REVIEW ARTICLE

The Role of Multiplex PCR in Respiratory Tract Infections in Children

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SUMMARY

Background: Infants, toddlers, and children of primary-school age without any special risk factors generally have three to ten febrile respiratory infections per year. Most such infections are of viral origin and self-limiting, but viral infection is often hard to distinguish from bacterial infection. The use of a multiplex polymerase chain reaction (PCR) to detect viruses in respiratory secretions is potentially beneficial, as it might help physicians avoid giving antibiotics unnecessarily.

Methods: This article is based on a selective review of the literature and on the findings of the authors' own investigations.

Results: Multiplex PCR is a highly sensitive, highly specific test for the detection of viral nucleic acids in respiratory secretions. If PCR reveals the presence of RNA derived from respiratory syncytial virus, human metapneumovirus, parainfluenza virus, or influenza virus, then an acute infection caused by the corresponding pathogen is probably present, and further treatment can be given accordingly. On the other hand, the nucleic acids of adeno-, boca-, rhino- or coronaviruses can be found in relatively trivial infections as well as in asymptomatic persons, probably reflecting either a prior infection or a current subclinical one. For children in particular, upper respiratory infections are so common in the winter months that acute and prior infections with these pathogens cannot be distinguished by multiplex PCR. The use of multiplex PCR in children has not been shown to shorten hospital stays or to lessen antibiotic consumption or overall cost.

Conclusion: The detectability of viral nucleic acids is an important contribution to the diagnostic assessment of children with severe respiratory infection. For these highly sensitive diagnostic tests to be used optimally, primary viral infections must be distinguished from bacterial superinfections.

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Infections of the respiratory tract are a common problem in the first decade of life. The yearly prevalence of respiratory tract infections in an otherwise healthy 3-year old child is about three to 10 infections (1–3). Although it has become an accepted fact among pediatricians and general practitioners that most respiratory tract infections are generally self-limiting, the use of antibiotics for this indication in Germany is high. Over a time period of 12 months, more than one third of all children and adolescents younger than 15 years of age takes antibiotics (4). The most common pathogens causing bacterial pneumonia in childhood—*Streptococcus pneumoniae* and *Haemophilus influenzae*—are part of the nasopharyngeal mucosal flora in healthy children (5). Confirmation of a viral infection by using traditional methods—viral cultures, direct immunofluorescence tests, and rapid antigen tests—are in many cases too laborious or unreliable: direct immunofluorescence tests and rapid antigen tests yield results within very few hours, but in many cases they lack sensitivity and specificity and are available for few viruses only (6). Serological tests are unsuitable for the diagnostic evaluation of acute respiratory infections.

Respiratory tract infections are caused clinically by a multitude of pathogens. Pathogen-specific clinical symptoms are often lacking. In view of 20 or more potential pathogens, investigations using specific polymerase chain reaction (PCR) for individual viruses (“monoplex-PCR”) are soon too time-consuming and elaborate for the laboratory. Multiplex-PCR methods enable testing for many pathogens in parallel in a single analysis and are therefore increasingly gaining in importance, since the required specimen volume, time expense, and cost expenditure hardly differ from monoplex-PCR, even though 20 or more different pathogens are analyzed simultaneously. This review article is restricted to multiplex-PCR and aims to summarize the current research status for multiplex-PCR for clinicians who treat pediatric outpatients and inpatients.

The results of currently available commercial multiplex methods are available within one to six hours, depending on the pathogen under investigation. The confirmation of viral or bacterial nucleic acid does, however, not enable any conclusions regarding the pathogens' ability to multiply or infectivity (7)

CASE REPORTS

● Case report 1

A 12 months old boy presents to the emergency outpatient department with cough and a fever up to 41°C, both of which he has had for a week. The blood count shows leukocytosis (35 G/L) with neutrophilia (78%). Opacification of the right middle lobe is radiologically visible. Multiplex-PCR testing for respiratory pathogens from nasopharyngeal secretions yields a positive result for human metapneumovirus (hMPV). The boy's high temperature, pronounced leukocytosis with neutrophilia, and the lobar opacification on the thoracic x-ray film do, however, raise urgent suspicions of a bacterial superinfection. For this reason, intravenous ampicillin treatment is started. The blood culture shows group A streptococci during the disease course.

● Case report 2

A 7 week-old baby girl born at term presents with fever, cough, and decreased oral intake for two days. She is somnolent but brightened up after being given an antipyretic. Her leukocyte count is in the normal range (13 G/L) without a left shift in the blood smear. The baby's urine status is normal, and no pulmonary infiltrates are seen on radiology. Multiplex-PCR of nasopharyngeal secretions confirms influenza A-H1N1-RNA. The child is admitted over night for close monitoring, but she is discharged home the following day in an improved general condition and without antibiotic treatment.

Generally, the analytical sensitivity and specificity of multiplex-PCR have become quite similar to that of monoplex-PCR. However, studies have shown a lower sensitivity for individual pathogens—for example, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), and enteroviruses (8, 9). Technologically, different test formats exist. To date, quantitative conclusions are not reliably established, neither for monoplex-PCR nor for multiplex-PCR. Some of the tests can be used to confirm not only viruses but also bacteria, such as *Mycoplasma pneumoniae* or *Bordetella pertussis*.

In the setting of multiplex-PCR, sampling and transporting specimens is also of major importance. Suitable specimens to confirm respiratory pathogens can be obtained from the upper or lower respiratory tract—that is, nasopharyngeal aspirate, pharyngeal swabs, nasopharyngeal swabs, or bronchoalveolar lavage fluid. The confirmation frequency of pathogens in children with acute respiratory symptoms may be in excess of 80%. In adults it is notably lower, with only 20% in some studies (10, 11). One of the reasons for this may be the fact that children have much higher viral titers. Furthermore, in view of the fact that respiratory tract infections in children occur in close succession during the winter season, it can be assumed that infections actually overlap. This may provide an explanation for the fact that viral nucleic acids of different viruses in one specimen are found virtually only in children—for example, in 8% of all specimens analyzed by the authors of this article (12).

Table 1 summarizes taxonomic characteristics, typical disease patterns, and specific treatment for selected respiratory viruses. Since the clinical symptoms of respiratory tract infections in children are very similar to one another, we structured the following sections not according to symptoms but by pathogen.

In addition to the benefit for individual patients, the use of multiplex-PCR is advantageous from the

perspective of hospital hygiene too: if there are not enough single rooms, the patients can be grouped according to their clinical symptoms. The advantage of putting together cohorts by pathogen is plausible and recommended for RSV (1) and influenzaviruses (e2) by the Robert Koch-Institute (RKI), even if studies have not sufficiently confirmed this.

Specific pathogens

Respiratory syncytial virus

RSV, a paramyxovirus, is the most common cause of bronchiolitis and pneumonia in the first year of life. 17 out of 1000 children younger than 6 months were treated for RSV as inpatients in a US study (e3). Furthermore, RSV causes apnea in neonates and young infants, even if patients had received prophylactic treatment with palivizumab (e4). In the first year of life, RSV is associated with more deaths than influenza (3.1 deaths v 0.3 deaths per 100 000 person-years [e5]). Bronchiolitis is a clinical diagnosis with respiratory failure and obstruction of the small airways. Many study authors recommend refraining from routine diagnostic evaluation (for example, microbiological testing, radiography of the thorax, and blood gas analyses) in children with bronchiolitis since these impose physical stress and lead to unnecessary inpatient admissions and treatment (13–18). These recommendations are only partly adhered to in German-speaking countries. In very small infants, virological diagnosis can contribute to avoiding further investigations (13, 16).

In suspected RSV, a rapid antigen test is a sensible first step. Because of the high specificity of rapid tests, the RSV diagnosis is regarded as confirmed when the result is positive (19). The reported sensitivity is 43–91% and is higher for nasopharyngeal lavage than for nasopharyngeal swabs (e6, e7). Serum and plasma are not suitable. In infants and young toddlers who became ill during the RSV season, the diagnostic validity was found to be notably higher than in older

TABLE 1

Taxonomic characteristics, typical disease patterns, and specific therapy in selected respiratory viruses

Virus	Family, genome	Sub-/serotypes	Disease/complication	Specific therapy
Human adenoviruses (e16)	Adenoviridae, dsDNA linear	> 50 serotypes with different disease patterns	Respiratory tract infections, gastrointestinal infections, conjunctivitis, hemorrhagic cystitis, hepatitis, hemorrhagic colitis, pancreatitis, nephritis, encephalitis; dissemination in immunosuppression	Cidofovir can be considered in severe cases of adenovirus infection
Human bocavirus (HBoV) (e17)	Parvoviridae, (+) and (–)ssDNA linear	Serotypes 1–4	Lower respiratory tract infections (HBoV 1)*, gastrointestinal infections (HBoV2–4)*, asthma exacerbations*	–
Coronaviruses (28)	Coronaviridae, (+)ssRNA linear	229E, OC43, NL63, HKU1, MERS, SARS	Primarily mild respiratory tract infections, non-diphtheric croup, febrile convulsions; MERS and SARS: atypical pneumonia with a severe disease course	–
Influenzavirus	Orthomyxoviridae, (–)ssRNA linear/segmented	A (mammals, birds), B (humans only), C (humans, pigs)	Sudden disease onset with a high temperature, headaches and aching limbs, gastrointestinal symptoms are possible, bacterial superinfection, rarely encephalitis, myositis, myocarditis	Neuraminidase inhibitors M2-inhibitors effective only in influenza A (but currently almost all isolates are resistant)
Human metapneumovirus (hMPV)	Paramyxoviridae, (–)ssRNA linear	Subtypes A and B	Second most common cause of bronchiolitis (after RSV), pneumonia; severe disease course in young children, older persons, and persons with immunosuppression; development of asthma	–
Parainfluenzavirus	Paramyxoviridae, (–)ssRNA linear	1–4 with different seasonal patterns	Febrile infection of upper and lower respiratory tract, non-diphtheric croup; more severe course in young children and persons with immunosuppression	After allogeneic stem cell transplantation, ribavirin and intravenous immunoglobulin if required (e19)
Rhinovirus (HRV)	Picornaviridae, (+)ssRNA linear	Species A to C, > 100 serotypes	Rhinorrhea, sore throat, cough, asthma exacerbations, pneumonia (HRV-type C)	Pleconaril (experimental, not available)
Human respiratory syncytial virus (RSV)	Paramyxoviridae, (–)ssRNA linear	Subtypes A and B	Bronchiolitis, pneumonia, upper respiratory tract infections; severe courses in premature infants, persons with cardiac and pulmonary disorders, and immunosuppressed persons; development of asthma* (e18)	In pneumonia after allogeneic stem cell transplantation, ribavirin and intravenous immunoglobulin if required (e19)

*The association between the virus and the diseases is still the subject of controversial discussion.

DNA, deoxyribonucleic acid; HRV, human rhinoviruses; MERS, Middle East respiratory syndrome; RNA, ribonucleic acid; RSV respiratory syncytial viruses; dsDNA, double strand DNA; SARS, acute respiratory syndrome; ssDNA, single strand DNA; ssRNA, single strand RNA

children and outside the RSV season (e1, e7). In cases where RSV infection is acutely suspected and the rapid RSV test yields a negative result, RSV-PCR can subsequently be undertaken. Since bronchiolitis can also be caused by a number of other pathogens (for example, hMPV), multiplex-PCR should be undertaken in such cases. Coinfection of RSV with other respiratory viruses is common and can increase the severity of the disease (20). Coinfection of RSV with *Bordetella pertussis* has also been observed (21). Lobar pneumonia due to *Staphylococcus aureus* (22) and *Streptococcus pneumoniae* (23) is a well-known complication of RSV infections.

Influenza

Some 6–12% of all children use healthcare services every year because of influenza (24). Additionally, many cases of infection with influenzavirus are not

diagnosed as such. In a large US study, only 58% of inpatients and 7% of outpatients with influenza were tested for this pathogen (24). In the US in every flu season, there are 0.5–3.8 deaths per 1 000 000 children and adolescents (25). Half of the children who die from flu do not have any of the well-known risk factors (25, 26), such as:

- Chronic heart disease
- Chronic lung disease (for example, asthma, cystic fibrosis)
- Metabolic diseases (for example, diabetes mellitus)
- Immunodeficiencies
- Neurological or neuromuscular disorders
- Obesity, or
- Pregnancy (25, 26).

If flu is suspected, a rapid antigen test can be performed as a first step. If the result is negative, diagnostic

TABLE 2

Benefits of confirmation of specific pathogens in certain patient populations

Patient population	Pathogen	Particular relevance	Notes
First months of life	Influenza	Trigger of apnea, oseltamivir treatment if required (27)	*1, *2
	RSV (human respiratory syncytial virus)	Trigger of apnea, preventing further, superfluous diagnostics and therapy (13–18)	*2
	Pertussis	Trigger of apnea, antibiotic treatment	
Chronic cardiac or pulmonary disorders, metabolic disorders (for example, diabetes mellitus), immunodeficiencies, neurologic or neuromuscular disorders, obesity or pregnancy (25, 26)	Influenza	Oseltamivir treatment if required (27)	*1, *2
Severely immunosuppressed patients	Adenovirus (e16)	Specific therapy (cidofovir) possible	PCR is the testing method of choice; Antigen tests lack sensitivity
Patients after allogeneic stem cell transplantation	Parainfluenza virus, RSV	Ribavirin and intravenous immunoglobulin if required (e19)	Severe courses likely (also in hMPV, adenoviruses)

*1 Oseltamivir in infancy is licence-approved in Europe only during pandemic flu outbreaks.

*2 Where flu or RSV are acutely suspected, a rapid antigen test can be undertaken first, as the result is available immediately and the test is less expensive than PCR-based diagnostic evaluation. If the test is negative, the authors of the current article will follow this up with multiplex-PCR.

hMPV, human metapneumovirus; RSV, human respiratory syncytial virus

evaluation by means of PCR testing makes sense. Early confirmation of influenzaviruses is helpful because treatment with neuraminidase inhibitors then becomes an option. Oseltamivir, the most commonly used neuraminidase inhibitor, is, however, at best of moderate effectiveness (27).

Patients with immunosuppression (for example, those with congenital immunodeficiencies or underlying oncological disorders) and patients with acute pulmonary deterioration in cystic fibrosis may also benefit from stringent flu diagnostics. In these patients, the indication for neuraminidase inhibitors could also justifiably be defined more generously. Confirmation of influenza RNA obviously cannot rule out superinfection with pneumococci, staphylococci, or *H. influenzae*. 5–6% of all cases of pneumococcal pneumonia are associated with flu, and this proportion is even higher in a time of increased flu activity (e8).

Coronaviruses

Six different human pathogenic coronavirus species (HCoV) are known to date. HCoV-229E and HCoV-OC43 were discovered as early as the 1960s. As a rule they cause benign disorders of the upper respiratory tract (28). After the SARS epidemic (SARS, severe acute respiratory syndrome) in 2003 with the SARS-CoV, HCoV-NL63 was described in 2004 and HCoV-HKU1 in 2005 (28). The Middle Eastern Respiratory Syndrome (MERS) is caused by MERS-CoV (29). Most of the multiplex-PCR methods in use can differentiate between HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1. HCoV-NL63 is assumed to be an important cause of non-diphtheric croup. Furthermore, coronaviruses can also cause febrile convulsions (28). If a patient's medical history gives

rise to a suspicion of MERS-CoV, specific diagnostic methods will have to be undertaken at the Robert Koch-Institute or in the reference laboratory at the University of Bonn (e9).

Persistence of nucleic acid of respiratory viruses

For a long time it was assumed that symptomatic infection with respiratory tract viruses was regularly shown by the confirmation of viral nucleic acids in respiratory secretions. However, in recent years, there have been increasing indications that this is not necessarily the case. Bocaviruses constitute an example: the clinical importance of these human parvoviruses, which were discovered in 2005, remains the subject of controversy. Bocavirus DNA can be confirmed in the respiratory tract of even asymptomatic children; a high concentration of these viruses in respiratory specimens seems to be associated with symptoms (30). Only quantitative monoplex-PCR or multiplex-PCR, which is usually not available, can provide information about virus concentration.

Adenovirus DNA is also often found in the respiratory secretions of asymptomatic children. For this reason, the confirmation of adenovirus DNA cannot exclude Kawasaki disease (31). Children with Kawasaki disease, in whom adenoviruses were confirmed, have a lower virus concentration than children who are symptomatic only because of adenovirus infection (31). Furthermore, nucleic acids from rhinoviruses (32) and coronaviruses (33) can persist for weeks and months. This is observed particularly in hypogammaglobulinemia (34). Rhinoviruses are often present in tumor patients and patients after stem cell transplantation, and they do not seem to be associated with increases in mortality in such patients (35).

The molecular confirmation of these four pathogens should always raise suspicions of infections in the more distant past that may not be directly related to the patient's current symptoms. This fact is important especially in small children, since they contract multiple infections in one winter season, some of which will have a short latency period between episodes of illness. Rhedin et al. showed that the nucleic acids of RSV, hMPV, and parainfluenza virus can be confirmed almost exclusively in symptomatic children and very rarely in asymptomatic children (33). A causal association between respiratory tract symptoms and the confirmation of RSV, hMPV, and parainfluenza virus therefore seems likely. In the same study, rhinovirus DNA was confirmed in 47.9% of symptomatic children, but also in 21.5% of the control group (33).

Effectiveness of virus diagnostics

The question of whether routine virological diagnostic evaluation based on antigen confirmation (not PCR) can reduce antibiotic consumption in the pediatric emergency setting, remained unanswered in a Cochrane meta-analysis, since studies with sufficiently large numbers of cases are lacking (36). Oosterheert et al. showed that the confirmation rate for infections of the lower respiratory tract in adults increased from 21% to 43% if quantitative PCR for respiratory pathogens is used, rather than conventional diagnostic tests. However, this study did not show any reduction in antibiotics or in costs (37). One limitation of the study is the fact that it dates back to 2005. The costs then incurred for PCR diagnostics, of 330.78 Euro/specimen, have been notably reduced since then (37).

Wishaupt et al. used PCR diagnostics for 17 different pathogens in the context of a controlled study (6) in patients younger than 12 years with respiratory tract symptoms. In the intervention group, clinicians were told the results within 12–36 hours after specimens had been taken, and in the control group, this was done after four weeks. RSV was the most common pathogen, at 55%. The average length of hospital stay and the duration of antibiotic therapy did not differ between groups. The intervention group, however, showed a significantly higher use of antibiotics. The reasons for this effect remain unclear, because the decision in favor of or against intravenous antibiotics was made on the basis of clinical assessment and before the PCR results were known. Since this study—and other, similar ones—excluded children with underlying diseases from participation, the results apply only to children who were previously healthy.

Atypical bacterial pathogens

An important observational study in children with infections of the upper respiratory tract, conducted in Rotterdam, was published in *PLoS Medicine* in 2013 (38). Spuesens et al. found *Mycoplasma pneumoniae* in 21.2% of asymptomatic children and in 16.2% of symptomatic children. Interestingly, the mycoplasma persisted for up to four months. The prevalence of my-

coplasma in this study in the summer of 2010 was up to 58%. Only 13.5% of examined patients were treated on an inpatient basis.

In the authors' own experience, confirmation of mycoplasma nucleic acid and DNA from other atypical pathogens in respiratory secretions by using multiplex-PCR is the exception. It also remains the subject of controversy whether the treatment of *Mycoplasma pneumoniae* with antibiotics is beneficial (39). Another atypical respiratory pathogen, *Chlamydomphila pneumoniae*, is found only rarely, in 1–2% of all cases of childhood pneumonia (e10). The Robert Koch-Institute regards PCR as the gold standard for the diagnostic evaluation of *Chlamydomphila pneumoniae* (e11). Fewer than 2% of infections with *Legionella spp.* manifest in childhood or adolescence, overwhelmingly in the first year of life and in teenagers (e12). The Robert Koch-Institute attests extraordinarily high sensitivity to PCR techniques for the confirmation of legionella bacteria from respiratory secretions and tissues, even though culture is still regarded as the gold standard (e13). The sensitivity of the widely used rapid urine antigen test depends on the severity of disease and the serotype (e14, e15). Especially in infants at acute risk because of apnea caused by pertussis, multiplex-PCR allows a substantial information advantage compared to the less sensitive culture. Furthermore, this method also captures other pathogens that cause paroxysmal coughing (40).

Conclusion

Only very few studies have investigated the costs or antibiotic usage while using multiplex-PCR for respiratory pathogens. So far, no benefit has been found for this diagnostic technique in children without risk factors. The potentially important rate of excretors of viral nucleic acids of specific pathogens that are not directly associated with the acute illness presents a problem in this setting. Generally, multiplex-PCR should be used whenever consequences for the treatment will result—for example, stopping a course of antibiotic treatment. So far, there are no algorithms for such an approach that have been validated in practice. The authors recommend the use of multiplex-PCR especially in the first months of life, in children with risk factors for influenza, and in immunosuppressed patients (for example, patients who have undergone an allogeneic stem cell transplant), as clinical consequences are more likely to be concluded from the results in such patients (Table 2). Infections with adenoviruses, parainfluenza viruses, or RSV often take a severe course in patients who have undergone allogeneic stem cell transplantation.

Further studies into the role of molecular pathogenic diagnostic evaluation in the prevention and detection of nosocomial chains of infection (“hygiene indication”) and in treating specific groups of patients (for example, cancer patients) are urgently needed. Confirmed DNA from hMPV, influenzavirus, parainfluenza virus, or RSV enables conclusions about the causative pathogen

in qualitative reverse transcriptase-PCR. For the evaluation of the confirmation of other pathogens—for example, bocaviruses, rhinoviruses, adenoviruses, or coronaviruses—a clinical evaluation, hopefully aided by quantitative PCR techniques in the future, is needed in order to distinguish acute infections from subclinical events with nucleic acid persistence.

Conflict of interest statement

PD Dr Panning has received travel expenses and speaker fees from Mikrogen.

The other authors declare that no conflict of interest exists according to the guidelines of the International Committee of Medical Journal Editors.

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KEY MESSAGES

- Quantitative multiplex-PCR testing of respiratory secretions is a highly sensitive method for the diagnosis of respiratory tract infections. In addition to viral pathogens, atypical bacterial pathogens can also be confirmed.
- Confirmation of nucleic acids from influenzavirus, parainfluenza virus, RSV, or hMPV usually allows the conclusion that an acute viral infection is present.
- Nucleic acid from bocaviruses, adenoviruses, rhinoviruses, or coronaviruses is often found in asymptomatic persons. Quantitative measurement and the study of the virus concentration over time might in the future contribute to distinguishing acute infections from protracted nucleic acid excretion.
- To date, it is not known whether using multiplex-PCR reduces the length of inpatient stay, the costs of inpatient treatment, or the administration of antibiotics. Especially studies of the nucleic acid confirmation guided cohort-formation of children with respiratory tract infections and for special patient populations (for example, cancer patients, immunosuppressed patients, patients with cystic fibrosis) are lacking.

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REVIEW ARTICLE

The Role of Multiplex-PCR in Respiratory Tract Infections in Children

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