes</td>
<td>mariPOC</td>
<td>54.3 (36.7-71.2)</td>
<td>98.6 (92.6-100.0)</td>
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<td>Jonckheere et al, 2015 (50)</td>
<td>Belgium</td>
<td>RSV</td>
<td>children</td>
<td>No</td>
<td>BD Veritor RSV</td>
<td>79.1 (71.8-85.2)</td>
<td>96.8 (91.1-99.3)</td>
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<td>Kanwar et al, 2015 (51)</td>
<td>USA</td>
<td>RSV</td>
<td>children</td>
<td>No</td>
<td>BD Veritor RSV</td>
<td>71.2 (61.5-79.6)</td>
<td>1000 (96.2-100.0)</td>
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<td>USA</td>
<td>H1N1</td>
<td>not reported</td>
<td>No</td>
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<td>48.8 (37.4-60.2)</td>
<td>96.6 (92.1-98.9)</td>
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<td>Kasper et al, 2011 (53)</td>
<td>Cambodia</td>
<td>influenza A+B</td>
<td>mixed</td>
<td>No</td>
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<td>52.1 (45.0-58.3)</td>
<td>99.0 (98.2-99.5)</td>
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<td>Keitel et al, 2011 (54)</td>
<td>Switzerland</td>
<td>H1N1</td>
<td>children</td>
<td>Yes</td>
<td>Influenzatop(^\circ)</td>
<td>64.0 (56.2-71.4)</td>
<td>99.3 (96.0-100.0)</td>
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<td>Kenmoe et al, 2014 (55)</td>
<td>Cameroon</td>
<td>influenza A+B</td>
<td>children</td>
<td>Yes</td>
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<td>29.4 (20.8-39.3)</td>
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<td>Khanom et al, 2011 (56)</td>
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<td>RSV</td>
<td>children</td>
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<td>100.0 (96.6-100.0)</td>
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<td>Kidata et al, 2008 (57)</td>
<td>Japan</td>
<td>hMPV</td>
<td>children</td>
<td>Yes</td>
<td>ICassay</td>
<td>70.6 (58.3-81.0)</td>
<td>95.5 (91.4-98.1)</td>
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<td>Kim et al, 2010 (58)</td>
<td>Korea</td>
<td>H1N1</td>
<td>children</td>
<td>Not reported</td>
<td>SD Bioline Influenza</td>
<td>58.9 (52.6-64.9)</td>
<td>98.4 (95.4-99.7)</td>
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<td>Koo et al, 2010 (59)</td>
<td>Australia</td>
<td>H1N1</td>
<td>not reported</td>
<td>No</td>
<td>QuickVue Influenza A+B</td>
<td>60.6 (54.4-66.5)</td>
<td>100.0 (98.4-100.0)</td>
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<td>Koul et al, 2015 (60)</td>
<td>India</td>
<td>influenza A+B</td>
<td>mixed</td>
<td>Not reported</td>
<td>QuickVue Influenza A+B</td>
<td>23.1 (17.3-29.9)</td>
<td>100.0 (99.1-100.0)</td>
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<td>Japan</td>
<td>RSV</td>
<td>children</td>
<td>Not reported</td>
<td>Directigen EZ RSV</td>
<td>69.6 (58.3-79.5)</td>
<td>91.3 (72.9-98.9)</td>
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<td>Kwon et al, 2011 (62)</td>
<td>Korea</td>
<td>H1N1</td>
<td>mixed</td>
<td>Yes</td>
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<td>68.5 (61.1-75.3)</td>
<td>98.4 (96.0-99.6)</td>
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<td>Landry et al, 2008 (63)</td>
<td>Korea</td>
<td>influenza A+B</td>
<td>mixed</td>
<td>No</td>
<td>BinaxNOW Influenza A&amp;B</td>
<td>53.0 (44.2-61.8)</td>
<td>98.1 (93.3-99.8)</td>
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<td>Lee et al, 2010 (64)</td>
<td>Korea</td>
<td>H1N1</td>
<td>not reported</td>
<td>No</td>
<td>Nano Sign Influenza A/B</td>
<td>79.4 (73.1-84.8)</td>
<td>97.2 (95.8-98.2)</td>
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<td>Korea</td>
<td>H1N1</td>
<td>mixed</td>
<td>Yes</td>
<td>SD Bioline Influenza</td>
<td>70.0 (67.4-72.6)</td>
<td>97.5 (96.3-98.4)</td>
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<td>Author et al., year, (x)</td>
<td>Country</td>
<td>Virus type</td>
<td>Population</td>
<td>Point-of-care</td>
<td>Index test</td>
<td>Sensitivity</td>
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<td>Leonardi et al., 2015 (66)</td>
<td>USA</td>
<td>influenza A+B mixed</td>
<td>No</td>
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<td>82.0 (74.3-88.3)</td>
<td>94.7 (91.4-97.0)</td>
<td>97.5 (95.0-99.0)</td>
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<td>Leonardi et al., 2015 (67)</td>
<td>USA</td>
<td>RSV mixed</td>
<td>No</td>
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<td>72.5 (56.1-88.4)</td>
<td>97.4 (94.0-99.1)</td>
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<td>USA</td>
<td>influenza A+B mixed</td>
<td>Not reported</td>
<td>Sofia Influenza A+B</td>
<td>81.5 (78.0-84.5)</td>
<td>97.1 (95.7-98.0)</td>
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<td>Li et al., 2012 (69)</td>
<td>UK</td>
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<td>No</td>
<td>Sofia FlU Cepheid</td>
<td>78.3 (66.7-87.3)</td>
<td>100.0 (95.6-100.0)</td>
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<td>Thailand</td>
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<td>No</td>
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<td>86.8 (83.8-89.5)</td>
<td>68.8 (62.9-74.2)</td>
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<td>Louie et al., 2010 (71)</td>
<td>USA</td>
<td>H1N1</td>
<td>mixed</td>
<td>QuickVue Influenza</td>
<td>65.8 (61.0-70.5)</td>
<td>83.6 (78.0-87.6)</td>
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<td>influenza A</td>
<td>mixed</td>
<td>No</td>
<td>QuickVue Influenza A+B</td>
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<td>98.8 (97.9-99.3)</td>
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<td>Miarka et al., 2014 (73)</td>
<td>Poland</td>
<td>H1N1</td>
<td>mixed</td>
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<td>60.7 (46.8-73.5)</td>
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<td>Miennyk et al., 2011 (74)</td>
<td>USA</td>
<td>RSV</td>
<td>mixed</td>
<td>No</td>
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<td>97.4 (94.5-99.1)</td>
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<td>Mills et al., 2011 (75)</td>
<td>UK</td>
<td>RSV</td>
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<td>83.1 (77.9-87.5)</td>
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<td>Ming et al., 2010 (76)</td>
<td>China</td>
<td>H1N1</td>
<td>mixed</td>
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<td>21.6 (15.8-28.4)</td>
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<td>Mitamura et al., 2013 (77)</td>
<td>Japan</td>
<td>influenza A+B (including H1N1)</td>
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<td>98.8 (95.3-100.0)</td>
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<td>Japan</td>
<td>H1N1</td>
<td>children</td>
<td>Not reported</td>
<td>Capilia FlU A + B</td>
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<td>97.7 (87.7-99.9)</td>
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<td>Munjal et al., 2011 (79)</td>
<td>USA</td>
<td>RSV</td>
<td>children</td>
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<td>99.6 (97.7-100.0)</td>
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<td>Nilsch-Oouch et al., 2012 (81)</td>
<td>Poland</td>
<td>H1N1</td>
<td>children</td>
<td>Yes</td>
<td>Directigen EZ FlU A+B</td>
<td>62.2 (46.7-76.2)</td>
<td>97.1 (91.9-99.4)</td>
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<td>Nitsch-Ouch et al., 2012 (82)</td>
<td>Poland</td>
<td>influenza A+B</td>
<td>children</td>
<td>Yes</td>
<td>Directigen EZ FlU A+B</td>
<td>36.8 (16.3-61.6)</td>
<td>99.2 (95.8-100.0)</td>
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<td>Nogueira et al., 2011 (83)</td>
<td>Spain</td>
<td>H1N1</td>
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<td>No</td>
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<td>Noh et al., 2015 (84)</td>
<td>South Korea</td>
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<td>adults</td>
<td>Yes</td>
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<td>95.9 (92.1-98.2)</td>
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<td>France</td>
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<td>France</td>
<td>H1N1</td>
<td>mixed</td>
<td>No</td>
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1. First author; 2. Year of publication; 3. Citation; 4. Country in which study was performed; 5. Analyzed respiratory virus; 6. Population in which test was performed; 7. If the test was evaluated at the point-of-care; 8. Name of the rapid test; 9. Calculated sensitivity; 10. Calculated specificity.
REFERENCES

15. Brotons P, Launes C, Inigo M, Peris N, Selva...


44. Hawkes M, Richardson SE, Ipp M, Schuh S, Adachi D, Tran D. Sensitivity of rapid


Supplemental Figure 1. Diagnostic accuracy estimates of included studies
Supplemental Figure I. (continued)

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Rapid tests for respiratory viruses
## Appendix A. Literature Search Strategy

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Rapid tests for respiratory viruses

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Deduplication in endote 3527 on dd 18-01-2016
Chapter 3

Evaluation of a rapid antigen detection point-of-care test for respiratory syncytial virus and influenza in a pediatric hospitalized population in the Netherlands


*Equal contribution as first authors
*Equal contribution as last authors

Diagnostic Microbiology and Infectious Disease (2014); 80(4):292-293
ABSTRACT

This pilot study evaluates the diagnostic performance of Sofia RSV Fluorescent Immunoassay Analyzer (FIA) and Sofia Influenza A+B FIA for rapid detection of respiratory syncytial virus and influenza A and B. Sofia had a lower-than-expected sensitivity for all viruses and a high rate of false-positive results for influenza B virus.
Respiratory viral infections are a serious public health concern and the most common cause for hospital admission of children in developed countries (1). Rapid and sensitive diagnosis of respiratory infections is needed to initiate timely and specific treatment, prevent misuse of antibiotics, and avoid infection transmission by optimizing infection control measures (2, 3). Due to its high sensitivity and high specificity, polymerase chain reaction (PCR) has become the reference method for diagnosing viral infections. However, performance of PCR is relatively expensive and may delay treatment as it requires technical expertise, and highly skilled laboratory personnel and equipment are not always directly available. As a result, rapid antigen detections tests or ‘point-of-care tests’ (POCT) are a promising alternative as a time saving method for viral detection (4). Sofia Fluorescent Immunoassay Analyzer (FIA) (Quidel, San Diego, CA) is a rapid fluorescence-based lateral flow immunoassay in which results are analyzed by a compact instrument (Sofia Analyzer). Sofia FIA is designed to be operated by both trained laboratory staff as well as bedside pediatric residents.

In this pilot study, we evaluated the performance of Sofia FIA for detection of respiratory syncytial virus (RSV) and influenza viruses compared to routinely used PCR and Binax blot, a rapid in vitro immunochromatographic assay for detection of RSV. Based on previously published results and information provided by the company, we expected sensitivity and specificity of Sofia FIA for RSV and Influenza in the order of 80% compared to PCR, which would be valued as acceptable (5). Secondly, we evaluated practical implementation and forthcoming clinical consequences of Sofia FIA testing.

The study was conducted at the pediatric intensive care unit (PICU) and infant ward (IW) of the Academic Medical Center (AMC), a university hospital located in Amsterdam, The Netherlands. After laboratory validation, samples were collected from children (aged 0-16 years old) with symptoms of respiratory illness admitted to either PICU or IW from December 2013 until February 2014. Nasopharyngeal swabs (Copan) were collected at the PICU and nasopharyngeal aspirates were taken at the IW. After collection, samples were immediately tested for RSV using Sofia RSV FIA and Binax and for Influenza using Sofia Influenza A+B FIA. An internally controlled multiplex real-time PCR was performed within 24 hours after sample collection using the same material (6). Two Sofia analyzers were provided by the manufacturers during study period for evaluation. Binax and PCR facilities were already operational at the AMC. Sofia testing was performed in accordance with the manufacturer’s instructions. Study sites were instructed to follow the package insert, and the Sofia analyzer was used in compliance with the manufacturer’s user manual. After diagnosis, pediatric residents and laboratory staff were asked to fill in a questionnaire to evaluate the practical benefits and limitations of Sofia
FIA. Influence of diagnosis on changes in isolation measures, discontinuation of antibiotics and initiation of antiviral agents were monitored.

Sixty-six samples were tested for RSV. Twenty-five were positive for RSV by PCR, 19 were positive by Sofia and 20 were positive by Binax. One sample (2.5%) was false positive using Sofia, and 1 was invalid. As compared to PCR results, Sofia had a sensitivity of 75% (95% confidence interval [CI]: 57.7-92.3%) and a specificity of 97.5% (95% CI: 92.7-100%), and Binax had a sensitivity of 80% (95% CI: 64.3-95.7%) and a specificity of 100% (Table 1).

Seventy-three samples were tested using Sofia Influenza A+B FIA. For Influenza A, 10 were positive by PCR and 8 were positive by Sofia. 2 samples (3.4%) were false positive using Sofia, and 5 were invalid. Compared to PCR, Sofia had a sensitivity of 66.7% (95% CI: 35.9-97.5%) and a specificity of 96.6% (95% CI: 92.0-100%) for Influenza A. For Influenza B, 11 of the 73 samples tested were positive by PCR, and 10 were positive by Sofia. Six samples (10.2%) were false positive for Sofia, and 5 were invalid. Compared to PCR, Sofia had a sensitivity of 40% (95% CI: 9.6-70.4%) and a specificity of 89.7% (95% CI: 81.8-97.5%) (Table 2).

Thirty questionnaires on clinical benefits and limitations were completed by pediatric residents. In 8 (26.6%), test result had consequences on isolation measures. In 1 case (3.3%) duration of administering antibiotics was reduced.

Our results show that performance of Sofia FIA, above all for sensitivity, is poorer than expected from other studies (7). Furthermore, rate of false positives is high, in particular for Influenza B. As mentioned by Olsen et al (2014) and Dunn et al (2014) the manufacturer of Sofia issued a recall on specific lots on December 3, 2012, due to issues with false-positive results (8-10). Despite that we used lots that were produced after the recall, our results demonstrate there still might be problems. Evaluation of Sofia by laboratory technicians showed as main limitation the numerous and time-consuming proceedings to perform the test, in contrast to Binax. Furthermore, additional material needed for on-site test implementation, e.g. vortex, clock and tube container, not routinely present on a medical ward, was not supplied. According to pediatric residents who were instructed by the company to perform the tests, Sofia was time consuming and difficult to perform. However, availability of a POCT was highly appreciated.
Table 1. Sensitivity and specificity calculations for Sofia FIA and Binax Blot, RSV.

<table>
<thead>
<tr>
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<th>PCR+</th>
<th>PCR−</th>
<th>Total</th>
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<tr>
<td>RSV</td>
<td></td>
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<td></td>
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<tr>
<td>Sofia+</td>
<td>18</td>
<td>1</td>
<td>19</td>
<td>Sensitivity Sofia: 18/(18 + 6) = 75% (95% CI: 57.7–92.3%)</td>
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<tr>
<td>Sofia−</td>
<td>6</td>
<td>39</td>
<td>45</td>
<td>Specificity Sofia: 39/(39 + 1) = 97.5% (95% CI: 92.7–100%)</td>
</tr>
<tr>
<td>Sofia invalid</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>41</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Binax+</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>Sensitivity Binax: 20/(20 + 5) = 80% (95% CI: 64.3–95.7%)</td>
</tr>
<tr>
<td>Binax−</td>
<td>5</td>
<td>41</td>
<td>46</td>
<td>Specificity Binax: 41/(41 + 0) = 100%</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>41</td>
<td>66</td>
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Table 2. Sensitivity and specificity calculations for Sofia FIA, Influenza A + B.

<table>
<thead>
<tr>
<th>Influenza A</th>
<th>PCR+</th>
<th>PCR−</th>
<th>Total</th>
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<tbody>
<tr>
<td>Sofia+</td>
<td>6</td>
<td>2</td>
<td>8</td>
<td>Sensitivity Sofia: 6/(6 + 3) = 66.7% (95% CI: 35.9 – 97.5%)</td>
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<tr>
<td>Sofia−</td>
<td>3</td>
<td>57</td>
<td>60</td>
<td>Specificity Sofia: 57/(57 + 2) = 96.6% (95% CI: 92.0 – 100%)</td>
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<tr>
<td>Sofia invalid</td>
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<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>63</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Influenza B</td>
<td>PCR+</td>
<td>PCR−</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Sofia+</td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>Sensitivity Sofia: 4/(4 + 6) = 40% (95% CI: 9.6–70.4%)</td>
</tr>
<tr>
<td>Sofia−</td>
<td>6</td>
<td>52</td>
<td>58</td>
<td>Specificity Sofia: 52/(52 + 6) = 89.7% (95% CI: 81.8–97.5%)</td>
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<tr>
<td>Sofia invalid</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>62</td>
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POCTs are a promising alternative as a simple, potentially time-saving, and cheaper method for viral testing. However, due to insufficient performance, POCT testing with Sofia FIA is not yet implemented at our center. For implementation of a POCT at a medical ward or emergency room, several logistic difficulties have to be overcome. Our advice for future implementation of POCTs is 3-fold. First, when implementing a POCT, a laboratory validation of the POCT is necessary. Second, it is strongly advised to provide sufficient training moments for both laboratory staff as well as other personnel involved in using the test to prevent problems with performing and interpreting test results. Third, confirmation of negative POCT results by more sensitive methods such as PCR would be a recommendable practice, and awareness for false-positive-results is necessary.
Conflict of interest

During the study period two Sofia analyzers were provided by the distributor (ITK Diagnostics) for evaluation. ITK Diagnostics had no involvement in the study design, data interpretation, or preparation of the manuscript.
REFERENCES

10. Administration UFD. Class 2 recall Sofia influenza A + B FICA, Kit#2028;2013.
Chapter 4

Detection and monitoring of human bocavirus 1 infection by a new rapid antigen test

Andrea H.L. Bruning, Petri Susi, Hanna Toivola, Andreas Christensen, Maria Söderlund-Venermo, Klaus Hedman, Heli Aatola, Aurelia Zvirbliene, Janne O. Koskinen.

New Microbes and New Infections (2016); 11:17-19
ABSTRACT

Clinically relevant diagnosis of human bocavirus 1 (HBoV1) is challenging, as the virus is frequently detected in asymptomatic patients, and cofindings with other respiratory viruses are common. The clinical value of current diagnostic methods, such as PCR, is therefore low, and alternative diagnostic strategies are needed. We describe for the first time the use of an antigen detection assay for the rapid identification of HBoV1 in a paediatric patient with respiratory tract infection symptoms. We estimate the duration of active HBoV1 infection to be 6 days.
INTRODUCTION

Human bocaviruses (HBoVs) are small single-stranded DNA viruses which belong to the family Parvoviridae (subfamily Parvovirinae). Currently, four HBoV species have been identified. Human bocavirus 1 (HBoV1) was described for the first time in 2005 in children suffering from respiratory tract infection (RTI) (1). Three other HBoVs, designated HBoV2, HBoV3, and HBoV4 have mainly been detected in faeces, but a causal relation with disease such as gastroenteritis is still unclear (2). Prevalence studies using nucleic acid amplification methods show that HBoV1 can be detected in 1.6% to 21.5% of children with symptoms of RTI, particularly during winter and spring (3). Seroepidemiology studies in the United States (4) and in Italy (5) indicated that over 90% of children had antibodies for HBoV by four years of age.

PCR amplification of viral nucleic acids is the most commonly used technique for the detection of HBoV1 in respiratory samples. However, the true clinical impact of a positive PCR result is often difficult to assess as HBoV1 DNA may persist for months in the respiratory tract. The DNA can be encountered in both symptomatic patients and asymptomatic individuals, as well as a cofinding in respiratory infections caused by other pathogens (6).

Nevertheless, causative associations between HBoV1 infection and severe illness, including unexplained severe lower RTI (7) and encephalitis (8), have been described. This emphasizes the need for novel diagnostic methods for accurate and rapid identification of clinically relevant HBoV1 infections. Several techniques for the diagnosis of HBoV1 infection have been developed. These include serology (9), mRNA reverse transcriptase (RT) PCR (10), and DNA PCR in serum and in nasal samples (11). However, these methods are cost and labour intensive and are only available in highly specialized diagnostic laboratories.

The mariPOC test system (ArcDia International Oy Ltd., Turku, Finland) provides a rapid alternative for HBoV1 detection. mariPOC is an automated and point-of-care compatible test for rapid and simultaneous detection of antigens of eight respiratory viruses (influenza A and B, respiratory syncytial virus, adenovirus, human metapneumovirus, parainfluenza type 1, 2, and 3 viruses) and Streptococcus pneumoniae from a single nasopharyngeal sample. Detection of antigens is based on separation-free two-photon excitation fluorometry (12). Recently, a method to detect HBoV1 antigen was added to the test panel. The new HBoV1 antigen test has an analytical sensitivity of 3 ng/mL for recombinant HBoV-like particles (VP2), and it has shown good correlation with HBoV1 mRNA PCR designed to detect acute infection cases only (13). Here we describe the use of the HBoV1 antigen test for the detection and monitoring of HBoV1 in nasopharyngeal samples.
Case Report

A previously healthy girl, 5 months of age, developed symptoms of rhinorrhoea, cough and fever (temperature up to 39°C). The consulting paediatrician diagnosed an upper RTI with no lower respiratory tract involvement or signs of otitis. After written parental consent was obtained, a nasopharyngeal swab was taken on day 2 of illness and tested by the mariPOC for virological diagnosis. Within 20 minutes the sample tested positive for HBoV1 antigen. Retrospective PCR testing confirmed the diagnosis: the nasopharyngeal sample tested positive for HBoV1 DNA by quantitative PCR (14) with a load of $7.7 \times 10^7$ copies/mL and for HBoV1 mRNA by RT-PCR (10) with a Ct value of 26. To follow the course of infection, nasopharyngeal samples were taken on day 3, 4 and 5 from the onset of symptoms. While rhinorrhoea and mild cough continued, the fever declined on day 3. An initial increase in mariPOC assay signal was followed by a gradual decrease (Figure 1). The viral antigen concentrations were extrapolated from a dose-response curve of a positive control dilution series. On day 6, the RTI symptoms were mild, which coincided with a decreasing HBoV1 antigen load.

![Figure 1](image-url)

**Figure 1.** Detection of human bocavirus 1 (HBoV1) antigen by mariPOC test (ArcDia International Oy Ltd., Turku, Finland) during course of respiratory infection. Dashed line indicates cutoff for positive finding. For each day of infection, nasopharyngeal sample was analysed by HBoV1 antigen test.

DISCUSSION

With the mariPOC rapid test system, we were able to *in vitro* diagnose and monitor for the first time the course of a primary HBoV1 RTI by directly targeting the virus proteins. Compared to standard laboratory diagnostics this rapid test is easy to perform and may allow for a clinically accurate diagnosis of HBoV1 infection. The initial HBoV1 antigen detection was confirmed by both DNA and mRNA PCRs. The costs of the mariPOC multianalyte tests are comparable with other antigen detection assays, such as direct fluorescent antibody testing. Compared to rapid
and easy to perform nucleic acid detection methods, e.g. PCR assays with integrated sample preparation, mariPOC immunoassay is a less expensive alternative.

mariPOC test results are typically reported as qualitative. In this study we followed the course of the HBoV1 infection by using the quantitative property of the underlying two-photon excitation technology. However, the nature of swab sampling limits the quantitativeness of the data to semi-quantitative. We showed the duration of this HBoV1 active infection to be approximately 1 week. The patient was HBoV1 antigen positive the morning after symptom onset, and HBoV1 antigen positivity lasted until day 5, when the signal barely exceeded the diagnostic threshold. No sample was available beyond day 5, while the decline in antigen since day 3 and the near-cut off result on day 5 strongly suggest that the patient would have been negative on day 6. These results are in line with the timespan of other respiratory viruses (15) and demonstrate once more that rapid testing should be done as soon as possible after the onset of symptoms and latest within 5 to 6 days.

While most HBoV1 infections are self-limited, they sometimes lead to life-threatening conditions. The availability of a rapid and accurate test for HBoV1 is likely to be useful in differential diagnoses and development of therapy, and will increase our understanding of the epidemiology and clinical impact of HBoV1 infections.

Acknowledgements

Supported in part by the Seventh Framework Programme of the European Union Marie Curie IAPP under contract PIAPP-GA-2013-612308 and partly supported by TEKES, the Finnish Funding Agency for Innovation, under the project name ‘Get it done!’, funding decision 534/14. We want to thank both the patient and her parents for participating in this study and enabling the researchers to precisely follow the course of the infection by testing multiple consecutive samples.

Conflict of Interest

HT, HA and JOK are R&D employees at ArcDia International Oy Ltd. The other authors declare no conflict of interest.
REFERENCES

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Chapter 5

Rapid detection and monitoring of human coronavirus infections


Manuscript in preparation
ABSTRACT

Human coronaviruses (HCoVs) are increasingly recognized as important respiratory pathogens associated with a broad range of clinical outcomes. This report describes a newly developed assay to rapidly detect HCoV infections. With the new rapid test we correctly identified and monitored four HCoV infections in patients with respiratory tract infection symptoms.
INTRODUCTION

Coronaviruses (CoVs) are large, enveloped, single-stranded, positive-sense RNA viruses which belong to the family of the Coronaviridae. Although the first two human CoVs, CoV-229E and CoV-OC43, were already discovered in the 1960s, no special attention was given to them as infections were primarily self-limiting and only associated with mild common cold symptoms (1). Since 2000, several new CoV types have emerged. In 2003, the World Health Organization issued a global alert about a deadly new infectious disease, Severe Acute Respiratory Syndrome (SARS), which turned out to be caused by a CoV (2). Late 2004, a novel CoV -NL63- was isolated from two children suffering from respiratory symptoms in the Netherlands, followed by the discovery of CoV-HKU1 in a patient with pneumonia. In 2012, the Middle East Respiratory Syndrome coronavirus (MERS-CoV) was identified and acknowledged as one of the most dangerous respiratory viruses for humans (3, 4).

As a result, CoVs are increasingly recognized as important pathogens associated with a broad range of clinical outcomes. Molecular techniques, specifically polymerase chain reaction (PCR), have been the method of choice for diagnosing CoV infections but encounter several disadvantages. (Commercial) PCR based methods are often relatively expensive, they require technical expertise, and the presence of viral RNA or DNA does not always reflect acute disease. Moreover, using PCR, CoVs are frequently co-detected with other respiratory viruses and the contribution of positive CoV PCR-results to disease severity is not always clear (5, 6).

Despite the high morbidity and mortality associated with infections caused by some specific CoVs and the frequent detection of CoV in patients with respiratory infections, there is currently no rapid method available that can detect clinically relevant CoVs in humans. The aim of this study was to increase our insight in clinically relevant CoV infections by monitoring antigen concentrations in confirmed CoV patients, using a newly developed assay for the rapid detection of CoV infections.

METHODS

During the influenza season 2015-2016, all employees from a work community of ten persons in Turku, Finland were asked to directly contact one of the research team members when respiratory tract infection symptoms developed. After verification of symptoms and informed consent nasopharyngeal swabs and information on clinical symptoms were collected daily from onset until disappearance of symptoms. After collection, swabs were immediately tested
with a mariPOC® respi test. mariPOC® (ArcDia Int. Ltd, Turku, Finland) is an automated and multianalyte antigen detection test system that enables rapid detection of acute infections. mariPOC® respi test is able to detect nine respiratory viruses (influenza A and B viruses, respiratory syncytial virus, adenovirus, human metapneumovirus, parainfluenzavirus type 1-3, human bocavirus) and Streptococcus pneumoniae from one nasopharyngeal sample at the point-of-care (7). An assay to detect CoV nucleoprotein antigens was recently added to the mariPOC® respi test for research use. This new CoV antigen test has an analytical sensitivity of 2 ng/ml for OC43 recombinant antigen. It cross-reacts with NL63 and 229E but not with other common respiratory pathogens or normal flora.

mariPOC® test results are typically reported as qualitative. For this study we used the semi-quantitative property of the mariPOC® analysis to report CoV-antigen-levels. For verification of results, samples were sent to two laboratories (Laboratory of Clinical Virology, Academic Medical Center, The Netherlands, and the National Institute for Health and Welfare (THL), Finland) for PCR-testing with respectively a multiplex RT-PCR (8) and a coronavirus-species-specific RT-PCR (5).

RESULTS

From December 2015 till March 2016 four out of ten otherwise healthy employees developed respiratory illness symptoms and tested positive for CoV in the mariPOC® assay. PCR confirmed these results. The four CoV were identified as CoV-OC43. Antigen measurement results from (almost) daily collected samples are shown in Figure 1. Antigen secretion correlated relatively well with symptom severity, especially with fever. Patient 1 secreted CoV-antigen at measurable levels for six days from onset of symptoms. Symptoms persisted for nine days and consisted of cough, fever, and fatigue. Fever decreased after day 5. Patient 2 suffered mainly from fever and cough, which diminished after day 6. In patient 3 only rhinitis persisted for six days, but cough was absent after day 4. Symptoms in patient 4 were only mildly present for four days with cough and fatigue, but no fever. All samples with measurable CoV-antigen levels in mariPOC® were also positive by PCR.
Rapid detection of human coronavirus

Figure 1. Detection of CoV antigen by mariPOC® in four patients with respiratory tract infection symptoms. Results are shown from the date of symptom onset. Bars marked with 'NEG' display samples with a mariPOC® signal below the cut-off of for a positive finding. One sample obtained in the middle of the positivity period and marked with an asterisk (*) was also negative with PCR suggesting that sample collection was unsuccessful.

**DISCUSSION**

In conclusion, during influenza season 2015-2016, four patients with confirmed coronavirus infection were monitored with the newly developed CoV antigen assay. The assay reacts with CoV-OC43, -229E and -NL63 but does not differentiate them. For the purpose of this study we further typed the CoVs and all four infections were CoV-OC43. This is in line with previous studies reporting CoV-OC43 as the most prevalent CoV in certain countries (9, 10).

Because of its frequent detection and the sometimes severe complications associated with CoV infection (11, 12) new diagnostic methods to rapidly identify these infections are needed. Here we showed that the new CoV test of the mariPOC® platform correctly identified and enabled monitoring of CoV infections. Compared to nucleic acid amplification based tests, the new antigen test could potentially identify patients in whom CoV is the real cause of the infection since it measures the virus itself and the antigen level needed for detection is achieved only during the acute phase of the infection. However, larger studies are needed to confirm these findings and further determine the diagnostic accuracy of the new assay, not only for CoV-OC43, -229E and -NL63, but also for the other CoVs, i.e. –HKU1, MERS- and SARS-CoV.
Monitoring of the antigen concentrations suggested that viral load peaked around the third and fourth day after symptom onset which confirms the findings in the experimental study by Adney et al (2014) in camels (13). Sampling in adults should therefore be done within the first four days of symptom onset in order to ensure maximum sensitivity of antigen detection testing. Prompt testing and diagnosis also maximizes the potential to affect treatment decisions such as prescribing virus specific drugs, and withholding prescription of antibiotics. The new rapid test might therefore be a valuable contribution to patient care.

Acknowledgements
The authors want to thank all patients for their voluntary contribution to this study.

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Conflict of interest
HA, HT and JOK are R&D employees at ArcDia International Oy Ltd. The other authors declare no conflict of interest.
REFERENCES

Chapter 6

Diagnostic performance and clinical feasibility of a point-of-care test for respiratory viral infections in primary health care


*Equal contribution as last authors

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ABSTRACT

Background: Inappropriately high levels of antibiotics are still prescribed in primary health care for respiratory tract infections (RTIs). Access to diagnostic point-of-care tests (POCTs) for RTIs might reduce this over-prescription.

Objective: The purpose of our study was to determine the diagnostic performance and clinical feasibility of a recently developed diagnostic POCT for respiratory viruses, the mariPOC®, in a Dutch primary health care setting.

Methods: In patients with RTI symptoms presenting to a family practice during the 2015-2016 winter season, we determined the test’s sensitivity and specificity relative to polymerase chain reaction (PCR) testing performed in a laboratory. The clinical feasibility of the POCT was evaluated by interviewing general practitioners (GPs).

Results: One or more respiratory viruses were detected in 54.9% of the patients (n=204). For influenza A virus (n=24), sensitivity of the POCT was 54.2% and specificity was 98.9%; for influenza B virus (n=18), sensitivity was 72.2% and specificity 99.5%; and for respiratory syncytial virus (RSV) (n=12), sensitivity was 50.0% and specificity 100%. In samples with higher viral load, sensitivity was 85.7% for influenza A, 78.6% for influenza B and 85.7% for RSV. The availability of a diagnostic test for respiratory viruses was appreciated by both patients and GPs.

Conclusions: Our study shows that diagnostic POCTs for respiratory viruses might contribute to a precise and evidence-based diagnosis of RTIs and could positively influence prescription of antibiotics by GPs. However, before implementation in primary health care, diagnostic accuracy of the POCT needs improvement and its impact on clinical decision making should be further assessed.
BACKGROUND

Inappropriate use of antibiotics contributes to antimicrobial resistance, increases health care costs and exposes patients to unnecessary risks of adverse drug events (1). The vast majority of antibiotics are prescribed in primary care (2-4). Respiratory tract infections (RTIs) are a frequent indication for prescription although many clinical guidelines recommend restrictive use of antibiotics as RTIs are mainly of viral origin. Despite interventions to reduce overuse of antibiotics, such as the introduction of clinical guidelines, C-reactive protein (CRP) testing and patient and doctor education, inappropriately high levels of antibiotics are still being prescribed (5-7).

Access to point-of-care tests (POCTs) to guide prescription might reduce over-prescription of antibiotics in primary care. POCTs are rapid, easy-to-use tests carried out near the patient by non-laboratory-trained personnel. A benefit of POCTs is their use in helping physicians to manage patients’ expectations for antibiotics and to encourage patients to self-care when suffering from a self-limiting condition (8). At the moment, general practitioners (GPs) in many countries only have access to POCTs for nonspecific biomarkers, such as CRP and cell count. Although these tests have been demonstrated to have an effect on antibiotic prescriptions, recent studies suggest that testing for both biomarkers and for viruses might further reduce these prescription rates (9, 10).

Therefore, new POCTs that can accurately detect pathogens associated with an RTI are required (11). To date, the mariPOC® (produced by ArcDia International Oy Ltd. in Turku, Finland) is the only multianalyte rapid diagnostic test available that is suitable as a point-of-care assay in primary care. While this POCT has been previously evaluated (12, 13), it has not yet been tested as a near-patient assay in a family practice. The purpose of our study was therefore to determine the diagnostic performance and clinical feasibility of this POCT in a Dutch primary healthcare setting.

Our research questions were as follows: How does mariPOC® perform in detecting respiratory viruses in patients with RTI symptoms? Using mariPOC® as a POCT, are GPs and their patients satisfied with the performance and ease-of-use of the test?

In order to answer these questions, we prospectively analyzed the clinical characteristics of all patients, both children and adults, who presented with RTI symptoms to GPs at a single practice during the 2015-2016 winter season. We determined whether or not patients had a respiratory virus using both the POCT and a reference test, the laboratory operated multiplexed polymerase
chain reaction (PCR) test. We calculated sensitivity, specificity, positive and negative predictive value (PPV, NPV) of the POCT and interviewed doctors about the added value of this POCT in primary health care.

**METHODS**

**Study population and sample collection**

This prospective study was conducted in a family practice in the neighborhood of the Academic Medical Center (AMC), Amsterdam, The Netherlands, from November 2015 until March 2016. This family practice serves approximately 10,000 patients and employs seven GPs. The family practice is one of the larger practices in the Netherlands and has been cooperating as a partner within our scientific network for more than fifteen years allowing optimal patient inclusion and study logistics.

All patients of any age with any underlying illness or medical condition presenting to the GP with RTI symptoms during regular opening hours of the family practice were eligible for inclusion. Patients needed to have at least two respiratory symptoms, e.g. cough, rhinorrhoea, headache, myalgia, wheeze, or fever (defined for purposes of this study as > 37.5°C measured by ear thermometer), for less than 7 days.

After giving written informed consent, patients were asked to fill in a questionnaire about their demographical and clinical background and symptoms, and to undergo posterior nasopharyngeal swab sampling. The swab was immediately tested at the primary health care practice using the POCT by a member of the study team. Prior to the start of the study, study team members had received one hour of training from the manufacturer in how to use the test. The remaining sample solution was transferred on the same day to the Laboratory of Clinical Virology at the AMC for reference testing with a multiplex PCR (14). PCR is considered the gold standard for respiratory virus detection (15).

The study was approved by the Medical Ethical Committee of the AMC. Informed consent was obtained from patients or their parents or caregivers before enrolment, with children providing assent if age appropriate.

**mariPOC® test system**

The mariPOC® is an automated, point-of-care compatible antigen test for the detection of nine respiratory viruses (influenza virus types A and B; respiratory syncytial virus (RSV);
parainfluenza virus (PIV) types 1, 2, and 3; human metapneumovirus (hMPV); human bocavirus (HBoV); and adenovirus), and *Streptococcus pneumoniae* in a single nasopharyngeal sample (ArcDia International Ltd, Turku, Finland). Detection of antigens is based on separation-free two photon excitation fluorometry. The signal response in this technique is directly proportional to the analyte concentration in the sample (16). After adding sample buffer to the swab sample, the sample can be inserted into the analyzer. Preliminary results are automatically reported after 20 minutes for medium and high positive samples. After 2 hours, the final results are automatically displayed on the computer. New samples can be inserted for analysis any given time. In our study, the final mariPOC® results were compared with the reference method. *S. pneumoniae* results were not included in this study.

**Reference method for detection of respiratory viruses**

A previously described multiplex PCR (14) was used to test all respiratory samples for the presence of respiratory viruses (influenza virus types A and B; RSV; PIV types 1, 2, 3 and 4; hMPV; HBoV; adenovirus; rhinovirus; human coronavirus (HCoV); enterovirus (EV); and human parechovirus (HPeV)). A threshold cycle (Ct) value of 40 or more was considered to be negative.

**Evaluation of the POCT in primary health care**

Study participants were asked their opinion on the availability of a rapid test for respiratory viruses in primary care on a scale with the following 4 options: ‘useless, no opinion, valuable, very valuable’. After the end of the study period the GPs were interviewed on the feasibility of this POCT in primary care. This structured interview was based on a standardized questionnaire covering the following topics: advantages and disadvantages of the POCT, additional value of the POCT in family practice, influence of the POCT on: prescription of antiviral medication, antibiotics, additional testing, and patient satisfaction.

**Sample size estimation**

To calculate the minimal number of patients needed for inclusion, we performed a sample size calculation. We assumed in this sample size calculation that sensitivity of the test was at least 85% for the two most important viruses, influenza and RSV. We decided, because of the limited information available and the different population in which the test was previously evaluated, to choose a precision (expressed as the maximum width of the 95% confidence interval for sensitivity) of 0.20. With an estimated prevalence of 25% for influenza and for RSV at least 196 patients had to be included.
Data analysis
Data were analyzed using SPSS statistical software (version 22.0, SPPS Inc., Chicago, IL). Sensitivities, specificities, and positive and negative predictive values with 95% confidence intervals (95% CI) were calculated for each virus separately. Readers of both the POCT and the reference standard were blind to the results of the other test and clinical information was not available for assessors of the reference standard. A two-sided p-value <0.05 was considered statistically significant. For data analysis, all infections were considered statistically independent.

RESULTS
Patient characteristics
From 11 November 2015 through 30 March 2016, a total of 204 patients were enrolled in the study. Patient characteristics are summarized in Table 1. The median age of the patients was 33 years (interquartile range (IQR) 13.8–55 years; range 0 to 83 years) and 78 were younger than 18 years of age (38.2%); 123 were female (60.3%). Sixty-four patients (31.4%) reported an underlying condition, the most frequent being an illness of the respiratory tract, e.g. asthma or COPD. The vaccination status was available for 200 patients, of whom 41 had received the annual influenza vaccination. Cough was the most frequently reported symptom, followed by rhinorrhoea and headache.

Respiratory viruses detected in samples
In 54.9% of the samples one or more respiratory viruses were detected by PCR. By POCT one or more respiratory viruses were detected in 18.6% of the samples. The distribution of all respiratory viruses detected by PCR and POCT is shown in Table 2. A total of two samples were positive for two viruses by POCT and eight samples were positive for two viruses by PCR. None of the samples were positive for more than two viruses.
Table 1. Patient characteristics of 204 patients with respiratory tract infection symptoms visiting the family practice from November 2015 until March 2016.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age in years (IQR)</td>
<td>33, 13.8 – 55 years</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>- Male</td>
<td>81 (39.7)</td>
</tr>
<tr>
<td>- Female</td>
<td>123 (60.3)</td>
</tr>
<tr>
<td><strong>Underlying condition</strong></td>
<td></td>
</tr>
<tr>
<td>- Respiratory</td>
<td>64 (31.4)</td>
</tr>
<tr>
<td>- Cardiac</td>
<td>19 (29.7)</td>
</tr>
<tr>
<td><strong>Influenza vaccine received</strong></td>
<td></td>
</tr>
<tr>
<td>- Yes</td>
<td>41 (20.1)</td>
</tr>
<tr>
<td>- No</td>
<td>159 (77.9)</td>
</tr>
<tr>
<td>- Unknown</td>
<td>4 (2.0)</td>
</tr>
<tr>
<td><strong>Clinical symptoms</strong></td>
<td></td>
</tr>
<tr>
<td>- Cough</td>
<td>177 (86.8)</td>
</tr>
<tr>
<td>- Rhinorrhea</td>
<td>168 (82.4)</td>
</tr>
<tr>
<td>- Headache</td>
<td>120 (58.8)</td>
</tr>
<tr>
<td>- Wheezing</td>
<td>83 (40.7)</td>
</tr>
<tr>
<td>- Fever (&gt; 37.5°C)</td>
<td>68 (33.3)</td>
</tr>
<tr>
<td>- Other, of which sore throat</td>
<td>64 (31.4)</td>
</tr>
</tbody>
</table>

Table 2. Respiratory viruses detected by PCR and POCT. Respiratory viruses included in the PCR, but not in the POCT are shown in italic.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of samples positive by PCR, n (%)</th>
<th>Number of samples positive by POCT, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>25 (12.3)</td>
<td>n/a</td>
</tr>
<tr>
<td>INFA</td>
<td>25 (12.3)</td>
<td>15 (7.4)</td>
</tr>
<tr>
<td>HCoV</td>
<td>22 (10.8)</td>
<td>n/a</td>
</tr>
<tr>
<td>INF B</td>
<td>18 (8.8)</td>
<td>14 (6.9)</td>
</tr>
<tr>
<td>RSV</td>
<td>12 (5.9)</td>
<td>6 (2.9)</td>
</tr>
<tr>
<td>hMPV</td>
<td>7 (3.4)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>HBoV</td>
<td>3 (1.5)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>ADV</td>
<td>2 (1.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>PIV-1</td>
<td>2 (1.0)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>PIV-2</td>
<td>2 (1.0)</td>
<td>2 (1.0)</td>
</tr>
<tr>
<td>PIV-4</td>
<td>1 (0.5)</td>
<td>n/a</td>
</tr>
<tr>
<td>EV</td>
<td>1 (0.5)</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>112 (54.9)</td>
<td>38 (18.6)</td>
</tr>
</tbody>
</table>

RV, rhinovirus; INFA, influenza A virus; HCoV, human coronavirus; INF B, influenza B virus; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; HBoV, human bocavirus; ADV, adenovirus; PIV, parainfluenzavirus; EV, enterovirus; n/a, not applicable.
Diagnostic performance of the mariPOC® test

A total of 202 samples were available for evaluation of the diagnostic performance of the POCT as two samples were excluded due to an invalid POCT analysis. Overall, the POCT had a sensitivity of 47.1% (95% CI 35.2-59.4), a specificity of 99.7% (95% CI 99.2-99.9), a PPV of 84.6% (95% CI 68.8-93.6), and a NPV of 97.9% (95% CI 97.1-98.5) for the panel of 9 viruses that it tests for. Sensitivities, specificities, and positive and negative predictive values for the three most frequently detected viruses are shown in Table 3. For influenza A virus (n=24), sensitivity of the POCT was 54.2% (95% CI 33.2-73.8) and specificity was 98.9% (95% CI 95.6-99.8); for influenza B virus (n=18), sensitivity was 72.2% (95% CI 46.4-89.3) and specificity 99.5% (95% CI 96.5-100); and for RSV (n=12), sensitivity was 50.0% (95% CI 22.3-77.7) and specificity 100% (95% CI 97.5-100). Due to the small number of infections with PIV types 1-3, hMPV, HBoV and ADV it was not possible to determine the sensitivity of the POCT for these viruses. Specificity calculations for these viruses resulted in a specificity of 99.0% (95% CI 96.0-99.8), 100% (95% CI 97.6-100), 99.5% (95% CI 96.8-100) and 100% (95% CI 97.7-100) for PIV 1-3, hMPV, HBoV and ADV, respectively.

The PCR technique generates results in terms of cycle threshold (Ct) values, which is a well-established semi-quantitative estimation of viral load (17), with lower Ct values representing higher amounts of virus. We examined whether the accuracy of the POCT depended on the viral load, i.e. whether inclusion of only those samples with a high viral load (defined as having a Ct value<30) could improve sensitivity. Indeed, the sensitivity of the test improved resulting in sensitivities for influenza A virus, influenza B virus and RSV of respectively 85.7% (95% CI 42.0-99.2), 78.6% (95% CI 48.8-94.3), and 85.7% (95% CI 42.0-99.2).

Table 3. Sensitivities, specificities, and predictive values of mariPOC® compared to PCR.

<table>
<thead>
<tr>
<th>Virus</th>
<th>mariPOC®</th>
<th>RT-PCR Positive</th>
<th>RT-PCR Negative</th>
<th>RT-PCR Sensitivity (95% CI)</th>
<th>RT-PCR Specificity (95% CI)</th>
<th>RT-PCR PPV (95% CI)</th>
<th>RT-PCR NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INFA</td>
<td>Positive</td>
<td>13</td>
<td>2</td>
<td>54.2 (33.2-73.8)</td>
<td>98.9 (95.6-99.8)</td>
<td>86.7 (58.4-97.7)</td>
<td>94.1 (89.4-96.9)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>11</td>
<td>176</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INFB</td>
<td>Positive</td>
<td>13</td>
<td>5</td>
<td>72.2 (46.4-89.3)</td>
<td>99.5 (96.5-100)</td>
<td>92.9 (64.2-99.6)</td>
<td>97.3 (93.6-99.0)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>5</td>
<td>183</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INFA or INFB</td>
<td>Positive</td>
<td>26</td>
<td>3</td>
<td>61.9 (45.7-76.0)</td>
<td>99.2 (94.9-99.8)</td>
<td>89.7 (71.5-97.3)</td>
<td>95.7 (93.0-97.5)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>16</td>
<td>359</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV</td>
<td>Positive</td>
<td>6</td>
<td>0</td>
<td>50.0 (22.3-77.7)</td>
<td>100 (97.5-100)</td>
<td>100 (91.7-100)</td>
<td>96.9 (93.1-98.7)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>6</td>
<td>190</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Clinical feasibility
All patients were asked their opinion on the availability of a rapid diagnostic test for respiratory viruses when visiting the GP with respiratory symptoms. Results were available for 202 patients. Most patients (n=151, 74.8%) were positive about the test, with 56.4% (n=114) considering it to be a valuable addition for primary care and 18.3% (n=37) considering it a very valuable addition. In cases where a respiratory virus was detected by the POCT, some patients reported being more confident about the self-limiting aspect of the illness. During the interviews, five out of 7 GPs considered the POCT helpful as they now had a tool to convince patients about the viral diagnosis. Five thought it would have influence on their antibiotic prescription behavior, not only during regular opening hours, but also at the out-of-hours department. According to the GPs, referral to a hospital would not be influenced by this POCT as this is often based on clinical symptoms and not on etiological diagnosis. All GPs thought implementing this POCT in primary health care would increase patient satisfaction, which might contribute to patients’ expectations and self-management, possibly influencing inappropriate referrals, a decrease in additional testing and extra visits. Disadvantages of the POCT are the extra costs and the time interval before final test results are reported, which requires a change in work flow. According to the personnel who performed the POCT, the test was rapid and easy-to-use.

DISCUSSION
In our study we evaluated for the first time the diagnostic performance and clinical feasibility of an automated and rapid test for respiratory viruses at the point-of-care in a primary health care setting in the Netherlands. The results of our study suggest that in the setting of Dutch family practice, the POCT is specific for detecting respiratory viruses in patients with respiratory tract symptoms, but sensitivity is lower than expected.

The fact that one or more respiratory viruses were detected by PCR in 54.9% of the patients in our study indicates that more than half of patients presenting to their GP with respiratory tract symptoms indeed have a viral infection. Our finding that rhinovirus and influenza A virus were the most frequently detected viruses is in line with the epidemiology of respiratory viruses in primary care reported in other studies (18, 19).

In terms of the diagnostic performance of the POCT, here we report sensitivities for influenza A virus, influenza B virus, and RSV that are lower than those reported in previous studies that evaluated this POCT (12, 13). This is likely due to the patients in our sample having a lower viral load than the patients in the other studies, a factor that – as demonstrated here – reduces
the sensitivity of the POCT. Three main factors might have influenced the lower viral load in most of our samples and thus the lower sensitivity. Firstly, our study population consisted mainly of adults, while other studies of this POCT were mainly conducted in children. Children often have higher viral loads which can explain better results in antigen detection tests (20). Secondly, while the previous studies were done in patients who were hospitalized or presented at an outpatient department, here we evaluated the POCT in primary care where patients tend to have less severe illness than those in hospital. Some studies suggest that disease severity correlates positively with viral load (21). Thirdly, many patients in our study visited the GP almost a week after symptoms had started (data not shown). Antigen tests are designed to detect pathogens in the acute stage of the infection, during the first 3-4 days after symptom onset, since this is when viral load is highest (22).

Despite the sensitivity of the mariPOC® test being lower than that found in the previous studies and compared to PCR, we have demonstrated that a substantial proportion of patients with a virus infection can be diagnosed correctly within two hours. The fact that specificity of the POCT ranged from 98.9-100% means that in almost all cases a positive test result correctly rules in a respiratory viral infection. However, correct identification of a viral infection does not rule out a bacterial infection. A limitation of our study is that it did not address the detection of concurrent infections with bacterial pathogens. The diagnosis of bacterial RTIs is notoriously difficult as the presence of respiratory bacteria in nasopharyngeal swabs makes it difficult for a test to distinguish between infection and colonization (23).

Several other limitations of our study need to be addressed. The small number of positive findings for certain viruses (i.e. hMPV, HBoV, PIV types 1-3, and adenovirus) meant we could not calculate the sensitivity of the POCT for each virus included in the panel, although we included more patients than the number suggested by our initial sample size calculation (see Methods). In our study, respiratory virus prevalence was unfortunately lower explaining the wide confidence intervals in the diagnostic accuracy calculations.

Although mariPOC® is the only multianalyte rapid test available that is suitable as a point-of-care assay for RTIs in primary care, the POCT would have had added value if the viral panel that it tests also included rhinoviruses, the most frequently detected virus. Another limitation might be a discrepancy between the number of eligible patients and the number of included patients. The research team continuously motivated GPs to refer patients for study participation, but some eligible patients might not have been recruited. However, compared to other primary care studies, the number of patients that we included was quite high, probably because of the fact that this study was performed in only one family practice and during just one respiratory season.
Our study shows that both GPs and patients consider diagnostic POCTs for respiratory viruses to be a valuable contribution to primary care. The implementation of such POCTs could help in confirming the presence of a viral infection and positively influence the rate of antibiotic prescription by GPs, in the Netherlands, but possibly also in other countries. This is in agreement with the results of an international survey among GPs which indicated an unmet clinical need for a more widely accessible range of POCTs to assist clinicians’ decisions, especially for acute conditions (24). A first explorative interview amongst the GPs revealed positive feedback on the usefulness of the POCT, but a more appropriate qualitative survey needs to increase insight in the potential additional value of POCTs in primary health care. Nevertheless, GPs should consider use of the POCT with care as the diagnostic performance of antigen tests depends on the viral load of the sample, which is in turn influenced by the severity of the illness and duration of symptoms. We have shown with the diagnostic test sensitivities estimated in our study that false-negative results are common. For clinical decision making, the test result should therefore always be interpreted in the light of the patient’s medical history and physical exam.

Our study paves the road to assess new diagnostic opportunities for RTIs in primary health care. To confirm our findings, assess the effect of the POCT on clinical decision making and determine whether using this POCT might positively affect antibiotic prescription rates additional studies are needed, preferably in the form of randomized controlled trials. Besides, it is important to note that multifaceted interventions to reduce overuse of antibiotics are more effective than single initiatives (25). We therefore emphasize that strategies to guide antibiotic prescription should be combined and we encourage the development of improved POCTs, preferably diagnostic POCTs that can detect both viruses and bacteria in combination with biomarkers such as CRP.

CONCLUSION

In summary, diagnostic POCTs for respiratory viruses might contribute to a precise and evidence-based diagnosis of RTIs. In this prospective study we determined the diagnostic performance and clinical feasibility of the mariPOC®, a POCT for the detection of respiratory viruses, in a Dutch family practice. Although the availability of a POCT was appreciated by both patients and GPs, sensitivity of the test was lower than expected. Before implementation of diagnostic POCTs in primary health care, diagnostic accuracy of the POCT need to improve and the impact of POCTs on clinical decision making should be further assessed.
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Declaration

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Ethical approval: The study was approved by the Medical Ethical Committee of the AMC.
Conflict of interest: Academic Medical Center Amsterdam and ArcDia International Oy Ltd are both part of AIROPico, a Seventh Framework Programme of the European Union.
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PART II

Epidemiology of respiratory viruses
Chapter 7

A molecular epidemiological perspective of rhinovirus types circulating in Amsterdam from 2007 to 2012

Lonneke van der Linden, Andrea H.L. Bruning, Xiomara V. Thomas, René P. Minnaar, Sjoerd P.H. Rebers, Janke Schinkel, Menno D. de Jong, Dasja Pajkrt, Katja C. Wolthers.

Clinical Microbiology and Infection (2016); 22(12):1002.e9-1002.e14
Chapter 7

ABSTRACT

Rhinoviruses (RVs) are frequently detected respiratory viruses that cause mild common cold symptoms, but may also lead to more severe respiratory tract infections. The large number of RV types, classified into species A, B and C, hampers clear insights in the epidemiology and clinical significance of each RV type. The aim of this study was to map the circulation of RV types in the Amsterdam area. RV-positive nasopharyngeal and oropharyngeal samples, collected from 2007 to 2012 in the Academic Medical Centre (Amsterdam, the Netherlands), were typed based on the sequence of the region coding for capsid proteins VP4 and VP2. RV-A, RV-B, and RV-C were found in a ratio of 52.4% (334/637), 11.3% (72/637), and 36.2% (231/637), respectively. We detected 129 of the 167 currently classified types. RVs circulated throughout the entire year with a peak in the autumn and a decline in the summer. Some RV types were observed throughout the entire sampling period and others more in a seasonal pattern. Nine RV-A and four RV-B novel provisionally assigned types were identified. This study provides an insight in the molecular epidemiology of RVs in the Amsterdam area. The RVs circulating are diverse and include several provisionally new types.
INTRODUCTION

Rhinoviruses (RVs) are causative agents of upper and lower respiratory tract infections (1). Symptoms range from a common cold to more serious infections such as bronchiolitis, and pneumonia. Also, RVs can cause acute exacerbations of asthma, chronic obstructive pulmonary disease, and cystic fibrosis and have been implicated in the pathogenesis of asthma (2).

RVs belong to the enterovirus (EV) genus of the picornavirus family, and currently 167 RV types have been classified into three species, named A, B, and C (3). Species RV-A and RV-B have been known since the 1950s (1), but species RV-C has only been discovered in 2006 upon the introduction of molecular techniques because these viruses cannot be cultured on standard immortalized cell lines (4–7).

Studies suggested that RV-C infections may elicit more severe disease compared with infections with RV-A or RV-B types (8). However, this has been refuted by several studies which did not observe a difference in outcome between RV-C and RV-A infections (8). Due to the large number of RV types, the severity of infections caused by individual RV types is even more elusive.

As a result of small sample sizes, short observation periods and the large number of RV types there is a lack of insight in the prevalence and circulation patterns of RV types. Compared to the other EVs, RVs co-circulate to a much larger extent, and seasonal patterns are less prominent (1,9,10). Increased insight into prevalence, circulation patterns and clinical significance is not only of importance for surveillance purposes, but also for the future development of antiviral therapy and vaccines. The aim of our study was therefore to investigate the prevalence of RV types in the hospital population of the Academic Medical Centre (Amsterdam, the Netherlands), by genotyping all RV-positive samples submitted for respiratory viral diagnostics from 2007 to 2012.

MATERIALS AND METHODS

Study design

This study was conducted at the Academic Medical Center (AMC) in Amsterdam, the Netherlands. From 2007 to 2012, a total of 6258 nasopharyngeal and oropharyngeal samples were submitted to the Laboratory of Clinical virology, department of Medical Microbiology of the Academic Medical Centre in Amsterdam for diagnostic evaluation. The Academic
Medical Centre receives mainly samples from the southeast area of Amsterdam. The samples were acquired from hospitalized patients and non-hospitalized patients visiting the outpatient clinics or emergence room. The reason for sampling was not systematically documented on the accompanying form for the laboratory and could therefore not be adequately monitored or analysed. Respiratory samples collected for research purposes and those that were not tested for RV were excluded from the analyses. A total of 1102 (17.6%) respiratory samples were positive for RV. There was no material available from five samples for additional gene sequencing. All available RV-positive samples were further characterised by sequencing the VP4/VP2 region. The sampling and virological testing were part of routine care and was executed according to hospital ethical guidelines and the Dutch code of conduct for responsible use of human tissue and medical research 2011.

**Virological assessments**

RNA was extracted from naso- and/or oropharyngeal samples with the MagnaPure LC instrument® using the total nucleic acid isolation kit (Roche Diagnostics). Samples were tested for the presence of RV, EV, human parechovirus (HPeV), influenzavirus A and B (InfA, InfB), para-influenzavirus 1 to 4 (PIV1-4), human bocavirus (hBoV), human coronavirus (hCoV: HKU1, NL63, 229E and OC43), respiratory syncytial virus (RSV), adenovirus (AdV) and human metapneumovirus (hMPV), with a multiplex real-time PCR as described previously (11). Primers used for the detection of RV were reported in Jaramillo-Gutierrez, 2013 (12). Ct values of ≥ 40 were considered negative.

**RV genotyping**

RV-positive samples were genotyped based on a 540-base pair fragment of the VP4/VP2 region as described previously (13) using a two-step semi-nested protocol with primers in the 5′-untranslated region (UTR) and in VP2 (Table 1). The VP4/VP2 sequences were phylogenetically compared to published reference sequences as proposed and provided by McIntyre et al (14). Sequences were analyzed using CodonCode Aligner version 3.7.1, aligned using Clustal X version 3.0.11 and edited using Genedoc version 2.7 software (15,16). Phylogenetic trees were constructed using neighbor-joining trees under a p-distance model as implemented in MEGA version 5.10 (17). Trees were unrooted and bootstrap values were determined from 100 bootstrap resamplings of the original data.

Sequences for which the genetic distance, i.e., nucleotide divergence, was above the threshold of 10%, 9.5%, and 10% for RV-A, RV-B, and RV-C, respectively (14), were submitted to Genbank.
Rhinovirus circulation in Amsterdam

(nr KP003842-KP003896 and KT272022-KT272030) and to the Picornavirus Studygroup to be designated a provisionally assigned type.

Table 1. Primers

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
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<tr>
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</tr>
<tr>
<td>Antisense</td>
<td>9565-reverse</td>
<td>GCA TCI GGY ARY TTC CAC CAN CC</td>
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<tr>
<td>Step 2</td>
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<td>HRV-VP4-2-forward</td>
<td>GGG GAC CAA CTA CTT TGG GTG TCC GTG T</td>
</tr>
</tbody>
</table>

Definitions

If multiple samples were available from the same patient, infections were defined as a new infection when the sample was the first RV-positive sample from that patient, when the sample yielded a different type than the previous sample from the same patient, and/or when the interval period between the two samples was >3.0 months.

As a result of the close genetic relationship between RVs and non-RV EVs, diagnostic detection assays may be cross-reactive and result in a false-positive outcome. An RV false-positive sample was defined as a sample that was RV-positive by real-time PCR and resulted in a non-RV EV sequence after phylogenetic analysis. A sample was only classified as an EV co-infection if the RV infection was verified by RV typing and if either of two EV typing protocols (18) yielded a non-RV EV sequence. The EV-A/EV-B protocol was adapted to a semi-nested PCR and used the same forward outer primer for the first and the second PCR for both EV-A and EV-B.

Statistical analysis

For data analysis, all new infections were considered statistically independent. Data were analyzed with the IBM SPSS Statistics software (version 22.0, IBM Corporation) and GraphPad Prism (version 5.01, GraphPad Software). Categorical variables were compared by means of chi-square test. Differences between continuous variables were determined using Student’s t-test or ANOVA if normally distributed and non-parametric tests if not normally distributed. A two-sided p-value of <0.05 was considered statistically significant.
RESULTS

RV genotyping

We analyzed 1098 RV-positive naso- and oropharyngeal samples. VP4/VP2 sequences were obtained for 745 samples (67.9%), yielding 709 samples with an RV sequence (64.6%, 64/1098) and 36 samples with a non-RV EV sequence (3.3%, 36/1098) (see web-only Appendix, Table A1).

A total of 637 new RV infections were observed in 557 patients of whom 310 (55.7%) were male and 247 (44.3%) were female. Overall, the patients were young children, with a median age at time of infection of 1.6 years (interquartile range (IQR) 0.5-17.6).

Distribution of RV species

Typing of the available samples revealed that 334 of 637 infections (52.4%) typed were caused by RV species A, 72 (11.3%) by species RV-B, and 231 (36.2%) by RV-C. RV-B contains the smallest number of RV types (32 types, compared to 80 RV-A types and 55 RV-C types) and thus one would expect a lower prevalence of RV-B infections if all RV types were equally prevalent. After correction for the skewed proportions of the different species, RV-B types were less prevalent than RV-A and RV-C types (chi-square test, p<0.0001).

Patients infected with RV-B types were significantly older (median 10.4 years, IQR 0.7-35.3) than those infected with a RV-A types (median 1.8 years, IQR 0.5-26.5) or RV-C (median 1.2 years, IQR 0.5-4.3) (Kruskal-Wallis test, p=0.007) (Figure 1). An analysis including only the first infection of each patient resulted in a similar outcome (Kruskal-Wallis test, p=0.002). To investigate whether the low median age of our population could explain the underrepresentation of RV-B infections, we calculated the ratio of RV species in patients over 10 years of age. In this selected patient population the underrepresentation of RV-B in our study was retained (chi-square test, p=0.005), indicating that the lower proportion of RV-B infections was not (solely) due to the lower detection of RV-B in the young children.

The detection of co-infections

In 130 RV infections (20.4%, 130/637) there was a viral co-infection (Figure 2). In the majority of these viral co-infections, there was one co-infecting viral agent (n=104), but up to four co-infecting viruses were detected, of which hBoV (n=42) and AdV (n=33) were the most prevalent.

Co-infection rates were similar between the different infecting RV species (22.4% [74/334] RV-A, 20.8% [15/72] RV-B and 17.7% [41/231] RV-C infected patients, chi-square test, p=0.44).
Figure 1. Age distribution of patients infected with rhinovirus type A (RV-A), RV-B or RV-C. The median and interquartile range are depicted as well as p-values calculated with Dunn-Bonferroni post hoc test.

Figure 2. Observed rhinovirus (RV) co-infections. Patient samples were tested with a multiplex real-time PCR for other respiratory viruses. The viral co-infections detected in RV-positive samples are depicted. Enterovirus (EV) infections were only included if EV genotyping yielded a non-RV EV sequence.
Circulation of RV types

In our population we could detect the majority of the currently classified 167 RV types (77.2% [128/167] of all RV types, 83.8% [67/80] of all RV-A types, 62.5% [20/32] of all RV-B types, and 74.5% [41/55] of all RV-C types) (Figure 3), suggesting that most types circulate ubiquitously. In addition, we detected eight previously described provisionally assigned types (PATs). These are virus strains predicted to be new types based on the VP4/VP2 sequence, but for which the VP1 sequence is not yet available (14). VP1 is regarded as the gold standard for type identification because it shows more sequence variation than VP4/VP2. Furthermore, we detected 31 strains which were classified as 13 novel PATs (9 RV-A and 4 RV-B). Phylogenetic trees including these PATs are provided as supplementary data (see web-only Appendix, Figure S1).

Most types were detected at low frequency, but some types, such as RV-A12 (n=23), RV-A78 (n=24), and RV-C2 (n=27), were present at a higher rate.

Figure 3. Detection of rhinovirus (RV) types. The frequency of all currently known RV types and all detected provisionally assigned types are indicated.
**Time of circulating species and types**

RVs circulated throughout the year, with a slightly higher number of infections occurring in the autumn and a decline in the summer (Figure 4 and web-only Appendix, Figure S2). In the second half of 2009 there was an exceptional large increase in RV-positive samples compared with other years (see web-only Appendix, Figure S2).

Over the year, the species distribution fluctuated slightly (Figure 4). RV-C tended to be more dominant in the winter months, while the rest of the year RV-A infections were more prevalent. RV-B showed low-level circulation throughout the year with a moderate increase of infections in the second half of the year.

Specific RV types (e.g., RV-A29) were consistently detected throughout the study period, while other RV types were mainly detected in peak seasons (see web-only Appendix, Figure S3). For example, RV-A12 was detected in clusters in the winters of 2007/2008, 2009/2010, and 2011/2012. Furthermore, RV-A101 was only detected during a small outbreak in August/September 2008 (n=11) and a few times in September/October 2011 (n=4). Part of these RV-A101 infections may have been hospital-acquired as deduced from the reason for admission and the time between the admittance of the patient and the sampling, but other RV infections were clearly contracted outside of the hospital (sample taken within one day of admittance to the hospital for respiratory illness). RV-C2 and RV-A78, the most commonly detected RV types in our population, were detected every year and followed a similar pattern as RVs in general: a higher detection rate in the autumn/winter and less infections in the summer.

![Figure 4](image.png)

**Figure 4. Frequency of rhinovirus type A (RV-A), RV-B and RV-C infections per month.** The amount of infections with RV species A, B and C observed in each month, pooling the years 2007-2012. The year 2009 was excluded, as RV circulation in that year was atypical.
DISCUSSION

In our study we described the prevalence of RV types in Amsterdam by typing RV-positive samples submitted for diagnostics in 2007-2012 to the Academic Medical Center. In our population we found a large number of different RV types, of which most belonged to species RV-A, and the least belonged to species RV-B. Relatively many RV strains were classified as novel PATs.

Among the 637 typed RV infections, 52.4% (334/637) belonged to RV-A, 11.3% (72/637) belonged to RV-B, and 36.2% (231/637) of the typed RV samples belonged to RV-C. The detection rates in our study are in line with previous studies that reported ratios of 47-64%, 2-13%, and 21-45% for respectively RV-A, RV-B, and RV-C (19–23). RV-A and RV-C infections are consistently reported to be more frequent than RV-B infections.

We could detect most of the currently classified types even though our samples were obtained in a geographically small area. These results suggest that most RVs are widespread, which is in agreement with previous reports that also describe the simultaneous circulation of a high number of types (20,24,25). Nevertheless, some types have repeatedly been detected at higher frequencies than other types over the world (14,22,26,27). Most of these types were also present at intermediate-to-high frequency in our population, such as RV-A12, RV-A78, RV-A101, RV-B104, RV-C2, RV-C16, and RV-C43. A limitation of our study was that we used population sequencing, which in most cases only detects the major type present and misses most RV-RV co-infections. However, there were no indications for RV-RV co-infections, such as sequence ambiguities.

4.8 percent (36/745) of all sequences obtained from RV-positive samples were characterised as an EV sequence. The finding of EV sequences is reflective of the well-reported cross-reactivity of the RV detection PCR for non-RV EVs, due to their close relationship and the conservation of the 5′-UTR (12,28). Almost half of the EVs (15/36) were EV-D68, an EV which is associated with respiratory diseases. The same holds true for EV-C104, which was detected in five samples (from four infections). The other EVs detected are found only occasionally in respiratory samples (8,29).

Our study monitored circulation of RVs over a relatively long time period, which allowed us to examine the circulation of RV species and types over six years, thereby limiting the influence of single outbreaks. Our observation that RVs circulate during the whole year has repeatedly been found (1,8). Interestingly, Linder et al. also reported a dominance of RV-C in the winter months.
We found that some types circulate persistently, but for other types we saw a different circulation pattern where the type was detected mainly in the winter months and occasionally early spring but not throughout the rest of the year. Further studies are required to determine if this circulation pattern is type-specific or due to the small sample size of specific RV types in our study.

We were able to detect no less than 13 putative new RV types. The finding of this many possible new types was unexpected compared to the 20 new RV types / PATs reported since 2010 (complete genome, VP1 or VP4/VP2 sequence published in 2010 or later, or sequence made available to the Genbank in 2010 or later if unpublished, www.picornastudygroup.com), though VP1 sequencing is in progress for our strains to confirm that these are new types. Theoretically, the large amount of new PATs could be due to local circulation of specific RV types, but as widespread occurrence has been reported for most RV types (14), we hypothesise that there may be many more RV types to be discovered and classified.

In July and August 2009 an atypical high number of RV infections was detected. This may be due to increased sampling as a result of the start of the influenza H1N1 epidemic (31). This emphasizes that one should be aware that the number of incoming samples can confound the estimated incidence when studying epidemiology by typing samples submitted for diagnostics. This is especially the case for a virus like RV, of which infections occur at high frequency and often asymptomatically.

Our study has some limitations. We were not able to perform associations between the clinical symptoms of the patients and the different RV types due to the retrospective character of the study and the limited information provided by the clinicians on the accompanying form to the laboratory. Because of the limited clinical information and heterogeneous composition of our study group, we were not able to evaluate the prevalence of various RV types in specific populations. A selection bias is that our study is composed of individuals attending the hospital and therefore might not completely reflect the prevalence of RV types in the general population. Nevertheless, the large variety in RV types in our population points towards continuous variations in RV types co-circulating in the community.

In summary, in this study we present an overview of the RV types circulating in the Amsterdam area in the Netherlands in the years 2007 to 2012. RV epidemiology is complex and many RV types are co-circulating simultaneously. RV-targeted vaccination and antiviral strategies should therefore aim for broad-spectrum activity and can probably not afford to focus on a selection of dominant RV types.
Conflict of interest and funding

L.L. and X.T. were completely and A.B. was partially funded by a grant from the Crucell Vaccine Institute (CVI). K.W. and A.B. are partially funded by the Seventh Framework Programme of the European Union IAPP (AIROPico, PIAPP-GA-2013-612308). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

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REFERENCES


Supplementary table A1. Enterovirus types detected

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<td>E6*</td>
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<td>EV-D68</td>
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*Typed by Blast analysis. * Including one repeat sample.
Supplemental Figure S1.
Supplementary Figure 2.
Supplementary Figure 3.
Chapter 8

Clinical, virological and epidemiological characteristics of rhinovirus infections in early childhood: a comparison between non-hospitalised and hospitalised children


*Equal contribution as first authors
±Equal contribution as last authors

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ABSTRACT

**Background**: Several studies have been published regarding the epidemiology and clinical significance of the different rhinovirus (RV) species (-A, -B and -C). However, data on RV types and the associations with clinical outcome in young children are limited. Here, we investigated the clinical, virological and epidemiological characteristics of RV infections in young children with mild or asymptomatic infection (non-hospitalised children) and in symptomatic young children admitted to the hospital.

**Objectives**: The aim of this study was to evaluate associations between different characteristics of RV infections and clinical outcome in young children.

**Study design**: RV-infected children were retrospectively selected from a Dutch birth cohort (EUROPA-study) and from hospitalised children admitted to the hospital because of respiratory symptoms. In total 120 RV-typed samples could be selected from 65 non-hospitalised and 49 hospitalised children between November 2009 and December 2012.

**Results**: RV-A was the predominant species in both study populations, followed closely by RV-C. RV-B was observed only sporadically. The distribution of the RV species was comparable in non-hospitalised and hospitalised children. In children with respiratory distress who required ICU-admission the distribution of RV species did not differ significantly from the non-hospitalised children. No predominant RV type was present in non-hospitalised nor hospitalised children. However, hospitalised children were younger, had more often an underlying illness, a higher RV load and more frequently a bacterial co-infection.

**Conclusions**: Clinical outcome of RV infected young children was not related to RV species or types, but may more likely be influenced by multiple (host-specific) factors.
INTRODUCTION

Rhinoviruses (RVs) are causative agents of frequently and recurrently occurring respiratory tract infections in young children. Symptoms range from a mild common cold to severe life-threatening pneumonia and about 1/3 of RV infections are asymptomatic (1). RVs are classified phylogenetically into three species: RV-A, RV-B, and RV-C, and are further divided into > 150 types (2).

Recently, several studies have been published regarding the molecular epidemiology and clinical significance of RVs, suggesting the infecting species might influence the clinical outcome (3, 4). RV-A and RV-B have been associated with mild disease, whereas RV-C may cause more severe disease (5). Particularly in children, RV-C seems to be associated with asthma exacerbations, acute lower respiratory tract infections, bronchiolitis, and pneumonia (6) (7).

However, RV species may not be the sole determinant of clinical outcome of an RV infection. Given the wide diversity of RV types within RV species, the RV type rather than the species may relate to severity of disease (8). Together with host susceptibility, the RV type and the cellular tropism, as partly determined by the virus receptor used, may be important factors contributing to disease and clinical outcome in young children (9). Up to date, data on the relation between RV types and clinical symptoms combined with the occurrence of RV types during various seasons in one year and circulation patterns are limited and often available either for hospitalised or asymptomatic children.

OBJECTIVES

This study describes the clinical, virological and epidemiological characteristics of RV infections in young children with mild or asymptomatic infection who do not need hospitalisation for their respiratory tract symptoms, as compared to young children in need of admittance to the hospital because of their respiratory tract disease. By comparing these groups associations between the different characteristics of RV infections, i.e. the infecting species and types, viral load, viral and bacterial co-infections, and seasonality, in relation to clinical outcome are studied.
STUDY DESIGN

Study population and sample selection

This retrospective study consisted of successfully sequenced RV-positive respiratory samples (naso- or oropharyngeal swabs and washes) from young children with mild or asymptomatic RV infection (non-hospitalised children) and from hospitalised children. For this study we selected children that met the following criteria: (i) date of birth between May 2008 and May 2010, (ii) sampling date between November 2009 and December 2012, (iii) a respiratory sample positive for RV in the 5'UTR multiplex PCR and successfully VP4/VP2 sequenced.

Non-hospitalised children were selected from the EUROPA-trial (Early Unbiased Risk Assessment of Paediatric Asthma), a prospective cohort study in Amsterdam, the Netherlands, which focused on the prediction of early signs of asthma (10). In this study, samples were obtained from children visiting the family physician with respiratory tract symptoms and from asymptomatic children. The population of hospitalised children consisted of retrospectively selected samples from children admitted to the Academic Medical Center (AMC), Amsterdam, because of respiratory tract disease (defined as a combination of one or more of the following symptoms: fever, rhinitis, cough, dyspnoea, tachypnoea, and/or wheezing). To investigate circulation of RV species and types in children with more severe illness we performed a subgroup analysis within the hospitalised population of children admitted to the Intensive Care Unit (ICU) due to respiratory distress.

Information about the children from the EUROPA-study was obtained from study report forms and all children with mild or asymptomatic infection were monitored for the development of severe disease that required hospitalisation at a later time point. The EUROPA-study was approved by the Medical Ethical Committee of the AMC (09/066) and the parents gave written informed consent. The EUROPA study is registered in the Dutch Trial Register (NTR-1955). The samples taken from the hospitalised children were part of routine diagnostic tests and were processed according to hospital ethical guidelines and the Dutch code of conduct for responsible use of human tissue and medical research in 2011. A standardized record form was designed to obtain the patients’ information from medical records, including demographic and clinical characteristics.

Virological and bacterial assessments

All respiratory samples were assessed within 24h after collection at the Laboratory of Clinical Virology of the AMC according to standard operating procedures for the presence of respiratory
tract associated viruses (enterovirus (EV), human parechovirus (HPeV), influenzavirus A and B, para-influenzavirus 1-4, human bocavirus (HBoV), human coronavirus, respiratory syncytial virus (RSV), adenovirus and human metapneumovirus), using a multiplex polymerase chain reaction (PCR) as described previously by Jansen et al (11) with adapted RV primers published elsewhere (12). A Ct-value of 40 or more was considered to be negative. The complete protocol for RV typing is provided as supplementary data (see Supplementary file). All RVs were divided in (i) the major group (which bind to the intercellular adhesion molecule 1 (ICAM-1)), (ii) the minor group (which use the low-density lipoprotein receptor (LDL-R) as a receptor), and (iii) the RV-C group which most probably uses the human cadherin-related family member 3 (CDHR3) receptor (13, 14). Information on bacterial co-infection was included in case a bacteriological culture was performed on the same day, or one day before or after virological sampling.

**Data analysis**

An infection was classified as a new infection in the event of a first RV-positive sample from a patient. If multiple samples were available from the same patient, infections were defined as a new infection when the sample yielded a different type than the previous sample, and/or when the time between the two samples was at least three months.

For data analysis, all infections were considered statistically independent. To avoid overrepresentation of data only the first RNA positive sample from a new infection was included. Data were analysed using SPSS statistical software (version 21.0, SPSS Inc, Chicago, IL). Categorical variables were compared by means of chi-squared test. Differences between continuous variables were determined using Student’s t test or ANOVA if normally distributed and non-parametric tests if not normally distributed. A two-sided p-value <0.05 was considered statistically significant.

**RESULTS**

**Patient characteristics**

Following our selection criteria we were able to include 120 RV-positive respiratory tract samples. A total of 68 RV-positive samples were included from children with mild or asymptomatic infection (non-hospitalised children) and 52 RV-positive samples were included from children who needed hospitalisation because of respiratory symptoms. Patient and virological characteristics are summarized in Table 1.
In the hospitalised group, 26 children were admitted to a general ward and 26 to the Intensive Care Unit (ICU) due to respiratory distress. Of the ICU-admitted children, 57.7% (n=15) had an underlying illness, most frequently prematurity (n=7), which is comparable with the hospitalised children who were not ICU-admitted (underlying illness: 46.2% (n=12)), of which seven were premature. No manifest underlying illness was present in the non-hospitalised children at inclusion. At the time of RV infection the hospitalised children were younger (median 0.8 years, interquartile range (IQR) 0.5 – 1.8) than the non-hospitalised children (median 1.5 years, IQR 1.0 – 2.2) (p<0.001).

Table 1. Patient and virological characteristics at time of infection

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<td>Co-infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral coinfection</td>
<td>34 (50.0)</td>
<td>23 (44.2)</td>
<td>9 (34.6)</td>
</tr>
<tr>
<td>Bacterial coinfection</td>
<td>1 (1.5)</td>
<td>14 (70.0)</td>
<td>12 (66.7)</td>
</tr>
<tr>
<td>(32 samples unknown)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Ct-value (SD)</td>
<td>28.3 (2.3)</td>
<td>26.0 (2.9)</td>
<td>25.7 (3.3)</td>
</tr>
</tbody>
</table>

RV, rhinovirus; ICU, Intensive Care Unit; IQR, interquartile range;
**RV species and types**

The prevalence of RV species, stratified by study group, is illustrated in Fig. 1. In both groups the most dominant species was RV-A, followed by RV-C and RV-B. The distribution of RV species was comparable between non-hospitalised and hospitalised children ($p=0.41$). Also, the distribution of RV species in the ICU-admitted children from the hospitalised group did not differ significantly from the non-hospitalised children ($p=0.84$). When dividing the RVs according to their receptor usage (ICAM-1, LDL-R and most probably CDHR3), Table 2 shows that the receptor use was comparable among the non-hospitalised and the hospitalised children, as well as among the ICU-admitted children.

When we further analysed the study groups for the occurrence of RV types, we did not observe skewing towards one type in either the hospitalised or non-hospitalised group (Fig. 2). We observed 19 RV types in both populations, while the other 48 RV types were detected in either hospitalised or non-hospitalised children. RV-A78 was the most prevalent type in non-hospitalised children, whereas in the hospitalised children these were RV-A78 and RV-A12. Within the RV-B species, RV-B6 and RV-102 were only seen in hospitalised children and RV-B27 solely in non-hospitalised children. Provisionally assigned type B4 (Bpat4), B5 (Bpat5) and B7 (Bpat7) were detected in both populations. For RV-C, the most prevalent type was RV-C2 in hospitalised children and RV-C43 in non-hospitalised children. The most commonly detected types in ICU-admitted children (RV-A12, RV-A28, RV-A89, and RV-C6) were all detected in non-hospitalised children as well.

![Figure 1. Distribution of RV species in non-hospitalised, hospitalised and ICU-admitted children. Distribution of RV species was comparable in all populations.](image-url)
Table 2. RV receptor

<table>
<thead>
<tr>
<th>Receptor (%)</th>
<th>New infections in non-hospitalised children (n =68), n (%)</th>
<th>New infections in all hospitalised children (n=52), n (%)</th>
<th>New infections in ICU admitted children (n=26), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1a (%)</td>
<td>38 (55.9)</td>
<td>24 (46.2)</td>
<td>13 (50.0)</td>
</tr>
<tr>
<td>LDL-Rb (%)</td>
<td>2 (2.9)</td>
<td>1 (1.9)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>CDHR3c (%)</td>
<td>22 (32.4)</td>
<td>21 (40.4)</td>
<td>9 (34.6)</td>
</tr>
<tr>
<td>Receptor to be determined</td>
<td>6 (10.3)</td>
<td>6 (11.5)</td>
<td>4 (15.4)</td>
</tr>
</tbody>
</table>

a ICAM, intercellular adhesion molecule 1.
b LDL-R, low-density lipoprotein receptor.
c CDHR3, human cadherin-related family member 3.

Figure 2. Frequency of RV types. For each species the prevalence of detected types in both populations is illustrated. White bars indicate non-hospitalised children and shaded bars indicate hospitalised children.
Viral load

Ct value is a well-established semi-quantitative measurement of viral load (15). The mean Ct-value of RV infections in children was significantly lower than the mean Ct-value of the samples from the non-hospitalised children (Ct-value 26.0 versus 28.3, \( p < 0.001 \)) (Table 1), implying a higher viral load in symptomatic children.

Viral and bacterial co-infections

In the non-hospitalised and hospitalised children, a viral co- or triple-infection was detected in 50.0% \((n=34)\) and 44.2% \((n=23)\) of the samples respectively. The most frequently detected co-infecting viruses in the non-hospitalised group were HBoV \((30.9\%, n=21)\), adenovirus \((13.2\%, n=9)\), and RSV \((13.2\%, n=9)\). In the hospitalised children, RSV was the most common viral co-pathogen \((19.2\%, n=10)\). A viral co-infection was detected in 34.6% \((n=9)\) of the ICU-samples, five of which were with RSV. There were no significant differences between the study groups regarding the number of viral co-infections and there was no significant difference in the number of viral co-infections detected in children infected with RV-A, -B, or –C.

A bacterial co-infection (as diagnosed by a positive bacterial throat or sputum culture) was present in only one of the non-hospitalised children, while positive bacterial cultures were detected in 14 \((70.0\%)\) of the 20 hospitalised children. From the remaining hospitalised children there was no information available on bacterial co-infections. The most frequently detected pathogens were Moraxella catarrhalis \((n=6)\) and Haemophilus influenzae \((n=5)\). Although the numbers of children with bacterial co-infections were small there was no skewing towards one RV species \((p=0.26)\). In ICU children a bacterial co-infection was present in at least 66.7% of the samples. The most frequently detected pathogens were M. catarrhalis \((n=5)\) and H. influenzae \((n=5)\).

Seasonal distribution of RV species and types

Combining both populations, RV infections were seen all year round with a peak in incidence in the autumn and winter of 2010 (Figure 3). Figure 4 shows that there is no distinct circulation pattern for the different RV types in time. Some trends could be observed. Type RV-A78, which was the most prevalent strain in the hospitalised population, was found only between July 2010 and July 2011. The second most prevalent type, RV-A12, was detected in the spring of 2010 and was sporadically detected again, but only in non-hospitalised children in the winter of 2011-2012. RV-C2, the most prevalent RV-C, was mainly observed during the autumn and the beginning of winter 2010-2011. In conclusion, despite small numbers no season-specific types could be observed.
**Figure 3.** Seasonal distribution of RV species. Stacked bars indicate the seasonal distribution of RV species.

**Figure 4.** Circulation of RV types in non-hospitalised and hospitalised children. The $x$-axis indicates the study period and the $y$-axis indicates the detected types from corresponding species detected during study period. White diamonds indicate non-hospitalised patients and black circles indicate hospitalised patients.
DISCUSSION

Here we described for the first time the clinical, virological and epidemiological characteristics of RV infections in young children with mild or no symptoms compared to symptomatic children hospitalised due to respiratory tract disease.

RV-A was the predominant species in both study populations, followed closely by RV-C. RV-B was observed only sporadically. These findings are in accordance with other studies (9, 16, 17) in both non-hospitalised and hospitalised children and suggest that our populations are representative for larger populations.

The distribution of the RV species was comparable in non-hospitalised and hospitalised children. Also in children with respiratory distress who required ICU-admission the distribution of RV species did not differ significantly from the non-hospitalised children. RV-C was not overrepresented in hospitalised children nor in ICU-admitted children suggesting that in our study populations an infection with RV-C alone might not be associated with more severe disease. Previous studies on disease severity showed that infection with RV-C was associated with more severe respiratory symptoms in children. However, these studies were mainly conducted in hospitalised patients where specific risk groups, such as immunocompromised patients, were overrepresented which might bias the results (5, 18). More recent studies are consistent with our findings (3, 19, 20). In addition, several studies in adults were in line with our results (21, 22).

We detected a large number of RV types by using the VP4/VP2 fragment for typing. A recent study by Martin et al. (2015) found that specific RV-A and RV-C types were associated with lower respiratory tract infections (8). We found several types in both populations and no predominant RV type could be found in severely ill children. However, due to the relatively small study population no definite conclusions can be made regarding specific RV types and clinical outcome.

When comparing the mild or asymptomatic RV infected children with the hospitalised children several differences between the study groups that might be important determinants for clinical outcome emerged. When evaluating the patient characteristics we found a high percentage of the hospitalised children having an underlying illness. Prematurity was the most common and a probable risk factor for more serious RV disease. Secondly, the median age in the hospitalised children was significantly lower than the median age at sampling for non-hospitalised children. As younger children are more vulnerable for hospitalisation this was not an unexpected finding.
However, as our study is not a matched case-control study we cannot exclude an age-specific
distribution of RV species and types. Ct-values differed significantly between non-hospitalised
and hospitalised children. We found a higher viral load (indicated by lower Ct values) in
hospitalised children, suggesting that a higher viral load might lead to more severe disease (15,
23). This has been described in other studies as well (24). Another influencing factor might
be infection with co-pathogens. The frequency of viral co-infections was comparable in both
groups, but a bacterial co-infection was detected more often in severely ill children. Our study
indicates that bacterial co-infection, and especially co-infection with respiratory pathogens such
as *M. catarrhalis* and *H. influenzae*, might be an important contribution to disease severity.

Some limitations of our study need to be considered. First, because of the retrospective study
design in combination with the relatively small sample size a matched case-control study was
not possible. By selecting children of comparable age and period of sample collection we made
the study groups as comparable as possible. A post hoc power analysis showed that with our
sample size we would be able to detect large differences between the study groups indicating
that our sample size was sufficient to support our main finding that RV species were equally
distributed among all study groups. Information on bacterial co-infections was not available
for all patients. Previous studies describing relations between molecular epidemiology of RV
infections and clinical outcome did not take bacterial co-infections into account (8, 25). Based
on the results from our study bacterial co-pathogens might be an important contributing factor
to disease severity and justify the inclusion and analyses of influences of these bacterial co-
pathogens in future studies.

In conclusion, our results support previous findings suggesting that clinical outcome is not
related to RV species or types alone, but may more likely be influenced by multiple (host-specific)
factors, such as age, chronic underlying illness, viral load and the presence of a bacterial co-
infection. A more detailed investigation of host-specific genetic factors, such as polymorphisms
in susceptibility genes (e.g. CDHR3) and alterations in chemokine genes would contribute to
an even better understanding in the relation between RV and clinical outcome. To further
elucidate this association, large longitudinal cohorts of RV-infected patients and samples from
both the acute period of infection as well as follow-up samples are needed.
Conflict of interest
All authors approved the final manuscript and they have no conflict of interest to declare.

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Authorship contribution
M.S., P.S., M.D., K.W. and D.P. contributed substantially to the conception of the work. A.B., X.T., L.L., J.W., R.J., and R.M. analysed and interpreted the data. A.B. and X.T. drafted the manuscript and all authors revised it critically for important intellectual content. All authors approved the final version of the manuscript for publication and agreed to be accountable for all aspects of the work.

Acknowledgements
We owe our gratitude to all the parents and children contributing to this study from the Emma Children’s hospital and the EUROPA-study. Furthermore we wish to acknowledge the significant contribution of Janke Schinkel, Simone Hashimoto, Aline Sprikelman, Wim van Aalderen and Eric Haarman, without whom this study would not have been possible. Part of this work is presented at the Scientific spring Meeting of the Dutch Society of Medical Microbiology and the European Society for Pediatric Infectious Diseases.

Appendix A. Supplementary data
Supplementary data associated with this article can be found in the Supplementary file.
REFERENCES

13. Schuler BA, Schreiber MT, Li L, Mokry M, Kingdon ML, Raugi DN, et al. Major and minor group rhinoviruses elicit differential signaling and cytokine responses as a function...


Supplementary material: RV typing

RV RNA was extracted from 200 µl RV-positive sample with the MagnaPure LC instrument® using the total nucleic acid isolation kit (Roche Diagnostics). Genotyping was performed by amplifying a 540-base pair fragment spanning part of the 5’- untranslated region (UTR), capsid protein VP4 and part of VP2 (VP4/VP2) of the RV-genome using a two-step semi-nested protocol (1). First, 6 µl of RNA was reverse transcribed and amplified with the SuperScript III one-step RT/Platinum Taq polymerase kit (Invitrogen) according to the manufacturer’s instructions using primers adapted from Savolainen et al (2). One µl of the combined RT-PCR product was then used as input for the second semi-nested PCR amplification. The reaction mix contained 1x PCR buffer, 2.5 mM MgCl₂, 0.5 µM of each primer, 200 µM of each dNTP, 0.1 µg/ml BSA, and 0.05 U of FastStart Taq polymerase (Roche) in a 20 µl-reaction volume. Cycling conditions were as follows: 94°C for 2 min and 30 cycles each consisting of 94°C (18 sec), 55°C (21 sec) and 72°C (90 sec). Amplicons were sequenced using primers used for the second step of the semi-nested protocol with the BigDye Terminator reaction kit (Applied Biosystems).

Species were determined by phylogenetically comparing VP4/VP2 sequences with published reference sequences as proposed and provided by McIntyre et al (1). Sequences were analysed using CodonCode Aligner version 3.7.1, aligned using Clustal X version 3.0.11 and edited using Genedoc version 2.7 software (3, 4). Phylogenetic trees were constructed using neighbour-joining trees under a p-distance model as implemented in MEGA version 5.10 (5). Trees were unrooted and bootstrap values were determined from 100 bootstrap resamplings of the original data.

Cross-reactivity of EV with RV was suspected when both EV and RV PCR were positive and typing resulted in an EV type or when only RV PCR was positive and typing resulted in an EV type. These samples were considered to be EV positive and RV negative.

For a complete overview of the number of samples screened in both study groups and the number of successfully sequenced samples see Table 1.
<table>
<thead>
<tr>
<th>Outcome of the typing assay</th>
<th>Non-hospitalised children</th>
<th>Hospitalised children</th>
</tr>
</thead>
<tbody>
<tr>
<td>- no RV (i.e. EV positive)</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>- successfully sequenced</td>
<td>68/101 (67.3%)</td>
<td>95/135 (70.4%)</td>
</tr>
<tr>
<td>- 'new infection' as defined in the Study Design</td>
<td>68</td>
<td>88</td>
</tr>
<tr>
<td>- Excluded because of absence of respiratory tract disease</td>
<td>n.a.</td>
<td>36</td>
</tr>
<tr>
<td>Included in final analyses</td>
<td>68</td>
<td>52</td>
</tr>
</tbody>
</table>

Table I.

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Non-hospitalised children</th>
<th>Hospitalised children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples screened for RV detection</td>
<td>236</td>
<td>548</td>
</tr>
<tr>
<td>Number of RV-positive samples</td>
<td>112</td>
<td>153</td>
</tr>
<tr>
<td>Number of RV-positive samples used in the typing assay</td>
<td>112</td>
<td>152</td>
</tr>
<tr>
<td>Outcome of the typing assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- no RV (i.e. EV positive)</td>
<td>11</td>
<td>17</td>
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<td>n.a.</td>
<td>36</td>
</tr>
<tr>
<td>Included in final analyses</td>
<td>68</td>
<td>52</td>
</tr>
</tbody>
</table>
REFERENCES


Chapter 9

Respiratory viruses in a primary health care facility in Amsterdam, the Netherlands


Manuscript in preparation
ABSTRACT

Background: Laboratory testing for respiratory tract infections (RTIs) is not routinely performed in primary care. Their etiology is usually unknown and although RTIs are mainly of viral origin, the contribution of different respiratory viruses is uncertain.

Objectives: Our study aims to increase our insight in the epidemiology of respiratory viruses in primary care and to evaluate the accuracy of the general practitioner’s (GP’s) clinical diagnosis of influenza virus infection.

Study design: We prospectively recruited patients who presented with RTI symptoms at a primary care facility in Amsterdam, the Netherlands, during the 2015-2016 winter season. Demographic and clinical characteristics of patients were summarized using a questionnaire. Nasopharyngeal swabs were collected and tested with a multiplex polymerase chain reaction (PCR) assay detecting 14 respiratory viruses.

Results: One or more respiratory viruses were present in 42.5% of the patients (n=353). The most frequently detected viruses were rhinovirus (11.6%), human coronavirus (8.8%), and influenza A virus (7.6%). Sensitivity of GPs clinical diagnosis for influenza virus infection was 52.6% and specificity 78.3%.

Conclusions: Despite the use of a sensitive PCR, a respiratory virus could be detected in less than half of the patients visiting the GP with RTI symptoms. It is difficult to clinically distinguish influenza from other causes of RTIs. Correct etiological diagnosis of RTIs is needed as it contributes to differential diagnosis, might give direction to development of specific antiviral therapies and vaccines, reduce unnecessary prescription of antibiotics and clarifies the clinical spectrum of the different respiratory viruses.
BACKGROUND

Respiratory tract infections (RTIs) continue to be a leading cause of morbidity and mortality worldwide. They are the commonest acute problem dealt with in primary health care (1). RTIs are mainly of viral origin and common respiratory viruses causing RTIs include influenza viruses, respiratory syncytial virus (RSV), human coronavirus (hCoV), rhinovirus (RV) and adenovirus (2-4). However, as all RTIs elicit similar symptoms, it is challenging to distinguish infection due to respiratory viruses from other causes, such as bacterial infections or a non-infective aetiology (5).

In the Netherlands, weekly surveillance data concerning RTIs in primary health care are automatically generated from automated, anonymized data extraction from electronic patient information systems based on consultation data. Although in the Netherlands as case definition for influenza-like-illness (ILI) the ‘Pel criteria’ (Pel 1965), i.e. 1) sudden onset of symptoms, 2) fever and 3) at least one of the following symptoms: cough, rhinorrhoea, sore throat, frontal headache, retrosternal pain or myalgia, are used, several case definitions for ILI and RTIs exist and no uniform definition is used. Patients suspected for ILI are not actively recorded nor are laboratory samples collected. Only patients with ILI from general practitioners (GPs) participating in ‘sentinel surveillance’ are actively reported, but diagnosis is at discretion of the GP. The population in this surveillance covers 0.7% of the Dutch population and is representative for age, sex, regional distribution and population density. From a random subset of these RTI patients a throat swab and a nose swab is collected and tested for influenza, RSV, rhinovirus and enterovirus (6).

As laboratory testing for RTIs is not routinely performed in Dutch primary health care, data on the etiology of RTIs in general practice are limited and diagnosis and treatment of RTIs is often based on clinical signs and symptoms. Only few studies have described the epidemiology of RTIs in the GP’s office, and these studies are often conducted in specific subpopulations of primary health care patients, e.g. children (7-10). Correct identification of respiratory viruses is helpful in order to distinguish influenza virus infections from the other respiratory viruses, to identify critically ill patients who might benefit from antiviral treatment and to reduce unnecessary prescription of antibiotics (11).
OBJECTIVES

The purpose of our study was to describe the epidemiology of viral RTIs in primary health care and to evaluate the accuracy of the GP’s clinical diagnosis of influenza virus infection. We prospectively recruited patients who presented with RTI symptoms to a primary health care facility in Amsterdam, the Netherlands, in the 2015-2016 winter season. We recorded clinical characteristics of included patients and analyzed nasopharyngeal swab samples for the presence of a respiratory virus using a multiplex polymerase chain reaction (PCR) assay. We compared the clinical characteristics of patients in whom no virus was detected, patients diagnosed with an influenza infection, and patients with respiratory virus infections other than influenza.

STUDY DESIGN

This study was performed as part of the PrimariPOC, a prospective study evaluating the diagnostic accuracy of a novel rapid test for respiratory viruses in primary health care. It was conducted in a family practice in Amsterdam, the Netherlands. The facility serves approximately 10,000 patients and employs seven GPs.

All patients of any age with any underlying illness or medical condition presenting to the GP with RTI symptoms were eligible for inclusion. Patients were recruited from November 2015 until March 2016 during regular opening hours of the facility. In order to ensure inclusion of all patients infected with a respiratory virus we used the following broad inclusion criteria: Patients needed to have at least two respiratory or flu-like symptoms, e.g. cough, rhinorrhea, headache, myalgia, wheeze, or fever (defined for purposes of this study as a temperature of >37.5°C measured by ear thermometer), for inclusion.

After providing written informed consent, patients were interviewed by a study team member using a standard questionnaire. The questionnaire included questions on demographic characteristics, general health status, and clinical symptoms of the present RTI episode. At the end of the interview a nasopharyngeal swab sample (Copan Swabs, Brescia, Italy) was collected. The nasopharyngeal sample was transferred on the same day to the Department of Medical Microbiology at the Academic Medical Center (AMC) for viral testing. The treating GP was asked separately, based on clinical signs and symptoms alone, if the patient was suspected for an influenza virus infection or not.

This study was approved by the Medical Ethical Committee of the AMC (2015_160). Informed consent was obtained from patients or their parents or caregivers before enrolment, with children providing assent for participation when age appropriate.
Respiratory viruses in a primary health care facility

Virological analysis
All nasopharyngeal samples were assessed for the presence of respiratory-associated viruses (influenza virus types A and B; Respiratory Syncytial Virus (RSV); Parainfluenzavirus (PIV) type -1, -2, -3, and -4; human Metapneumovirus (hMPV); human Bocavirus (hBoV); adenovirus; rhinovirus; enterovirus; human coronavirus (hCoV); and human parechovirus (hPeV)) using a multiplex PCR assay as described previously by Jansen et al (12). A threshold cycle (Ct) value of 40 or more was considered to be negative.

Data analysis
Data were analysed using SPSS statistical software (version 22.0, SPPS Inc., Chicago, IL). The base line characteristics of patients in whom no virus was detected, patients diagnosed with an influenza infection, and patients with respiratory virus infections other than influenza were compared by means of chi-squared test. Differences between continuous variables were determined using Student’s t test or ANOVA if normally distributed and non-parametric tests if not normally distributed. A two-sided p-value <0.05 was considered statistically significant. If during the study period the same patient visited the GP several times with RTI symptoms, only the results of the first visit were included as samples obtained from the same person cannot be counted as independent observations.

RESULTS
Patient characteristics
From 11 November 2015 through 30 March 2016, a total 353 patients with RTI symptoms was included. Of these, 145 (41.1%) were male and 208 (58.9%) were female. The median age of the patients was 45 years (interquartile range (IQR) 21.5 – 60 years; range 0 to 88 years). A total of 124 patients (35.1%) reported an underlying condition of which an illness of the respiratory tract, e.g. COPD or asthma, was the most frequently reported.

Detection of respiratory viruses
At least one virus was detected in 150 of the 353 patients (42.5%). RV was the most frequently detected virus (n=41, 11.6%), followed by hCoV (n=31, 8.8%), influenza A virus (n=27, 7.6%), and influenza B virus (n=17, 4.8%). A viral double-infection was present in 11 (3.1%) of the patients. Of these, six patients (54.5%) were co-infected with RV. An overview of the detected respiratory viruses is shown in Table 1. RSV was significantly more often detected in children ≤ 4 years of age than in patients > 4 years of age (p<0.001). Older patients more often had no virus.
detected; in 60.8% of the children ≤ 17 years of age a respiratory virus was present compared to only 37.6% of the adult population. In patients >65 years of age significantly less viruses were detected compared to patients ≤ 65 years (p=0.005). The percentages of detected respiratory viruses per age group are presented in Figure 1.

**Table 1.** Respiratory viruses detected by multiplex PCR in patients with respiratory tract infection symptoms in a primary health care facility in Amsterdam, the Netherlands, winter 2015-2016.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of samples positive by PCR, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhinovirus</td>
<td>41 (11.6)</td>
</tr>
<tr>
<td>Human coronavirus</td>
<td>31 (8.8)</td>
</tr>
<tr>
<td>Influenza A virus</td>
<td>27 (7.6)</td>
</tr>
<tr>
<td>Influenza B virus</td>
<td>17 (4.8)</td>
</tr>
<tr>
<td>Respiratory Syncytial Virus</td>
<td>15 (4.2)</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>15 (4.2)</td>
</tr>
<tr>
<td>Human bocavirus</td>
<td>5 (1.4)</td>
</tr>
<tr>
<td>Parainfluenza virus type 1 - 4</td>
<td>5 (1.4)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>4 (1.1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>150 (42.5)</strong></td>
</tr>
</tbody>
</table>

**Figure 1.** Detected respiratory viruses per age group
Seasonality

Influenza virus activity started mid-January. Influenza A virus was mainly found at the beginning of the 2015-2016 influenza season. Influenza B virus started to circulate from February 2016 till the end of the study period. The other two most frequently detected viruses, hCoV and RV, were present during the whole study period.

Clinical characteristics

In order to assess differences between patients with no respiratory virus detected, patients with influenza virus detected, and patients with respiratory viruses other than influenza detected, we compared the demographic and clinical characteristics of these three patient groups. A detailed overview is presented in Table 2.

In patients in whom symptoms lasted more than one week, significantly less respiratory viruses were detected (p<0.001) than in patients with more acute symptoms. In patients with influenza, the clinical symptoms headache, fever and myalgia were significantly more often present as compared to the other two groups (p<0.001, p<0.001, p=0.002, respectively).

Overall, at least 83 patients (23.5%) had received the annual influenza vaccination. An influenza virus was detected in 7 (8.4%) of them versus 36 (13.6%) of the 264 patients that had not received the annual influenza vaccination. This difference was not statistically significant.

Clinical prediction of influenza virus infection

From 11 January 2016 till the end of the study period, when influenza virus circulated, the treating GPs of all included patients were asked if they clinically suspected the patient of an influenza virus infection. Information on clinical prediction of an influenza infection was available for 199 of the 353 included patients. Of these, 55 patients were clinically diagnosed with influenza according to the treating GP, of whom 20 were confirmed by PCR. In total 38 patients tested positive for either influenza A or B by PCR. Following this, sensitivity of the clinical diagnosis of influenza by the treating GP was 52.6% (95% confidence interval (CI), 36.0 – 68.7%), and specificity was 78.3% (95% CI, 70.9 – 84.2%) resulting in a positive predictive value of 36.4% (95% CI, 24.1-50.5%) and a negative predictive value of 87.5% (95% CI 80.7-92.2%).
Table 2. Clinical characteristics of patients with no respiratory virus, patients with influenza virus, and patients with respiratory viruses other than influenza detected

<table>
<thead>
<tr>
<th></th>
<th>No virus</th>
<th>Influenza virus</th>
<th>Other respiratory virus</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>203 (57.5)</td>
<td>44 (12.5)</td>
<td>106 (30.0)</td>
<td>353</td>
<td></td>
</tr>
<tr>
<td>Median age in years (IQR)</td>
<td>52 (29-62)</td>
<td>30.5 (7-51)</td>
<td>34.5 (9-56)</td>
<td>45 (22-60)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age categories</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 4 years of age</td>
<td>16 (7.9)</td>
<td>6 (13.6)</td>
<td>20 (18.9)</td>
<td>42 (11.9)</td>
<td></td>
</tr>
<tr>
<td>5-18 years of age</td>
<td>13 (6.4)</td>
<td>9 (20.5)</td>
<td>10 (9.4)</td>
<td>32 (9.1)</td>
<td></td>
</tr>
<tr>
<td>18-65 years of age</td>
<td>146 (71.9)</td>
<td>27 (61.4)</td>
<td>71 (67.0)</td>
<td>244 (69.1)</td>
<td></td>
</tr>
<tr>
<td>&gt;65 years of age</td>
<td>28 (13.8)</td>
<td>2 (4.5)</td>
<td>5 (4.7)</td>
<td>35 (9.9)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>86 (42.4)</td>
<td>15 (34.1)</td>
<td>44 (41.5)</td>
<td>145 (41.1)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>117 (57.6)</td>
<td>29 (65.9)</td>
<td>62 (58.5)</td>
<td>208 (58.9)</td>
<td></td>
</tr>
<tr>
<td>Underlying condition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
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<tr>
<td>Yes</td>
<td>80 (39.4)</td>
<td>13 (29.5)</td>
<td>31 (29.2)</td>
<td>124 (35.1)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>123 (60.6)</td>
<td>31 (70.5)</td>
<td>75 (70.8)</td>
<td>229 (64.9)</td>
<td></td>
</tr>
<tr>
<td>Influenza vaccine received</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Yes</td>
<td>58 (28.6)</td>
<td>7 (15.9)</td>
<td>18 (17.0)</td>
<td>83 (23.5)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>141 (69.5)</td>
<td>36 (81.8)</td>
<td>87 (82.1)</td>
<td>264 (74.8)</td>
<td></td>
</tr>
<tr>
<td>Duration of symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≤7 days</td>
<td>89 (44.5)</td>
<td>37 (84.1)</td>
<td>65 (61.3)</td>
<td>191 (54.1)</td>
<td></td>
</tr>
<tr>
<td>&gt;7 days</td>
<td>111 (55.5)</td>
<td>7 (15.9)</td>
<td>41 (38.7)</td>
<td>159 (45.0)</td>
<td></td>
</tr>
<tr>
<td>Clinical symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cough</td>
<td>178 (87.7)</td>
<td>43 (97.7)</td>
<td>96 (90.6)</td>
<td>317 (89.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Rhinorrhoea</td>
<td>145 (71.4)</td>
<td>37 (84.1)</td>
<td>97 (91.5)</td>
<td>279 (79.0)</td>
<td>0.006</td>
</tr>
<tr>
<td>Headache</td>
<td>106 (52.2)</td>
<td>33 (75.0)</td>
<td>56 (52.8)</td>
<td>195 (55.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Wheezing</td>
<td>97 (47.8)</td>
<td>19 (43.2)</td>
<td>45 (42.5)</td>
<td>161 (45.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Fever (&gt; 37.5°C)</td>
<td>34 (16.7)</td>
<td>22 (50.0)</td>
<td>23 (21.7)</td>
<td>79 (22.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Myalgia</td>
<td>82 (40.4)</td>
<td>28 (63.6)</td>
<td>43 (40.6)</td>
<td>153 (43.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>Other (most common: sore throat)</td>
<td>129 (63.5)</td>
<td>32 (72.7)</td>
<td>69 (65.1)</td>
<td>230 (65.2)</td>
<td>N/A</td>
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DISCUSSION

In this prospective study we presented an overview of respiratory viruses detected in a primary health care facility in the Netherlands during the 2015-2016 winter season. Of all RTI episodes, at least one virus was present in 42.5% of the episodes. RV, hCoV, and influenza A virus were the most frequently detected viruses. In children under five years of age viruses were more often detected compared to patients > 65 years.

Our respiratory virus detection rates were lower than reported in previous studies conducted in primary health care (9, 13) but comparable with a recent study on the incidence of viral RTIs in the United States (14). Several factors might explain the relatively high proportion of patients in whom no virus was detected. Almost half of the patients visited the GP’s office more than a week after symptom onset. Although PCR is more sensitive than other virus detection methods such as immunofluorescence and culture, it might be possible that viral load had already declined below the detection limit. Our study population consisted mainly of adults and the median age of included patients was quite high. Viral load is inversely associated with age: children often have higher respiratory virus viral loads than adults and therefore higher detection rates (15).

RV, hCoV, and influenza viruses are the most frequently detected viruses in other primary care studies as well (16-19). According to the National Institute for Health and the Environment (RIVM), the Dutch influenza epidemic started on 20 January 2016 and ended in the beginning of April 2016, which is in agreement with our data. Influenza virus type A(H1N1)pdm09 was the predominant influenza virus strain (20).

Because all respiratory viruses cause similar symptoms, GP’s clinical diagnosis for specific causes is not very accurate. When influenza was diagnosed on clinical grounds, sensitivity was only around 52.6% and specificity 78.3%. These calculations suggest that influenza cases are often missed and non-influenza cases are incorrectly diagnosed as influenza. This is in agreement with other studies in which clinical prediction rules for influenza were evaluated (21-23). Symptom perception is subjective and differences are probably too subtle to clinically distinguish infection due to influenza viruses from infections caused by other respiratory viruses (24). However, headache, fever, and myalgia were more often present in influenza cases and can help in establishing an influenza virus diagnosis.

Our study is one of the few studies describing an extensive panel of respiratory viruses in primary health care in both children and adults simultaneously and thereby increasing our knowledge of the epidemiology of respiratory viruses. Despite these strengths, several limitations of our
study need to be addressed. Although we tried to include patients consecutively, some eligible patients might not have been recruited. We did not recruit control patients for our study. Some virus detection could have been associated with asymptomatic infection or shedding from a previous or forthcoming illness episode. In addition, our study was performed in one primary care facility during one respiratory season which limits generalizability of our results.

Although influenza viruses were one of the most frequently reported viruses in primary health care, it is difficult to clinically distinguish influenza virus infection from the other respiratory viruses.

Correct etiological diagnosis of RTIs is needed as it contributes to differential diagnosis, improved surveillance, might give direction to development of specific antiviral therapies, reduce inappropriate use of antibiotics, and clarify the clinical spectrum of the different respiratory viruses. Our results show that although some clinical symptoms are predictive for an influenza virus infection, GPs cannot diagnose influenza on clinical symptoms alone. To correctly identify patients with influenza or other respiratory virus infections rapid diagnostic tests, e.g. point-of-care tests, are needed.

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**Competing interests:** None declared

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REFERENCES


Acute respiratory tract infections are one of the most frequent reasons for health care consultations and hospitalizations, and a leading cause of morbidity and mortality worldwide. Respiratory tract infections can be caused by many different pathogens. Although bacteria were historically considered the main pathogens causing severe infections, the role of viruses is increasingly recognized. Rapid and accurate etiological diagnosis of respiratory tract infections is important for clinical patient management, public health surveillance, and infection prevention. In recent years, the diagnostic opportunities for the detection of respiratory viruses have advanced rapidly. There is a clear trend towards faster diagnostics. Increasing numbers of rapid tests designed for use at the point-of-care have been developed. The aim of this thesis was 1) to evaluate the use and diagnostic accuracy of rapid tests for respiratory viruses in the hospital setting and in primary health care; 2) to increase our insight in the epidemiology and clinical relevance of respiratory viruses.

**PART 1: RAPID DETECTION OF RESPIRATORY VIRUSES**

In chapter 2 we provided a systematic literature overview of the rapid tests that are currently available for the detection of respiratory viruses. Following our strict inclusion criteria, in which a rapid test was defined as any commercially available quick (up to two hours) and easy-to-use test requiring little or no additional equipment or technological skills, we included 125 articles evaluating 50 different rapid tests in our systematic review. Although our search strategy contained search terms for all common respiratory viruses, most rapid tests were capable of detecting only one virus, either influenza virus, or Respiratory Syncytial Virus (RSV). Overall, pooled sensitivity and specificity for detection of influenza was 61.1% (95% confidence interval [CI], 53.3 to 68.3) and 98.9% (95% CI, 98.4 to 99.3), respectively. Performance for RSV was higher in general, with a pooled sensitivity of 75.3% (95% CI, 72.6 to 77.8), and a pooled specificity of 98.7% (95% CI, 97.3 to 99.4). Even though all rapid tests studied in this review were designed to be performed by non-laboratory trained personnel at the point-of-care, many studies were not evaluated at the point-of-care. Quality assessment of included studies revealed that because of the frequently incomplete reporting of study characteristics, risk of bias for included studies was often unclear. Although the newer tests seem to be more sensitive, there is a lack of high quality evaluations of these tests.

According to our systematic review, one of the rapid tests with the best diagnostic performance characteristics was Quidel’s Sofia Fluorescent Immunoassay. In chapter 3 we evaluated the clinical use of the Sofia FIA for the detection of influenza and RSV in our own hospital, the Academic Medical Center (AMC) in Amsterdam, in a pediatric hospitalized population at the...
point-of-care. Using our in-house multiplex PCR as reference test, Sofia had a sensitivity of 75% (95% CI, 57.7-92.3) and a specificity of 97.5% (95% CI, 92.7-100) for RSV. For influenza A, sensitivity was 66.7% (95% CI, 35.9-97.5) and specificity 96.6% (95% CI, 92.0-100) and for influenza B sensitivity was 40% (95% CI, 9.6-70.4) and specificity 89.7% (95% CI, 81.8-97.5). Diagnostic performance of the test was lower than expected, but the availability of a rapid test at the point-of-care was appreciated by pediatric residents.

As described in Chapter 2 many of the available point-of-care tests for respiratory viruses can detect only one single virus, i.e. influenza virus or RSV. In the past decade, new respiratory viruses have been discovered, such as human bocavirus 1 in 2005 and several new coronaviruses. Causative associations between infection with these respiratory viruses and severe disease, e.g. unexplained severe lower respiratory tract infections or encephalitis, have recently been described. It is therefore important to rapidly identify acute, clinically relevant infections caused by these viruses. The mariPOC\textsuperscript{®} test system (ArcDia International Oy Ltd., Turku, Finland) might be a suitable option for rapid detection of acute infections. MariPOC\textsuperscript{®} is an automated and point-of-care compatible test for rapid and simultaneous detection of antigens of eight respiratory viruses (influenza A and B, RSV, adenovirus, human metapneumovirus, and parainfluenza type 1, 2, and 3 viruses) and \textit{Streptococcus pneumoniae} from a single nasopharyngeal sample. In chapter 4 and chapter 5, we described the addition of two new targets, human bocavirus 1 and coronavirus, on the mariPOC assay. We demonstrated that the new tests correctly identified and enabled monitoring of respectively human bocavirus and coronavirus infections. It should be noted that both studies were proof-of-principle studies. The exact performance characteristics of the new targets need to be evaluated in larger validation studies.

As previously described in the introduction of this thesis not only laboratory validation studies are needed to evaluate the diagnostic accuracy and use of rapid tests. In chapter 6 we therefore evaluated not only the diagnostic performance, but also the clinical feasibility of a rapid test. In patients with respiratory tract infection symptoms presenting to a family practice during the 2015-2016 winter season, we determined the sensitivity and specificity of the mariPOC\textsuperscript{®} respi test relative to PCR testing performed in our laboratory. The clinical feasibility of the rapid test was evaluated by interviewing study participants and general practitioners. One or more respiratory viruses were detected in 54.9% of the included patients with respiratory tract infection symptoms (n=204). Rhinovirus and influenza A virus were the most frequently detected viruses. Overall, the mariPOC\textsuperscript{®} had a sensitivity of 47.1% (95% CI, 35.2-59.4), a specificity of 99.7% (95% CI, 99.2-99.9), a positive predictive value of 84.6% (95% CI, 68.8-93.6), and a negative predictive value of 97.9% (95% CI, 97.1-98.5) for the panel of 9 viruses that it
tests for. For influenza A virus, sensitivity of the mariPOC was 54.2% (95% CI, 33.2-73.8); for influenza B virus, sensitivity was 72.2% (95% CI, 46.4-89.3) and for RSV, sensitivity was 50.0% (95% CI, 22.3-77.7). Specificity was high, ranging from 98.9% to 100.0%. In samples with higher viral load, i.e. Ct-value below 30, sensitivity was 85.7% for influenza A virus, 78.6% for influenza B virus and 85.7% for RSV. The availability of a diagnostic test for respiratory viruses was appreciated by both patients and general practitioners with more than two-third of the patients considering it to be a valuable or very valuable addition for primary care. Patients reported being more confident about the self-limiting aspect of the disease and general practitioners considered the rapid test helpful as they now had a tool to convince patients about the viral diagnosis.

PART 2: EPIDEMIOLOGY OF RESPIRATORY VIRUSES

Part 2 of this thesis focused on the epidemiology and clinical relevance of respiratory viruses, in particular rhinoviruses. Rhinoviruses are the most frequently detected respiratory viruses in humans and the predominant cause of the common cold. Although most of the time responsible for relatively mild respiratory illness, rhinoviruses can also cause severe lower respiratory tract infections. Rhinoviruses, members of the family *Picornaviridae* and the genus *Enterovirus*, can be classified into three species, RV-A, RV-B, and RV-C, and more than 150 types. It is unclear whether pathogenicity and clinical significance differ between the three rhinovirus species, let alone between the individual rhinovirus types.

In chapter 7, we investigated the prevalence of rhinovirus types in the patient population of the AMC by genotyping all rhinovirus-positive samples submitted for respiratory viral diagnostics from 2007 to 2012. In total, 52.4% of the samples (n=637) belonged to RV-A, 11.3% to RV-B, and 36.2% to RV-C. The majority of the currently classified rhinovirus types could be detected in our population. Some types were more frequently present than others, such as RV-A12, RV-A78, and RV-C2. Furthermore, we detected eight previously described provisionally assigned types, i.e. virus strains predicted to be new rhinovirus types. Rhinoviruses circulated the whole year around, with a slightly higher frequency in autumn, and a decline in summer. Some rhinovirus types could be detected intermittently during the whole study period, while others were detected mainly in winter and early spring. A limitation of this study was that we could not investigate associations between the clinical symptoms of patients and the different rhinovirus types.
Because of the limitation described above, we set up a study linking different characteristics of rhinovirus infections to clinical outcome. In chapter 8 we describe the results of this study which compared the clinical, virological and epidemiological characteristics of rhinovirus infections in young children with mild or asymptomatic infection to young children admitted to the hospital because of their respiratory illness. RV-A was the most frequently detected rhinovirus species in both study populations, followed closely by RV-C. The distribution of the rhinovirus species was comparable in non-hospitalised and hospitalised children. A subgroup analysis was performed to investigate the circulation of rhinovirus species and types in children with more severe respiratory disease, i.e. children admitted to the ICU due to respiratory distress. Also, in this latter group, distribution of rhinovirus species did not significantly differ, which is in contrast with previous studies suggesting that infection with RV-C is associated with more severe respiratory tract disease. No predominant rhinovirus type could be found in severely ill children. We therefore conclude from our study that clinical outcome is not related to rhinovirus species or types alone, but may more likely be influenced by multiple (host-specific) factors, such as age, chronic underlying illness, viral load and the presence of a bacterial co-infection.

Rhinoviruses are the most frequently detected respiratory viruses in hospitalized patients with respiratory tract infection symptoms, but for primary health care patients this is uncertain as diagnostic testing of respiratory tract infections is not routinely performed in primary health care. The contribution of different respiratory viruses is unclear. The purpose of chapter 9 was to describe the epidemiology of viral RTIs in primary health care and to evaluate the accuracy of the GP’s clinical diagnosis of influenza virus infection. This study was part of the study described in chapter 6, in which we prospectively recruited patients who presented with RTI symptoms to a primary health care facility in Amsterdam, the Netherlands, in the 2015-2016 winter season. We used the results of our multiplex PCR assay to determine the presence of a respiratory virus. We compared the clinical characteristics of patients in whom no virus was detected, patients diagnosed with an influenza virus infection, and patients with respiratory virus infections other than influenza. At least one virus was present in 42.5% of the patients with RTI symptoms. Rhinovirus was the most frequently detected virus, followed by coronavirus, and influenza A virus. Although influenza viruses were among the most frequently detected viruses, we showed that it is difficult to clinically distinguish influenza virus infection from other respiratory viruses as sensitivity of GP’s clinical diagnosis for influenza was only 52.6% (95% CI, 36.0 – 68.7) and specificity 78.3% (95% CI, 70.9 – 84.2).
In conclusion, the aim of this thesis was 1) to evaluate the use and diagnostic accuracy of rapid tests for respiratory viruses; 2) to increase our insight in the epidemiology and clinical relevance of respiratory viruses, in particular rhinoviruses. There is a growing need for rapid and accurate diagnostics for respiratory tract infections. Rapid tests have the potential to fulfil this need. However, sensitivity of rapid tests needs improvement and the development of new rapid tests, preferably tests that can detect viruses and bacteria in combination with host response markers, should be encouraged. Before implementation in the clinic, high quality evaluation studies on the impact of these tests on clinical patient management should be performed.
Chapter 11

General Discussion
Respiratory viruses are thought to be responsible for the vast majority of respiratory tract infections and account for more episodes of respiratory tract illness than any other microbial pathogens (1). Rapid identification of viral respiratory tract infections has several advantages. Prompt diagnosis may reduce unnecessary additional testing, avoid inappropriate prescription of antibiotics, lead to rapid implementation of infection control measures, and early administration of antiviral medication (2, 3). As a result, a decrease in hospital stay and a reduction in health care costs might be accomplished (4). However, making both a rapid and accurate viral diagnosis is challenging. In the next paragraphs, the current challenges with rapid testing that emerged from this thesis will be addressed and the implications for future research and clinical practice discussed.

**CHALLENGE 1. Limited virus detection range and disappointing sensitivity of rapid tests**

Clinical signs and symptoms of respiratory viruses overlap (5). The etiological diagnosis therefore requires a syndromic approach as multiple viruses might be the cause of the infection. The results of chapter 9 demonstrated that based on clinical symptoms alone influenza cases are often missed and non-influenza cases are incorrectly diagnosed as influenza. To correctly and timely identify patients with a respiratory virus, rapid diagnostic tests - preferably tests capable of detecting multiple pathogens - are needed. Unfortunately, the number of rapid tests capable of detecting multiple viruses is limited. Our systematic review showed that most rapid tests have the possibility to either detect influenza viruses or RSV. In addition, their diagnostic sensitivity is often unsatisfactory low, with an over-all pooled sensitivity of 61.1% for rapid tests for influenza. Sensitivity was even lower when including only high quality studies. Lower-than-expected sensitivities for rapid tests were demonstrated as well in chapter 3 and 6.

**Unmet need: Rapid, sensitive and specific multiplex test platforms**

Rapid, sensitive and specific multiplex rapid tests are needed. More sensitive molecular assays that have the ability to detect a broad range of viral and bacterial pathogens in a short time span are currently in development. At the moment, fully automated molecular methods which require minimal hands-on-time are commercially available (6). A major drawback of these newer diagnostic devices are the high costs and the low sample throughput (7, 8), which limits their potential for direct ‘point-of-care’ use. Molecular methods have the advantage that they are highly sensitive, but the interpretation of molecular test results is not always straightforward. Often the question remains whether high sensitivity provides more reliable information about the true etiology of a given infection as PCR detects viral nucleic acids regardless of the presence
of viral antigens or replicating infectious virus (9). The presence of viral DNA or RNA does not always reflect acute disease since positive PCR results may represent a previous infection or asymptomatic carriage. Furthermore, with PCR often more than one respiratory virus is detected and the contribution of the positive result to disease severity is not always clear. Quantification of viral load might help in determining the clinical relevance of the infection, but at the moment clinically relevant cut-off values for respiratory viruses in randomly collected samples are limited (10). Finally, as with all molecular methods, there is a danger of contamination and mistakes in the pre-analytical phases (9). Also in the upcoming years, non-molecular rapid tests will therefore still be of importance in clinical patient management. Their possible role in identifying clinically relevant infections, as proposed in chapters 4 and 5, should be further examined as no large clinical studies with rapid tests comparing asymptomatic and symptomatic infections are available.

As already mentioned in chapter 6, not only direct viral testing, but also analysis of the host response to infection might help in correctly diagnosing respiratory tract infections (11). Various laboratory tests that aid in differentiating viral from bacterial infections are currently in use, such as white blood cell count, C-reactive protein (CRP) and procalcitonin concentrations. However, effects of these tests on antibiotic prescription rates are inconsistent (12). Recently, the combination of CRP, tumour necrosis factor-related, apoptosis-inducing ligand, and interferon gamma-induced protein-10 has been investigated (13), and new approaches towards monitoring the host response in viral and bacterial infections, such as gene expression profiling, continue to evolve (14-19). Although results are promising, and assays in which the detection of viruses, bacteria, and host responses are combined might provide new diagnostic opportunities, there is still a long road ahead before routine use of these newer devices in clinical practice. First, results of these assays should be confirmed in larger studies with different patient populations (20).

**CHALLENGE 2. Collecting the correct sample for diagnosing respiratory tract infections**

A second difficulty of accurately diagnosing respiratory tract infections is collecting the correct sample. To identify the cause of the respiratory tract infection lower respiratory tract samples, e.g. sputum or broncho-alveolar lavages, are the preferred sample type. However, these are difficult to obtain, especially in children and the most commonly used samples today are therefore upper respiratory tract samples, such as nasal swabs and aspirates. The problem
with upper respiratory tract samples is that the virus detected might be from a resolving upper respiratory tract infection and not the cause of the lower respiratory tract infection.

Unmet need: Improved, standardized methods for respiratory sample collection
Respiratory virus detection highly depends on the type of sample collected, the time of collection after the onset of clinical symptoms, and the transport and storage of the sample before testing. Several sampling methods are currently approved for rapid tests, such as nasal swabs and nasopharyngeal aspirates (21). At the moment, nasopharyngeal flocked swabs are recommended for detecting respiratory viruses (22), but improved, standardized methods for reliable respiratory sample collection for diagnosing respiratory tract infections are warranted, and the search for better sample collection methods for lower respiratory tract infections should continue.

CHALLENGE 3. Lack of well-designed diagnostic test accuracy studies
To determine the diagnostic performance of a rapid test diagnostic test accuracy studies are needed. Our systematic review showed that the same rapid test has different sensitivity and specificity estimates in different diagnostic accuracy studies. Diagnostic accuracy calculations are extremely sensitive to the design of the study and are influenced by many factors, such as virus prevalence, time from disease onset to sample collection, sample type, quality of the collected sample, and age of the patient (23, 24). A remarkable finding of the quality assessment of the studies included in our review was that many of the study characteristics influencing the risk of bias were not clearly reported or simply missing.

Another major finding was that although all rapid tests studied in this review were designed to be performed by non-laboratory trained personnel at the point-of-care, many studies were not evaluated at the point-of-care. The setting or the personnel that performed the test was rarely properly described. The actual diagnostic performance of the rapid tests in the setting of daily clinical practice might therefore be different than the accuracy estimates reported in the studies.

Unmet need: High quality diagnostic test accuracy studies performed at the point-of-care
To increase their quality, diagnostic test accuracy studies should be reported according to the Standards for Reporting Diagnostic accuracy studies (STARD-guidelines) (25). Despite that
these guidelines have already been developed in the beginning of the 2000s, adherence to the STARD is still moderate and should be encouraged (26).

Besides, laboratory validation studies of rapid tests are not sufficient to evaluate the diagnostic accuracy and use of rapid tests. As shown in chapter 3, the implementation of POCTs requires novel strategies. For implementation of a rapid test, logistic difficulties must be overcome and it is therefore important to provide sufficient training moments for personnel for an adequate operation of the rapid tests and interpretation of the test results. In July 2015, a general practitioners’ guideline was published in the Netherlands on how to deal with POCTs in general practice as also in the field of general practice there is a clear trend towards point-of-care testing (27). One of the main messages from this guideline underscored the rise in numbers of available POCTs and introduction of these tests in primary health care settings without proper evaluation and implementation studies being performed. In chapter 6 we therefore not only evaluated the diagnostic performance, but also the clinical feasibility of a rapid test. Our study is one of the few that addresses this item and hopefully a stimulant for other researchers to take this important topic into account as well when evaluating a new test.

CHALLENGE 4. Lack of clinical impact studies from both the doctor's and the patients perspective

Despite the strict inclusion criteria for our systematic review, we could still include 125 articles that evaluated the accuracy of one or more rapid tests. On the contrary, the number of studies evaluating the clinical impact of rapid test results on patient management is limited (28-31). Besides, in recent years the concept of patient-centered care has received attention. Patient-centered care underscores the importance of better understanding the experience of illness and the patients’ needs (32). In chapter 6 we showed that patients highly appreciated the availability of rapid test for respiratory viruses in primary health care. To our knowledge, ours is the only study in which the patient’s perspective on rapid testing was addressed.

Unmet need: Randomized controlled trials to determine the impact of point-of-care tests

In addition to high quality diagnostic accuracy studies performed at the point-of-care there is a need for studies that evaluate the clinical impact of rapid tests (33). Several studies did report on the association of rapid tests on prescription of antibiotics, initiation of antiviral therapy, implementation isolation measures, and/or additional testing. Some reported a positive influence of rapid testing on clinical outcome (31, 34), but others did not report any significant
differences compared to routine diagnostics. However, many of these studies were retrospective studies (35) or studies in which the turn-around-time of the evaluated test was still quite long (36).

Only with well-designed randomized controlled trials, the true impact of rapid tests on clinical patient management can be assessed. We therefore encourage the planning of randomized controlled trials that assess the clinical relevance of rapid tests of terms of clinically relevant outcomes, such as use of antibiotics, prescription of antiviral medication, use of additional ancillary testing, and length of hospital stay. Cost-effectiveness evaluations are an important component of these studies. Furthermore, for optimal patient management, to encourage self-management and reduce the number of unnecessary doctor visits it is important to know the needs and views of patients themselves with regard to rapid testing and how this should be implemented.

RAPID DIAGNOSTICS TO PAINT THE EPIDEMIOLOGICAL PICTURE

Despite the limitations of the current rapid tests addressed in the previous paragraph and the challenges that are faced with point-of-care testing, faster and more accurate virus diagnostics are warranted. As we have shown in chapters 6 and 9, viruses could not be detected in almost half of the patients with a respiratory tract infection in primary health care. Similar results were described in a recent study by Jain et al (2015), in which in nearly two-third of the hospitalized patients no disease-causing pathogen could be identified in those with radiologically confirmed lower RTI (37). Improved rapid diagnostics can aid to bridge this gap in knowledge and increase our insight in the epidemiology of respiratory viruses. For example, with the introduction of PCR also non-culturable viruses, e.g. rhinovirus C and human bocavirus 1 were more easily identified. This has contributed significantly to our understanding of the viruses causing respiratory tract infections. As shown in chapters 4, 7 and 8, the general view of these viruses as just ‘common cold’ viruses has been adjusted since especially rhinoviruses are increasingly recognized as important respiratory pathogens capable of causing severe lower respiratory tract infections as well (38, 39).

In addition, not only will enhanced detection of respiratory viruses increase our insight in the epidemiology, it might also give direction to development of antiviral therapy as exemplified for RVs (40-42). The availability of rapid, sensitive, and inexpensive diagnostic tests that can detect respiratory pathogens might boost the interest of pharmaceutical companies in drug
development as the disease spectrum of the different viruses becomes more clear and targeted therapy can become a realistic option.

However, in the near future a complete epidemiological picture is not yet feasible and a substantial proportion of the respiratory tract infections will probably remain undiagnosed. In the absence of a detectable pathogen, host-targeted approaches - such as the above mentioned host protein-based assays and gene expression profiling - might help in differentiating viral from bacterial infections.

FUTURE PERSPECTIVE: CATCHING THE COMMON COLD?

The studies presented in this thesis focused on two themes: 1) development and evaluation of rapid tests for the detection of respiratory viruses; 2) epidemiology and clinical relevance of respiratory viruses, in particular rhinoviruses. In the era of emerging respiratory viruses, there is a growing need for rapid, sensitive and specific identification of these viral pathogens. Rapid tests have the potential to fulfil these needs, but one should be aware of their current limitations in diagnostic performance and pathogen identification. The development of improved rapid tests, preferably tests that can detect both respiratory viruses and bacteria in combination with host response markers, should therefore be encouraged. Before these tests become commercially available, high quality evaluation studies are required. These evaluation studies should not only focus on diagnostic accuracy, i.e. if the new test correctly identifies the pathogen and pathogen-specific host response, but also on the interpretation, implementation, and impact of the rapid test on clinical patient management (43). This thesis showed that point-of-care testing involves more than just performing a rapid test at the point-of-care. Implementation of point-of-care testing requires novel strategies for logistics and organization and for successful implementation of rapid tests in the clinic high quality evaluation studies at the point-of-care are required. Furthermore, studies on the impact of rapid tests should not only be measured from the doctor’s perspective, but also from the patient’s perspective.
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Appendices

Nederlandse samenvatting
List of co-authors
Author contributions
PhD portfolio
List of publications
Acknowledgements
About the author
NEDERLANDSE SAMENVATTING

Luchtweginfecties komen wereldwijd veel voor en zijn één van de voornaamste redenen om een dokter te consulteren. Luchtweginfecties kunnen veroorzaakt worden door micro-organismen, zoals virussen en bacteriën. De meeste luchtweginfecties worden door virussen veroorzaakt. Toch wordt er vaak, ondanks dat antibiotica niet tegen virussen werken, antibiotica voorgeschreven. Dit draagt bij aan de ontwikkeling en toename van antibiotica-resistentie. Het is niet alleen voor de individuele patiënt, maar ook voor de algemene gezondheidszorg belangrijk om snel duidelijk te hebben welk micro-organisme de luchtweginfectie veroorzaakt, zodat onnodig antibioticagebruik kan worden voorkomen, maar zodat bijvoorbeeld ook de juiste isolatiemaatregelen kunnen worden getroffen.

In de afgelopen jaren zijn de diagnostische mogelijkheden voor het aantonen van micro-organismen behoorlijk toegenomen. De diagnostiek wordt steeds sneller en er worden steeds meer sneltesten ontwikkeld die niet in een gespecialiseerd laboratorium, maar direct dichtbij de patiënt kunnen worden uitgevoerd. Deze sneltesten worden ook wel ‘point-of-care’-testen genoemd. Ook voor de virussen die luchtweginfecties kunnen veroorzaken - de zogenaamde 'respiratoire virussen', zoals bijvoorbeeld het influenza virus (griepvirus) en het respiratoire syncytieel virus (RSV) - zijn recent veel nieuwe sneltesten ontwikkeld.

Het doel van dit proefschrift was daarom 1) het evalueren van de kwaliteit (‘diagnostische accuratesse’) en het gebruik van sneltesten voor respiratoire virussen in zowel het ziekenhuis als de huisartsenpraktijk en 2) het vergroten van onze kennis van de epidemiologie en de klinische relevantie van respiratoire virussen.

DEEL 1: Sneldiagnostiek voor respiratoire virussen

Hoofdstuk 2 van dit proefschrift is een zogenaamd systematic review, een literatuuronderzoek waarbij op systematische wijze gezocht is in verschillende databases naar originele onderzoeksartikelen die een antwoord kunnen geven op een bepaalde onderzoeksvraag. Onze systematic review geeft antwoord op de vraag welke sneltesten voor respiratoire virussen op dit moment beschikbaar zijn voor artsen en wat de gemiddelde diagnostische accuratesse, dus de sensitiviteit (‘gevoeligheid’) en specificiteit, van deze testen is. In de review bespreken we 125 artikelen die in totaal 50 verschillende soorten sneltesten evalueren. Het blijkt dat de meeste sneltesten zo ontwikkeld zijn dat ze ofwel influenza ofwel RSV kunnen detecteren. De gemiddelde sensitiviteit van sneltesten voor influenza is 61.1% (95% betrouwbaarheidsinterval
Nederlandse samenvatting

[BI], 53.3 tot 68.3) en de specificiteit 98.9% (95% BI, 98.4 tot 99.3). Sneltesten voor RSV zijn over het algemeen accuratere testen, met een gemiddelde sensitiviteit van 75.3 % (95% BI, 72.6 tot 77.8), en een gemiddelde specificiteit van 98.7% (95% BI, 97.3 tot 99.4). Hoewel alle sneltesten in onze review in principe bedoeld zijn om als 'point-of-care' test te fungeren, was maar een klein deel van de testen ook daadwerkelijk uitgevoerd op de 'point-of-care'. Als we kijken naar de kwaliteit van de geïncludeerde artikelen wordt duidelijk dat de opzet van veel onderzoeken en ook de manier waarop de onderzoeksresultaten zijn geanalyseerd niet duidelijk worden beschreven, wat het risico op 'bias', dus een onjuiste interpretatie van de onderzoeksuitkomsten kan vergroten. Hoewel de nieuwere sneltesten een betere sensitiviteit lijken te hebben, ontbreekt het aan kwalitatief goed uitgevoerde evaluaties van deze nieuwere testen. Dit is de reden waarom we, zoals beschreven in hoofdstuk 3 en hoofdstuk 6, zelf twee sneltesten hebben onderzocht.

Uit onze review bleek dat Quidels’ Sofia Fluorescent Immunoassay één van de sneltesten was die het beste presteerde. In hoofdstuk 3 hebben we daarom deze test onderzocht op de kinderafdelingen van ons eigen ziekenhuis, het Academisch Medisch Centrum in Amsterdam. De Sofia had - in vergelijking met de referentie standaard, onze multiplex PCR test die in het laboratorium wordt gedaan - een sensitiviteit van 75% (95% BI, 57.7-92.3) en een specificiteit van 97.5% (95% BI, 92.7-100) voor RSV. Voor influenza A virus was deze sensitiviteit 66.7% (95% BI, 35.9-97.5) en de specificiteit 96.6% (95% BI 92.0-100) en voor influenza B virus was de sensitiviteit 40% (95% CI, 9.6-70.4) en de specificiteit 89.7% (95% BI, 81.8-97.5). Hoewel de sensitiviteit van de test lager was dan verwacht, werd de mogelijkheid om de sneltest op de ‘point-of-care’ uit te voeren, dus op de kinderafdeling zelf, door dokters als erg prettig ervaren.

Uit hoofdstuk 2 bleek dat veel sneltesten slechts één virus kunnen detecteren, vaak ofwel influenza ofwel RSV. Het wordt echter steeds duidelijker dat andere, minder bekende en recent ontdekte virussen, zoals het bocavirus en een aantal coronavirussen ook belangrijke verwekkers kunnen zijn van ernstige luchtweginfecties. Het is dus belangrijk dat ook infecties veroorzaakt door deze virussen snel ontdekt kunnen worden. Het mariPOC® platform (ArcDia International Oy Ltd, Turku, Finland) zou een mogelijkheid kunnen bieden om acute infecties door deze virussen aan te tonen. MariPOC® is een geautomatiseerde sneltest die acht verschillende respiratoire virussen (influenza A en B, RSV, adenovirus, humaan metapneumovirus, en parainfluenza virus type 1, 2 en 3) kan detecteren. In hoofdstuk 4 en 5 beschrijven we hoe we twee nieuwe tests, namelijk voor het aantonen van humaan bocavirus 1 en humaan coronavirus, aan het mariPOC® platform hebben toegevoegd. We lieten zien dat deze nieuwe tests de virussen op de juiste wijze in patiënten konden aantonen. Wel dient opgemerkt te worden dat deze studies
zogenaamde ‘proof-of-principle’ studies waren en dat de uiteindelijke werkzaamheid van de test onderzocht moet worden in grotere validatie studies.

Zoals in de introductie van dit proefschrift al is beschreven, zijn niet alleen laboratorium validaties van belang bij het evalueren van de kwaliteit en het gebruik van een test. In hoofdstuk 6 hebben we de diagnostische accuratesse, maar ook de praktische bruikbaarheid van een sneltest onderzocht. Bij patiënten die met klachten van een luchtweginfectie – bijvoorbeeld hoesten, verkoudheid, of koorts - naar de huisarts gingen, hebben we de sensitiviteit en de specificiteit van de mariPOC® test vergeleken met onze PCR. De klinische en praktische toepasbaarheid van de test hebben we onderzocht door zowel patiënten als huisartsen te interviewen over wat ze vonden van de sneltest. Eén of meer respiratoire virussen werden gedetecteerd bij 54.9% van de patiënten. Het rhinovirus en influenza virus type A waren de meest voorkomende virussen. De sneltest had, als alle virussen bij elkaar werden genomen, een sensitiviteit van 47.1% (95% BI, 35.2-59.4) en een specificiteit van 99.7% (95% BI, 99.2-99.9), een positief voorspellende waarde van 84.6% (95% BI, 68.8-93.6), en een negatief voorspellende waarde van 97.9% (95% BI, 97.1-98.5). Voor influenza A virus was de sensitiviteit 54.2% (95% BI, 33.2-73.8), voor influenza B virus 72.2% (95% BI, 46.4-89.3), en voor RSV 50.0% (95% BI, 22.3-77.7). Specificiteit was voor alle virussen hoog, variërend van 98.9% tot 100.0%. In patiënten materialen met een hoge virale load, namelijk een Ct-waarde onder de 30, was de sensitiviteit 85.7% voor influenza A virus, 78.6% voor influenza B virus en 85.7% voor RSV. Zowel huisartsen als patiënten vonden het prettig dat er een sneltest als extra hulpmiddel werd gebruikt, waarbij meer dan twee-derde van de patiënten het een waardevolle toevoeging voor de huisartsenpraktijk vond. Patiënten rapporteerden dat ze na de uitslag van de test meer overtuigd waren van het ‘self-limiting’-aspect van hun ziekte en de huisartsen vonden de test met name behulpzaam ter verduidelijking aan patiënten dat hun klachten veroorzaakt werden door een virus en dat een antibiotische behandeling niet nodig was. Wel blijkt dat de kwaliteit van de sneltest op dit moment nog niet goed genoeg is en verbeterd moet worden. Daarnaast is het belangrijk dat er ook sneltests ontwikkeld worden die niet alleen virale verwekkers van luchtweginfecties kunnen aantonen, maar ook bacteriële verwekkers en zelfs informatie zouden kunnen geven over de immuunrespons van de patiënt.

DEEL 2: Epidemiologie van respiratoire virussen

Deel 2 van dit proefschrift beschrijft de epidemiologie en klinische relevantie van respiratoire virussen, in het bijzonder de rhinovirussen. Rhinovirussen komen heel veel voor en zijn de belangrijkste veroorzaker van verkoudheid ofwel ‘common cold’. Hoewel rhinovirussen
meestal maar milde klachten veroorzaken, blijkt dat ze ook verantwoordelijk kunnen zijn voor ernstige lage luchtweginfecties, zoals longonsteking. Rhinovirussen, lid van de familie van de Picornavirussen en van het genus Enterovirus, kunnen worden onderscheiden in drie soorten, RV-A, RV-B, en RV-C, die vervolgens weer bestaan uit meer dan 150 subtypes. Het is onduidelijk in hoeverre de verschillende subspecies, maar ook de verschillende subtypes, klinisch verschillende klachten kunnen geven. Sommige onderzoeken suggereren dat RV-C ernstigere klachten zou kunnen veroorzaken dan de andere RV-species.

In hoofdstuk 7 hebben we het voorkomen van de verschillende rhinovirus subtypes in de patiëntenpopulatie van het AMC onderzocht door alle rhinovirus-positieve patiënten materialen tussen 2007 en 2012 te genotyperen. Van alle rhinovirus-monsters (n=637) behoorde 52.4% tot RV-A, 11.3% tot RV-B, en 36.2% tot RV-C. Het overgrote deel van alle momenteel bekende rhinovirus types werd geobserveerd in onze populatie. Sommige subtypes kwamen vaker voor dan andere, zoals RV-A12, RV-A78, en RV-C2. Daarnaast hebben we waarschijnlijk ook acht nieuwe types ontdekt. Sommige rhinovirus types kwamen het hele jaar door voor, terwijl anderen vaker in de winter en het vroege voorjaar gezien werden. Een beperking van dit onderzoek was dat we geen associaties tussen klinische symptomen en het rhinovirus species of subtype konden onderzoeken.

De hierboven beschreven beperking is de reden dat we een studie hebben opgezet die specifiek keek naar de virologische kenmerken van de rhinovirus infectie in relatie tot het klinisch beloop. In hoofdstuk 8 beschrijven we de resultaten van deze studie, waarbij we de klinische, virologische en epidemiologische kenmerken van rhinovirus infecties in jonge kinderen met geen of milde luchtwegklachten vergelijken met kinderen die zijn opgenomen in het ziekenhuis vanwege hun luchtweginfectie. RV-A kwam het vaakst voor in beide onderzoeksgroepen, op de voet gevolgd door RV-C. De verdeling van de rhinovirus species was vergelijkbaar tussen de twee groepen. We hebben een zogeheten subgroup-analyse gedaan om te kijken of er verskil was tussen rhinovirus species en types in kinderen met ernstige luchtwegklachten, namelijk die kinderen die op de IC terecht waren gekomen. Ook hier verschilde de verdeling van de rhinovirus species niet, wat in contrast was met de eerdere studies die lieten zien dat een infectie met RV-C voor ernstigere klachten zou zorgen. In onze studie kon geen overheersend rhinovirus type worden gevonden in ernstig zieke kinderen. We concluderen daarom dat het klinisch beloop waarschijnlijk niet het gevolg is van het rhinovirus species of subtype, maar waarschijnlijk beïnvloed wordt door een aantal factoren, zoals leeftijd, onderliggend liden, virus load en het wel of niet aanwezig zijn van een bacteriële co-infectie.
Rhinovirussen zijn de meest voorkomende virussen in patiënten met luchtwegklachten in het ziekenhuis. Dit is waarschijnlijk ook zo in de eerste lijn, maar omdat daar bijna nooit specifiek op microbiologische verwekkers van de luchtweginfectie wordt getest, is het voorkomen van de verschillende respiratoire virussen in luchtweginfecties in de huisartsenpraktijk minder bekend. Het doel van hoofdstuk 9 was om de epidemiologie van virale luchtweginfecties in de eerste lijn te beschrijven. Daarnaast hebben we onderzocht of huisartsen influenza kunnen onderscheiden van een infectie met een ander respiratoir virus op basis van klinische symptomen alleen. Deze studie was onderdeel van de studie die we hebben beschreven in hoofdstuk 6, waar we patiënten met luchtwegklachten die zich presenteerden bij de huisarts includeerden. We gebruikten de resultaten van onze multiplex PCR om te kijken of er sprake was van een virale luchtweginfectie. Daarnaast hebben we de klinische kenmerken van patiënten waarbij geen virus was gedetecteerd, vergeleken met de patiënten waarbij influenza of een ander respiratoir virus werd aangetoond. Tenminste één virus was aanwezig bij 42.5% van de patiënten met luchtwegklachten. Rhinovirus kwam het vaakste voor, gevolgd door coronavirus, en influenza A virus. Hoewel influenza virussen vaak voorkomen, toonden we aan dat huisartsen het moeilijk vinden om influenza klinisch te onderscheiden van andere respiratoire virussen. De sensitiviteit van de klinische blik van de huisarts om een luchtweginfectie veroorzaakt door een influenzavirus te onderscheiden van een infectie door een ander of geen respiratoir virus was namelijk 52.6% (95% BI, 36.0-68.7) en de specificiteit 78.3% (95% CI, 70.9-84.2).

Concluderend hebben we in dit proefschrift een aantal onderzoeken besproken die tezamen als doel hadden om 1) het gebruik en de diagnostische accuratesse van sneltesten voor respiratoire virussen te evalueren en 2) onze kennis van de epidemiologie en de klinische relevantie van respiratoire virussen te vergroten.

Er is een groeiende behoefte aan betere en snellere diagnostiek van luchtweginfecties. Sneltesten voor respiratoire virussen kunnen voorzien in deze behoefte, maar op dit moment blijkt over het algemeen de kwaliteit en met name de gevoeligheid van de sneltesten nog te laag. Daarnaast is gebleken dat veel van deze sneltesten die bedoeld zijn om als ‘point-of-care’ test te gebruiken niet op de juiste manier worden onderzocht. Het is dus belangrijk dat er nieuwe sneltesten ontwikkeld worden. Bij voorkeur zouden dit sneltesten moeten zijn die tegelijkertijd meerdere - bacteriële en virale - ziekteverwekkers kunnen aantonen, maar die ook informatie zouden kunnen verschaffen over hoe (het immuunsysteem van) de patiënt zelf reageert in de luchtweginfectie. Deze sneltesten zouden vervolgens geëvalueerd moeten worden in goed opgezette studies die bij voorkeur uitgevoerd zijn op de ‘point-of-care’ zelf, zodat een representatief beeld ontstaat van de kwaliteit, maar ook van de klinische toepasbaarheid van deze sneltesten.
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Chapter 3: K.D. and K.W. designed the study. K.D., J.W., and F.K. collected the data. A.B., K.D., H.E., G.K., D.P., and K.W. interpreted the data. A.B. and K.D. drafted the manuscript. All authors critically revised the manuscript and approved the final version for publication.

Chapter 4: A.B. and J.K. were involved in the design of the study. A.B., H.A., and J.K. collected the data. P.S., H.T., A.C., M.S., K.H. and A.Z. analysed the data. A.B., H.A. and J.K. interpreted the data and wrote the manuscript. All authors critically revised the manuscript and approved the final version for publication.

Chapter 5: A.B., H.A. and J.K. contributed to the conception of the study. H.A., and J.K. collected the data. H.A., H.T., N.I., C.S., S.B. analysed the data. A.B., H.A., J.K., D.P. and K.C. interpreted the data. A.B. drafted the manuscript. All authors critically revised the manuscript and approved the final version for publication.

Chapter 6: A.B., H.W., W.W., M.D., K.W. and D.P. contributed substantially to the conception of the study. A.B, W.K. and W.W. collected and analysed the data. A.B., H.W., M.D., D.P. and K.C. interpreted the data. A.B. wrote the manuscript and all authors revised it critically for important intellectual content. All authors approved the final version of the manuscript for publication.

Chapter 7: L.L., M.D., D.P., and K.W. designed the study. L.L., A.B., X.T., R.M., S.R., and J.S. collected and analysed the data. L.L, A.B. and X.T. interpreted the data and drafted the manuscript. All authors critically revised the manuscript and approved the final version for publication.

Chapter 8: M.S., P.S., M.D., K.W. and D.P. contributed substantially to the conception of the work. A.B., X.T., L.L., J.W., R.J., and R.M. analysed and interpreted the data. A.B. and X.T. drafted the manuscript and all authors revised it critically for important intellectual content. All authors approved the final version of the manuscript for publication and agreed to be accountable for all aspects of the work.
Chapter 9: A.B., H.W., A.V., M.D., K.W. and D.P. contributed substantially to the conception of the study. A.B., W.K., H.W., K.W. and D.P. analysed and interpreted the data. A.B. drafted the manuscript and all authors revised it critically for important intellectual content. All authors approved the final version of the manuscript for publication.
PHD PORTFOLIO

Name: Andrea Hubertina Lena Bruning
PhD period: May 2014 – November 2016
Promotor: Prof. Dr. M.D. de Jong, Prof. Dr. T.W. Kuijpers
Copromotores: Dr. K.C. Wolthers, Dr. D. Pajkrt
Department: Medical Microbiology / Paediatric haematology, immunology and infectious diseases.

PHD TRAINING

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## Appendices

### Presentations (oral)

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<th>Presentation</th>
<th>YEAR</th>
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<tr>
<td>Point-of-care diagnostiek voor virale luchtweginfecties in de eerste lijn - Werkgroep Moleculaire Diagnostiek voor Infectieziekten, Utrecht</td>
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<td>Detection and monitoring of HBoV1 and CoV infections by mariPOC® - Scientific Spring Meeting for Infectious Disease Researchers, Turku, Finland</td>
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<td>Sneldiagnostiek in de huisartsenpraktijk - LOVAH congres, Maarssen</td>
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<td>PAediatric Causes of Encephalitis and Meningitis, the first PACEM results – Amsterdam Kinder Symposium</td>
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<td>Jaaroverzicht 2015 Infectieziekten – NVK, Veldhoven</td>
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<tr>
<td>Prevalence of different human rhinovirus species in infected hospitalized and non-hospitalized children in The Netherlands – Amsterdam Kinder Symposium</td>
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### Presentations (poster)

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<tr>
<td>Rapid diagnosis of respiratory viral infections in primary health care – European Society of Clinical Virology, Lisbon</td>
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<td>Detection and monitoring of human bocavirus 1 infection by a new rapid antigen test - 26th European Congress of Clinical Microbiology and Infectious Diseases, Amsterdam</td>
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<tr>
<td>Rhinovirus infections in early childhood: a comparison between hospitalised and non-hospitalised children – Scientific Spring Meeting Dutch Society of Medical Microbiology, Papendal</td>
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<td>Evaluation of an Antigen Detection Point-of-Care Test for RSV and Influenza In a Pediatric Hospitalized Population in The Netherlands – European Society of Clinical Virology, Prague</td>
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### (Inter)national conferences

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<tr>
<td>Werkgroep Moleculaire Diagnostiek Infectieziekten, Utrecht</td>
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<td>European Society of Clinical Virology, Lisbon</td>
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<td>Dutch Annual Meeting of Family Doctors in Training, Maarssen</td>
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<td>European Congress of Clinical Microbiology &amp; Infectious Disease, Amsterdam</td>
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<td>Amsterdam Paediatric Symposium, Amsterdam</td>
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<td>Dutch Association of Pediatrics, Veldhoven</td>
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<td>Amsterdam Paediatric Symposium, Amsterdam</td>
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TEACHING

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<th>Lecturing</th>
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<tr>
<td>Workshop 'Leren presenteren' for bachelor thesis students</td>
<td>2016</td>
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<th>Tutoring, mentoring, supervising</th>
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<tr>
<td>Supervising 9 students involved in the PrimariPOC® study</td>
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<tr>
<td>Lisa Kolodziej, bachelorthesis</td>
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<tr>
<td>Celine Busch, bachelorthesis</td>
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<tr>
<td>Hannah Vos, bachelorthesis</td>
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<td>Willeke de Kruijf, wetenschappelijke stage</td>
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PARAMETERS OF ESTEEM

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<tr>
<td>Travel Grant ESCV 2016, Lisbon</td>
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<tr>
<td>Spinoza grant, Universiteitsbeurs, University of Amsterdam</td>
<td>2016</td>
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<tr>
<td>Marie Curie Early Stage Research Fellowship (in the framework of AIROPico, an FP7 Marie Curie Industry-Academia Partnerships and Pathways network), 5 months secondment to Turku, Finland</td>
<td>2014-2016</td>
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LIST OF PUBLICATIONS

This Thesis

International publications


Other

International publications


**National Publications**


**Book chapters**

ACKNOWLEDGEMENTS

The writing and completion of this thesis would not have been possible without the advice, assistance and support of many. I wish to express my sincere gratitude to:

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Susan and Bart, our paths have crossed so often that it feels natural to have the two (actually three!) of you standing next to me as paranimfen. I feel grateful that what started out as a professional relationship (groepje 17 and ANIOS Kindergeneeskunde) has turned into a friendship that I value very highly.
Lieve pappa en mamma, lieve vrienden en familie. Met dit gedrukte proefschrift is het doel van mijn PhD-traject bereikt. Maar zoals mij al van jongs af aan is geleerd: het gaat niet om het doel, maar om de reis er naar toe. En wat voel ik mij gezegend en gelukkig met jullie als reisgenoten. Dank je wel dat jullie stuk voor stuk op je eigen prachtige manier mijn wereld een beetje mooier maken.

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ABOUT THE AUTHOR

Andrea Bruning (1987, Maastricht, the Netherlands) graduated in 2005 cum laude from the Porta Mosana College in Maastricht. Afterwards, she studied Bèta Gamma at the Institute for Interdisciplinary Studies of the University of Amsterdam. In addition, she started to study Medicine at the Academic Medical Center in Amsterdam in 2006.

In 2010, for her medical research internship, Andrea spent five months at the Department of Infectious Diseases of the Istituto Superiore di Sanità in Rome, Italy. After two years of clinical rotations she travelled to Dehradun, India, for her final internships in Pediatrics and Public Health. She obtained her medical degree cum laude in 2013.

After graduation, Andrea has worked for one year as a resident at the Pediatric Department of the Sint Lucas Andreas Ziekenhuis (currently OLVG-West). In 2014, she started her PhD research on respiratory viruses at the Department of Pediatric Infectious Diseases and the Department of Medical Microbiology under supervision of Dr. Dasja Pajkrt, Dr. Katja Wolthers, Prof. dr. Taco Kuipers and Prof. dr. Menno de Jong. As part of her research, she lived for five months in Turku, Finland as a Marie Curie Research Fellow. During her PhD research, she became infected with the microbiology-virus. As a result, Andrea has started her training to become a medical microbiologist at the Academic Medical Center in Amsterdam in December 2016.
Acute respiratory tract infections are a leading cause of morbidity and mortality worldwide. Symptoms can be mild, for example those of the ‘common cold’, but severe complications such as pneumonia may develop. Respiratory viruses are thought to be responsible for the vast majority of respiratory tract infections. Rapid identification of these viruses is important for clinical patient management, public health surveillance, and infection prevention. In recent years, the diagnostic possibilities for the detection of respiratory viruses have advanced rapidly. There is a clear trend towards faster diagnostics. Increasing numbers of rapid tests designed for use at the point-of-care have been developed. The aim of this thesis is 1) to evaluate the use and diagnostic accuracy of rapid tests for respiratory viruses in the hospital setting and in primary health care; 2) to increase our insight in the epidemiology and clinical relevance of respiratory viruses.