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ORIGINAL ARTICLE

Comparison of diagnostic tools with multiplex polymerase chain reaction for pediatric lower respiratory tract infection: A single center study

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Background/Purpose: Acute respiratory tract infections are a leading cause of morbidity and mortality in children worldwide. Most have a viral etiology, with pneumococcus as an important pathogen. This single-center study compared the use of conventional diagnostic tools and two multiplex polymerase chain reaction (PCR) examinations for determining pathogens in lower respiratory tract infections (LRTIs) among children aged <5 years.
Methods: From July to October 2010, 45 patients aged 2 months to 60 months and diagnosed as having LRTIs were enrolled. Their nasopharyngeal aspirates were evaluated through viral culture and two multiplex PCR examinations. The patients' clinical course, symptoms, signs, and laboratory findings were recorded and analyzed.
Results: Among the 45 patients, 38 (84.4%) had detectable pathogens. Conventional viral and blood cultures had 35.6% positive rate, which increased to 51.1% when the quick antigen tests (Influenza A+B test and respiratory syncytial virus) and urine pneumococcal antigen test were combined. The positive rate further increased to 84.4% when the two multiplex PCR methods were combined. Twelve patients had co-infection, including 10 detected by the multiplex PCR methods. The co-infection rate was 26.7% (12/45).

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Conclusion: Most LRTIs in children have a viral etiology. Multiplex PCR tests are rapid assays that can increase the diagnostic yield rate and detect slow-growing viruses and can detect more pathogens than conventional viral culture to enable, thereby helping clinicians to provide appropriate and timely treatment.

Introduction

Acute respiratory infections are a leading cause of morbidity and mortality in children worldwide,[1,2] imposing significant disease burden.[3] Most respiratory tract infections among children aged under 3 years have a viral etiology and children with co-infection are hospitalized more frequently than those with a single viral infection.[4] Respiratory syncytial virus (RSV), influenza virus, parainfluenza virus, and adenovirus are the most common causes of lower respiratory tract infections (LRTIs) in children,[5,6,7] whereas *Streptococcus pneumoniae* is a frequent cause of bacterial community-acquired pneumonia in Taiwan.

Conventional diagnostic tools such as viral, blood, and sputum cultures have high false negative rates such that some pathogens are often underestimated. Bacterial LRTIs require prompt diagnosis for early proper antibiotic therapy. The early recognition of pathogens in such infections helps in determining more appropriate treatment actions.

This study aimed to survey pathogens of LRTIs in children aged $\leq$5 years in a single center using two kinds of multiplex polymerase chain reaction (PCR) methods and to compare these with conventional diagnostic tools.

Methods

Participants

Children with LRTI admitted to the pediatric ward of Kaohsiung Veterans General Hospital from July to October 2010 were enrolled. In addition to detailed history taking and physical examinations, chest plain x-rays were taken to assist diagnosis. Lower respiratory tract infection was defined as a diagnosis of bronchitis, bronchiolitis, bronchopneumonia, or pneumonia. Children with asthma and laryngo-trachitis were excluded.

The hospital's institutional review board and ethics committee approved the study (VGHKS 99- CT-7-13) and the patients' parents or guardians provided written informed consent.

Specimens

Within 48 hours after admission, nasopharyngeal aspirates were collected from each patient and sent for viral culture and two kinds of multiplex PCRs. Other laboratory examinations included complete blood cells with differential counts, levels of C-reactive protein, blood urea nitrogen, serum creatinine, aspartate aminotransferase, and alanine aminotransferase, and sputum and blood cultures.

Pathogen identification

The specimens underwent conventional viral culture and nucleic acid extraction for multiplex PCR amplification examinations. The viral culture utilized a series of primary cell lines (human hibroblast, rhesus monkey kidney) and continuous cell lines (A549 human lung carcinoma) selected for their ability to support the replication of a wide variety of clinically relevant viruses. The specimens were inoculated onto these cell culture monolayers and monitored by light microscopy for the cytopathic effect, which were the visible cellular changes that occurred in response to viral infection.

The QIAGEN QIAamp RNA and DNA mini kits (Qiagen, Valencia, CA, USA) were used for extraction of nucleic acids from pathogens in the nasopharyngeal samples, and prepared these extracted nucleic acids for the two multiplex PCR methods: the Respiratory Viral Panel (RVP; Luminex Molecular Diagnostics, Totronto, Canada) for respiratory virus and the ResPlex I panel (Qiagen) for adenovirus and bacteria. These two multiplex PCR methods could detect pathogens from nasopharyngeal specimens within one day.

For RVP, the extracted nucleic acids from the QIAamp RNA mini kits were reverse transcribed to complimentary DNA for testing. Specific primers to each target virus were mixed for multiplex target-specific PCR and to rapidly create multiple copies of DNA. The amplified DNA was mixed with short sequences (TAG primers) of DNA that were specific to each viral target. If the target was present, the primer would bind and be lengthened through a process called target-specific primer extension. During this process, a label was incorporated. Amplified products were labeled while color-coded beads were added for the easy identification of tagged primers. They were then placed in the Luminex 200 instrument (Luminex, Austin, TX, USA) and the color-coded beads were analyzed by lasers (Luminex xMAP technology). The lasers identified the color of the beads (specific to a virus or subtype) and the presence or absence of the labeled primer. If a particular virus was present, it would generate a signal and be identified by the associated data analysis software (xPONENT software, Luminex) as positive. This method detected viral pathogens like adenovirus, entero-rhinovirus, influenza A and B, para-influenza 1, 2, 3, and 4, RSV, and human metapneumovirus.

In the ResPlex I panel method, the HotStarTaq Master Mix, ResPlex I SuperPrimers, extracted nucleic acids and RNase-free water were prepared for amplification reaction. A negative control (without template DNA) was also prepared. The GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA), running in 9600 emulation mode, was then used to program the thermal cycle. After

the thermal cycle, the PCR product was mixed with Genaco detection buffer and ResPlex I bead mix. After hybridization, dilute streptavidin-phycoerythrin and Genaco stopping buffer were added before sample detection. These products were then analyzed on the Luminex 200 instrument using the QIAplex Molecular Differential Detection Software. The ResPlex I panel detected 10 different pathogens, including *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila*, as well as *Neisseria meningitidis*, *Haemophilus influenzae* (all strains), and adenovirus (types 3, 4, 7, and 21).

The Influenza A + B quick antigen test was done using the QUICKVUE Influenza A+B test kit (QUIDEL, San Diego, CA, USA) and by nasopharyngeal sterile swab (COPAN Diagnostic Inc., Murrieta, CA, USA). Specimens were collected by sterile swab and sent to the microbiology laboratory for the QUICKVUE Influenza A+B test immediately.

The RSV quick antigen test was done using the BinaxNOW RSV test kit (Inverness Medical Innovations, USA). Nasopharyngeal aspirates were collected as specimens. This test was a rapid immuno-chromatographic assay for the qualitative detection of RSV fusion protein antigen.

Results of identified pathogens from conventional cultures and from multiplex PCR methods were considered as true infections by the patient's primary physician based on their respective clinical correlations.

Clinical data

The clinical data included demographic data (i.e., age, gender, body height and weight, and underlying disease), symptoms and signs, physical examinations, duration of fever, length of hospital stay, treatments, and outcomes.

Statistical analysis

Continuous variables were analyzed by independent *t* test while categorical data were compared by χ^2 test or Fisher's exact test, as appropriate. A *p*-value <0.05 was considered statistically significant. Data were analyzed using the SPSS software for Windows (17.0; SPSS Inc., Chicago, Illinois, USA).

Results

Study population and demographics

There were 45 patients enrolled (26 males), with mean age of 2.76 ± 1.32 years (range 2 to 60 months). Their mean body mass index was 15.8 ± 1.92 kg/m^2 and mean length of hospitalization was 6.9 ± 3.2 days. Ten patients had symptoms of dyspnea on admission (Table 1).

Patients and pathogens

Among the 45 patients, 38 had detectable pathogens (84.4%). The positive rate was 42.2% (19/45) for multiplex PCR via RVP method and 42.2% (19/45) via ResPlex I panel method.

In this study, conventional cultures (e.g., viral culture and blood culture) had 35.6% positive rate, which increased to 51.1% when the RSV quick antigen test, Influenza quick antigen test and urine pneumococcal antigen test were added. The positive rate further increased to 84.4% when the two multiplex PCR methods were added (Table 2).

Twelve patients (26.6%) had co-infections, including 10 with more than one pathogen detected by the two multiplex PCR methods, even though the conventional viral culture showed only one pathogen in each positive culture.

Adenovirus was the most commonly isolated pathogen in the LRTIs, followed by *Streptococcus pneumoniae* (Table 3). The two multiplex PCR methods detected more positive results of adenovirus than those detected by conventional viral culture, with significant difference ($p < 0.001$).

Table 1 Demographic data of patients with LRTIs

	Patient no. (%)	Mean ± SD
Age (year)		2.76 ± 1.32
Gender (male)	26 (57.8)	
BMI (kg/m^2)		15.8 ± 1.92
Hospitalization days		6.9 ± 3.2
Total fever days		4.9 ± 2.74
Patients with dyspnea	10 (22.2)	
WBC ($\times 10^9$/L)		11.3 ± 5.1
Band form >5%	1	
CRP (mg/dL)		3.53 ± 5.81

BMI = body mass index; CRP = C-reactive protein; SD = standard deviation; WBC = white blood cell.

Relationship between clinical symptoms and single infection and co-infections

In terms of demographic data (i.e., age, sex, and body mass index), there was no significant difference between single and multiple infections. In clinical symptoms, signs, and laboratory data, only breath sounds, particularly rales, were significantly different between single and multiple infections (Table 4).

Table 2 Comparison of positive results between pathogen detection methods

Methods	Patient number	Positive patient number (%)
(a) Multiplex PCR, ResPlex	45	19 (42.2)
(b) Multiplex PCR, RVP	45	19 (42.2)
(a) + (b)	45	32 (71.1)
(c) Viral culture	45	16 (35.6)
(d) Blood culture	36	0
(c) + (d)	45	16 (35.6)
(e) Influenza A + B	28	2 (7.1)
(f) RSV	5	4 (80)
(g) Urine pneumococcal Ag	20	3 (6)
(c)+ (d) + (e) + (f) + (g)	45	23 (51.1)
(a) + (b) + (c) + (d) + (e) + (f)	45	38 (84.4)

Ag = antigen; PCR = polymerase chain reaction; RVP = Respiratory Viral Panel.

Table 3 Number of different pathogens detected by the two multiplex PCR methods and conventional viral culture

Pathogens (number)	(1) Viral culture	(2) RVP	(3) ResPlex[c]	(2) + (3)	*p* value[d]
Adenovirus	6	9	5	10	<0.001
Entero-rhinovirus[a]	2	6	NA	6	0.57
Influenza virus (A, B)	2	6	NA	6	0.12
Parainfluenza virus	1	2	NA	2	0.88
HSV	2	NA	NA	0	NA
RSV	3	2	NA	2	0.07
hMPV	0	2	NA	2	NA[e]
All virus	16	27	NA	28	0.58
S pneumoniae	NA[b]	NA	9	9	NA
C pneumoniae	NA	NA	0	0	NA
L pneumophila	NA	NA	0	0	NA
N meningitidis	NA	NA	0	0	NA
H influenzae	NA	NA	5	5	NA
M pneumoniae	NA	NA	2	2	NA
Total positive results	16	27	16	43	NA

[a] Viral culture detected two positive results of enterovirus and no rhinovirus; enterorhinovirus were detected together in RVP method.
[b] Pathogens not detected by the method were labeled "NA", not available.
[c] ResPlex detected adenovirus and bacterial pathogens listed in above table.
[d] Denote Chi-square test; $p < 0.05$ = statistically significant.
[e] No statics are computed because of no human meta-pneumovirus detected by conventional viral culture.
C pneumoniae = *Chlamydia pneumoniae*; *H influenzae* = *Haemophilus influenzae;* hMPV = human meta-pneumovirus; HSV = herpes simplex virus; *L pneumophila* = *Legionella pneumophila*; *M pneumoniae* = *Mycoplasma pneumoniae*; *N meningitidis* = *Neisseria meningitidis*; NA = not available; RSV = respiratory syncytial virus; RVP = respiratory viral panel; *S pneumoniae* = *Streptococcus pneumoniae*.

Discussion

The pathogens of LRTIs in young children are not easily identifiable from infected sites. Direct evidence of pathogens may be established via culture or PCR of lung biopsy of pulmonary empyema, but these invasive procedures are seldom performed in young children.

Table 4 Association of characteristics between single and multiple infections

Characteristics	Single infection ($n = 26$)	Co-infection ($n = 12$)	*p*[a]
Age	2.99 ± 1.32	2.35 ± 1.03	0.37
Gender	Male: 17	Male: 6	0.19
BMI	15.7 ± 1.86	16.2 ± 2.01	0.67
Fever days	4.88 ± 2.40	5.42 ± 3.55	0.97
Cough	1	1	1.0
Dyspnea (patient number)	5	3	0.88
Rales (patient number)	0	3	0.02
Wheezing (patient number)	5	4	0.51
WBC	11624.6 ± 5649.4	9695.8 ± 3224.4	0.39
CRP	4.18 ± 6.49	1.92 ± 2.00	0.14

[a] Mann-Whitney *U* test; $p < 0.05$ = statistically significant.
BMI = body mass index; WBC = white blood cell; CRP = C-reactive protein; SD = standard deviation.

In the present study, single infection and multiple infections in children (age <5 years) with LRTI have no obvious differences in clinical symptoms (except for breath sounds of rales) and laboratory data. However, it is still difficult to determine if a patient has more than one pathogen. Respiratory viruses have been detected in 64.4% (29/45) of children with LRTI, which is consistent with those of previous reports.[4,8,9,10,11] The co-infection rate is 26.7% and is comparable to the 23.5% found by Sung et al (children with LRTI aged <36 months)[12] and 27% found by Cilla et al (children with pneumonia aged <36 months).[4]

There was no co-infection noted by conventional viral and blood cultures in this study and only 4 patients had co-infection when the quick antigen test (Influenza A + B, RSV, urine pneumococcal) was added. Ten patients had co-infections when the two multiplex PCR methods were used.

Conventional viral culture is time-consuming and may overlook some possible pathogens. In the current study, RVP detected more pathogens than conventional viral culture (27 vs. 16 positive results) and had better detection rates than conventional viral culture in adenovirus (9 vs. 6) and influenza virus (6 vs. 2; Table 3). Moreover, RVP detected human meta-pneumovirus in two patients even though it was not yet winter-spring season.[13,14] Human meta-pneumovirus is an important pathogen for acute respiratory tract infection in pediatric patients, especially those younger than five years old.[15,16,17,18] The virus replicates poorly in conventional cell cultures and is clinically indistinguishable from RSV.[19] Early detection may guide clinicians in appropriate decision-making and treatment.[16]

It is not easy to clinically differentiate bacterial and nonbacterial infections in children. Of the 36 patients with blood cultures performed, none had bacterial growth in this

study. Blood culture is time-consuming and has very low positive growth rates, especially in children.[20,21] In clinical practice, the decision to prescribe antibiotics may be influenced by the patient's clinical presentation and stress from their parents. In infants, antibiotics are usually prescribed because of their young age, which makes evaluation even more difficult, especially in identifying infants with serious bacterial infections.[22,23] The early recognition of possible pathogens in LRTIs of children may help clinicians treat them more promptly and appropriately.[24]

In this study, 29 patients received antibacterial agents even though only 11 had positive bacterial examination results and 15 had positive viral examination results. Pathogens detected from nasopharyngeal or throat swabs may not truly reflect the host infectious status,[25,26] and clinicians should still rely on the patient's disease course, history, symptoms, signs, physical examinations, and laboratory results to avoid unnecessary antibiotic use.[24]

Streptococcus pneumoniae is another important pathogen in bacterial LRTI among children.[27] The direct evidence of pneumococcal infection is cultures or PCR from infected sites. Urine pneumococcal antigen test has high false positive rates in young children due to the high nasopharyngeal carriage rate[28,29] and is not considered useful for diagnosing children with acute pneumococcal pneumonia.[30,31] Blood culture rates of children is also low.[21] In *this* study, RVP detected *Streptococcus pneumoniae* in seven patients, including six considered as true infection based on their clinical symptoms, signs, and disease course.

Viral infections are the mostly frequent pediatric LRTIs. Most can be treated with supportive care. However, because of the difficulty in differentiating bacterial and viral infections, more medications, more laboratory examinations, more hospitalizations, and even more sick leaves taken by parents are inevitable. Multiplex PCR can increase diagnostic yield[32] and may help clinicians minimize such problems. In this study, conventional diagnostic tools (viral, blood and sputum cultures, Influenza A + B quick test, RSV quick antigen test, and urine pneumococcal antigen test) had 35.6% positive rate. This rate increased to 46.7% when the quick antigen tests were added and further to 84.4% when the two kinds of multiplex PCR methods were incorporated (Table 2).

In conclusion, multiplex PCR can detect more respiratory pathogens than conventional diagnostic tools and the results can be obtained very quickly. The detection rate of pathogens can be increased dramatically by integrating conventional diagnostic tools. This is the future trend in clinical diagnosis and may someday replace some conventional laboratory examinations, thereby decreasing the burden of disease on society.

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Conflicts of interest

All authors declare that they have no conflicts of interest relevant to this article.

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