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# Application of a nucleic acid-based multiplex kit to identify viral and atypical bacterial aetiology of lower respiratory tract infection in hospitalized children

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**Abstract**

**Introduction**. Lower respiratory tract infections (LRTIs), particularly those acquired in hospitals, are an important cause of childhood morbidity and mortality. Understanding the aetiology and epidemiology of LRTIs is necessary for clinical management, reduction of antibiotic usage, vaccine development and prevention of nosocomial infection.

**Aim**. In this study, we aimed to detect 13 viruses and atypical bacteria in nasopharyngeal secretion specimens from hospitalized children with LRTIs.

**Methodology**. From January 2014 to December 2016, nasopharyngeal secretion specimens were prospectively collected from 3232 children aged between 1 and 72 months. Nucleic acid was extracted and analysed using the SureX13 respiratory pathogen multiplex kit as per the manufacturer's instructions.

**Results**. A total of 2874 (88.9 %) children tested positive for viral and/or atypical bacterial infections, and 965 (29.9 %) were co-infected with multiple pathogens. The most frequently detected respiratory tract pathogens (RTPs) were rhinovirus, respiratory syncytial virus, parainfluenza virus and adenoviruses. The rates of RTP and co-infection positivity in the toddler group were significantly higher than those in the infant and preschool groups.

**Conclusion**. The SureX13 respiratory pathogen multiplex kit has the ability to effectively detect a range of RTPs in hospitalized paediatric patients with LRTIs.

## INTRODUCTION

Lower respiratory tract infections (LRTIs) are common in children and are associated with high morbidity and mortality rates worldwide [1]. The majority of LRTIs are caused by viruses and the atypical bacteria *Mycoplasma pneumoniae* (Mp) and *Chlamydia pneumoniae* (Cp). The current pharmacological interventions for viral LRTIs are limited, and self-care and symptomatic treatment are the typical therapeutic recommendations. However, although antibiotic use may not be beneficial in patients with viral LRTIs and is associated with a high risk of adverse effects, antibiotics are still commonly prescribed.

Understanding the aetiology and epidemiology of LRTIs is necessary for clinical management, reduction of antibiotic usage, vaccine development and prevention of nosocomial infections. Nevertheless, it is difficult to confirm the aetiology of LRTIs without laboratory tests. Viral culture used to be the gold standard for diagnosis, but it has since been replaced by molecular methods. Nonetheless, viral culture remains important for discovering new unknown viruses, but its use is limited because it is a tedious and time-consuming procedure, and not applicable to all viruses [2]. Similarly, serology has limited capability for accurate diagnosis due to its low sensitivity [3]. Meanwhile, although antigen-based assays, such as the indirect immunofluorescence antibody and direct immunofluorescence antibody methods, are widely used because of their affordability and ease of use, they have limited sensitivity and specificity. The nucleic acid amplification test (NAAT) has played an increasingly important role

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Keywords: co-infection; hospitalized children; lower respiratory tract infections; multiplex RT-PCR; virus.
Abbreviations: AdV, adenoviruses; Ch, *Chlamydia*; CoV, coronavirus; H3, seasonal H3N2 virus; 09H1, pandemic H1N1 2009; HBoV, bocavirus; HMPV, metapneumovirus; HRV, rhinovirus; InfB, influenza B virus; Mp, *Mycoplasma pneumoniae*; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

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in accurate detection of the aetiological agents of infectious diseases, because it yields immediate results and has high sensitivity and specificity [2]. Thus, the NAAT has become the new standard method for detecting respiratory viruses. Recently, multiplex reverse transcription polymerase chain reaction (RT-PCR) assays have been developed to detect respiratory tract pathogens (RTPs) [4, 5], and some, such as xTAG RVP, are commercially available [6].

We aimed to assess 13 type/subtypes of RTPs in nasopharyngeal secretion specimens from hospitalized children with LRTIs in Wenzhou using the SureX 13 respiratory pathogen multiplex kit based on advanced fragment analysis [7]. The tested RTPs were influenza A virus (InfA), pandemic influenza A virus-2009 (09H1), seasonal H3N2 virus (H3), influenza B virus (InfB), respiratory syncytial virus (RSV), adenoviruses (AdVs), rhinovirus (HRV), bocavirus (HBoV), metapneumovirus (HMPV), parainfluenza virus (PIV, including PIV-1, 2, 3 and 4), coronavirus (CoV, including OC43, 229E, NL63 and HKU1), *Chlamydia* [Ch, including *Chlamydia trachomatis* (Ct) and Cp] and Mp.

## METHODS

### Compliance with ethical standards

All aspects of the study were performed in accordance with the ethical standards of the institutional research committee and in line with the 1964 Helsinki Declaration, and the study was approved by the Ethics Committee of the Second Affiliated Hospital and Yuying Children's Hospital, Wenzhou Medical University (number: 2014–59).

### Specimens

Nasopharyngeal secretion specimens (NPSs) were collected from hospitalized children aged 1 to 72 months who, based on clinical assessment, presented with LRTIs to the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Zhejiang Province, PR China, from January 2014 to December 2016. Our hospital is the only tertiary paediatric hospital for Wenzhou, and serves as the major referral centre for the region, which has a population of >9 million people. The LRTIs were diagnosed according to the clinical symptoms of tachypnea (wheezing; severe cough; fever; and respiratory distress symptoms, such as nasal flaring, retraction and cyanosis ), abnormal auscultatory findings (wheezing and crackles) or radiological evidence indicative of LRTIs. The clinical syndromes of bronchitis, bronchiolitis and pneumonia were categorized as LRTIs. Children whose guardians refused to provide demographic data or those for whom sufficient specimens for pathogen detection were not available were excluded from the study. Approximately 1 ml of respiratory tract secretion was collected from each participant and stored at −80 °C.

### Nucleic acid extraction

Total RNA/DNA was extracted from 300 µl respiratory tract secretion using Viral Total Nucleic Acid Extraction (Taiwan Advanced Nanotech, Taiwan, ROC) on Smart LabAssist-32 (Taiwan Advanced Nanotech, Taiwan, ROC) according to the manufacturer's instructions. The extracts were eluted into 80 µl of DNase- and RNase-free water and stored at −80 °C.

### Multiplex RT-PCR assay

The SureX 13 respiratory pathogen multiplex kit (Ningbo Health Gene Technologies Ltd, Ningbo, PR China) was used to detect the following RTPs simultaneously: InfA, AdV, HRV, HBoV, 09H1, PIV, Ch, HMPV, InfB, Mp, H3, CoV and RSV. Primer pairs targeting the 13 tested RTPs and 3 internal controls (human RNA control, human DNA control and RT-PCR control) were amplified in a single tube. The amplification products were analysed via advanced fragment analysis [7]. In brief, one tube of 25 µl PCR mixture containing 14 µl ResP mixture, 1 µl RT-PCR enzyme mix and 5 µl extracted nucleic acid was subjected to the following conditions: 25 °C for 5 min, 50 °C for 15 min and 95 °C for 2 min, followed by five cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min (the annealing temperature was decreased by 1 °C in each cycle), and then 25 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and a final incubation at 72 °C for 10 min. The reactions were analysed on an Applied Biosystems 3500Dx Genetic Analyzer with LIZ500 as the internal standard and were analysed with GeneMapper 4.1 software (Thermo Fisher Scientific, Waltham, MA, USA).

### Identification of Ct and Cp by Sanger sequencing

For Ch-positive samples, the extracted nucleic acid was amplified using JumpStart *Taq* DNA polymerase (Sigma-Aldrich)

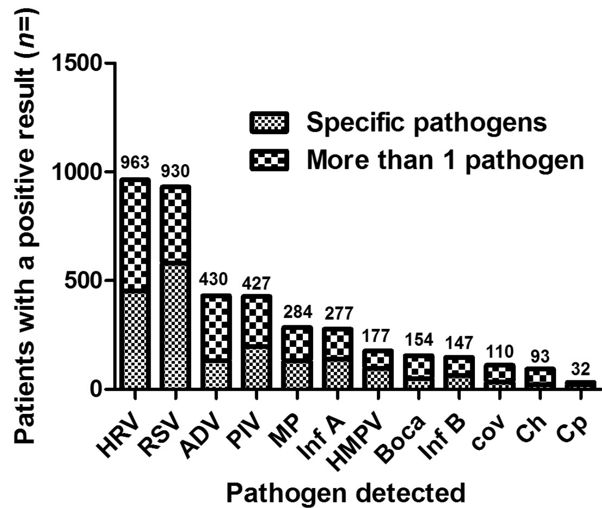
**Table 1.** Primers for identification of Ct and Cp by Sanger sequencing

| Pathogen | Target gene | Amplification primers | Product length | Annealing temperature |
|---|---|---|---|---|
| Ct | Cryptic plasmid ORF1 gene | 5′-AGGGTTTTCCCAGTCACGGATGATTTGAGCGTGTGTAGCG-3′ | 265 bp | 60 °C |
| | | 5′-GAGCGGATAACAATTTCACACTACGAGCCAGCACTCCAATTTC-3′ | | |
| Cp | omp9 | 5′-AGGGTTTTCCCAGTCACGTCTCCATAGCTTACGTTCCCGA-3′ | 260 bp | 60 °C |
| | | 5′-GAGCGGATAACAATTTCACACAAGATCGATAGCATAGCTACGAGAAG-3′ | | |

The underlined sequences are M13-47 and M13-48, respectively, which were used as sequencing primers.
Cp, *Chlamydia pneumoniae*; Ct, *Chlamydia trachomatis*.

**Table 2.** Patient demographics and clinical characteristics (*n*=3232)

| Characteristics | *n* (%) |
|---|---|
| Male gender | 2141 (66.2) |
| Age group | |
| Infant (1 to <12 months) | 1675 (51.8) |
| Toddler (12 to <36 months) | 998 (30.9) |
| Preschool (36 to <72 months) | 550 (17.0) |
| Children with unclear birth date | 9 (0.3) |
| Clinical signs/symptoms on presentation | |
| Fever | 2136 (66.1) |
| Cough | 3002 (92.9) |
| Cyanosis | 254 (7.9) |
| Nasal flaring | 185 (5.7) |
| Wheezing | 2006 (62.1) |
| Tachypnea | 1565 (48.4) |




AQ1 **Fig. 1.** Viruses and atypical bacteria detected in the nasopharyngeal secretion specimens from paediatric hospitalized patients with LRTIs.

and the primer pairs listed in Table 1. The PCR conditions were as follows: 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and a final incubation at 72 °C for 10 min. The amplicons were sent to Sangon (Sangon, Shanghai, PR China) for sequencing using universal primers M13-47 and/or M13-48. The retrieved sequences were analysed by alignment on NCBI BLAST (https://blast.ncbi.nlm.nih.gov).

### Statistical analysis

All statistical analyses were performed using SPSS software (version 22.0; SPSS, Inc., Chicago, IL, USA). Statistical comparisons were performed using the chi-square test. A probability (*P*) value less than 0.05 was considered statistically significant.

## RESULTS

### Testing of the respiratory tract secretion specimens

In this study, a total of 3232 respiratory tract secretion specimens were collected. The median age of the study participants was 11.0 months. Of the 3232 patients, 2141 (66.2 %) were boys and 1091 (33.8 %) were girls. All specimens collected from paediatric inpatients with LRTIs were tested via the SureX 13 respiratory pathogen multiplex kit. Information on the demographics and clinical characteristics of the enrolled children is shown in Table 2. A total of 2874 patients (88.9%) tested positive for at least 1 pathogen, and 965 (29.9 %) of these were co-infected with multiple pathogens. All of the tested viruses and atypical bacteria were detected in this study. The most common viruses were HRV and RSV (Fig. 1), accounting for 29.8 % (963/3232) and 28.8 % (930/3232) of the isolated pathogens, respectively, followed by AdV, PIV, Mp and InfA. The least common aetiological agent discovered in this study was CoV, which tested positive in 110 cases (3.4%).

### RTP prevalence in different age groups

The rate of positive detection in boys (1927/2141; 90.0%) was higher than that in girls (947/1091; 86.8%) (*P*=0.0061). For further comparison, patients were categorized into three age groups, i.e. infant (<12 months), toddler (12–<36 months) and preschool (36–72 months), and sample positivity was compared between these groups. Nine children with an unclear date of birth were excluded from further analyses. The positivity rates for the infant, toddler and preschool groups were 87.7%, 91.7% and 88.7%, respectively (Table 3).

The rate of positivity for each RTP is shown in Table 4. HRV was the most common RTP. The prevalence of RSV was the highest in infants, accounting for 50.39 %. The same trend was observed for PIV and Ch. Mp was the most common pathogen among preschoolers.

### Co-infection with different pathogens

There were 965 cases (29.9%) with co-infection. As shown in Table 3, the positivity rates and the co-infection positivity rates in the toddler group were significantly higher than those in the infant and preschool groups, respectively. Of the 965 cases of mixed infection, 792 cases were co-infected with 2 pathogens, (Table 5), 158 cases were co-infected with 3 pathogens and 14 cases were co-infected with 4 pathogens. HRV was the most common RTP detected among those with co-infection.

### RTP prevalence in different months

Monthly distribution analysis revealed which RTPs were observed most frequently in each month. HRV was more

**Table 3.** Prevalence of respiratory tract pathogens in the three age groups (*n*=3223)

| Age group | Total no. of samples | Total no. of negative samples (%) | Total no. of positive samples (%) | Total no. of co-infection samples (%) |
|---|---|---|---|---|
| Infant (1 to <12 mo) | 1675 | 206 (12.3) | 1469 (87.7)# | 479 (28.6)# |
| Toddler (12 to <36 months) | 998 | 83 (8.3) | 915 (91.7)* | 350 (35.1)** |
| Preschool (36 to <72 months) | 550 | 62 (11.3) | 488 (88.7) | 136 (24.7) |
| Total | 3223 | 351 (10.9) | 2872 (89.1) | 965 (29.9) |

#, *P*<0.01 compared with the toddler group; *, *P*<0.05 compared with the preschool group; **, *P*<0.01 compared with the preschool group.

common in May and June, while RSV was more common in August, September and December. PIV was common from April to August (Fig. 2). The pandemic H1N1 2009 (09H1) was common in January, while H3N2 was common in July, August and September.

## DISCUSSION

LRTIs are among the most common health problems worldwide, and they are also one of the leading causes of death among children younger than 5 years in China. Viruses and some atypical pathogens such as Mp are the primary pathogenic causes of LRTIs in children, and are also common causes of childhood pneumonia and bronchiolitis in many countries [8, 9]. Herein, a total of 2874 samples (88.9%) were positive for at least 1 pathogen. HRV and RSV were the most commonly detected pathogens, followed by AdV, PIV, Mp and InfA.

RSV is the causative pathogen for over 30 million new episodes of acute LRTI in children aged under 5 years every year, and causes more than 3.4 million hospital admissions and 160 000 deaths annually. RSV is also the main causative pathogen of LRTIs in China [10]. Our previous study found that RSV was more common in children under 3 years, particularly in those under 1 year. These data are consistent with those of other studies conducted among children in China [11, 12]. RSV infection has seasonal characteristics. In temperate regions of the northern hemisphere, the infection season starts in October, peaks in January to February, and ends in April [13]. From a global perspective, despite the differences in peak and popular duration, the annual prevalence in each country is

**Table 4.** Prevalence of each respiratory tract pathogen in the three age groups

| Respiratory tract pathogen | Positive sample no. (%) in the three age groups | | | *P* |
|---|---|---|---|---|
| | Infant (*n*=1675) | Toddler (*n*=998) | Preschool (*n*=550) | |
| HRV | 491 (29.31) | 313 (31.36) | 157 (28.55) | 0.41 |
| RSV | 614 (36.66) | 257 (25.75)$^{\#\#}$ | 59 (10.73)$^{**++}$ | <0.01 |
| PIV | 262 (15.64) | 126 (12.63)# | 39 (7.09)$^{**++}$ | <0.01 |
| AdV | 154 (9.19) | 162 (16.23)$^{\#\#}$ | 112 (20.36)$^{**+}$ | <0.01 |
| HBoV | 85 (5.07) | 47 (4.71) | 20 (3.64) | 0.39 |
| HMPV | 85 (5.07) | 65 (6.51) | 27 (4.91) | 0.23 |
| InfA | 103 (6.15) | 111 (11.12) $^{\#\#}$ | 64 (11.64) $^{**}$ | <0.01 |
| 09H1 | 44 (2.63) | 57 (5.71) $^{\#\#}$ | 41 (7.45) $^{**}$ | <0.01 |
| H3N2 | 55 (3.28) | 53 (5.31)# | 22 (4.00) | 0.04 |
| InfB | 47 (2.81) | 53 (5.31) $^{\#\#}$ | 47 (8.55) $^{**+}$ | <0.01 |
| CoV | 49 (2.93) | 48 (4.81)# | 13 (2.36) $^{+}$ | 0.01 |
| Mp | 65 (3.88) | 119 (11.92) $^{\#\#}$ | 100 (18.18) $^{**++}$ | <0.01 |
| Ch | 90 (5.37) | 22 (2.20) $^{\#\#}$ | 12 (2.18) $^{**}$ | <0.01 |

#, *P*<0.05, $^{\#\#}$, *P*<0.01, toddler compared with infant; $^{*}$, *P*<0.05, $^{**}$, *P*<0.01, preschool aged compared with infant; $^{+}$, *P*<0.05, $^{++}$, *P*<0.01, preschool aged compared with toddler.

AdV, adenoviruses; Ch, *Chalmydia*; CoV, coronavirus; 09H1, pandemic influenza A virus-2009; HBoV, bocavirus; H3N2, seasonal H3 virus; HRV, rhinovirus; InfA, influenza A virus; InfB, influenza B virus; Mp, *Mycoplasma pneumoniae*; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

**Table 5.** Prevalence of co-infection with different pathogens

| | HRV | RSV | PIV | AdV | HBoV | HMPV | InfA | InfB | CoV | Ch |
|---|---|---|---|---|---|---|---|---|---|---|
| RSV | 125 | | | | | | | | | |
| PIV | 59 | 24 | | | | | | | | |
| AdV | 72 | 38 | 26 | | | | | | | |
| HBoV | 24 | 16 | 10 | 5 | | | | | | |
| HMPV | 26 | 4 | 7 | 10 | 2 | | | | | |
| InfA | 19 | 23 | 8 | 17 | 5 | 4 | | | | |
| InfB | 12 | 12 | 9 | 7 | 1 | 1 | 7 | | | |
| CoV | 11 | 9 | 11 | 13 | – | 1 | 1 | – | | |
| Ch | 12 | 16 | 8 | 10 | 2 | 2 | 3 | 10 | | |
| Mp | 39 | 13 | 17 | 19 | 3 | 3 | 5 | 7 | 3 | 1 |

AdV, adenoviruses; Ch, *Chlamydia*; CoV, coronavirus; 09H1, pandemic influenza A virus-2009; HBoV, bocavirus; H3N2, seasonal H3 virus; HRV, rhinovirus; InfA, influenza A virus; InfB, influenza B virus; Mp, *Mycoplasma pneumoniae*; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

consistent [14]. There is a clear correlation between climate and RSV infection, with the peak of infection usually occurring in rainy and cold seasons [8]. Indoor crowded conditions and high population density may facilitate the spread of the virus [15]. In several Asian countries, including Malaysia, Cambodia and Vietnam, the rainy season is conducive for RSV infections [16–18]. Our data showed that RSV presented two peaks in August and December. Wenzhou is located on the east coast of China, where the rainy season starts in August and the cold season is in December and January, which coincides with the peak RSV infection periods. RSV bronchitis has also been reported to have double peaks in the year in spring and autumn in Taiwan [19].

This study showed that HRV is the primary viral aetiology of LRTIs (29.8%, 963/3232), followed closely by RSV; such results are similar to those reported in Shanghai [20]. Despite the ongoing debate, there is growing evidence that HRV is one of the causes of LRTIs [21]. Furthermore, HRV plays an important role in asthma attacks and respiratory infections in premature infants [22]. In China, however, HRV-caused LRTIs have not yet gained sufficient attention from paediatricians. Unlike RSV infection, HRV infection can be seen in different age groups, and there is no apparent seasonal difference. We found that HRV infection peaked in May, when RSV showed low incidence. However, this correlation still requires further study.

The development of molecular technology has improved research on LRTI-causing pathogens. In addition to RSV and HRV, other viruses, such as HMPV, CoV, HBoV, influenza virus, ADV and PIV, can cause pneumonia and bronchiolitis. All of the target viruses and atypical bacteria tested positive in this study. HPIV and RSV are common in infants, while the incidence of ADV and influenza virus infections increases with age. However, no significant difference in the age of those infected with HBoV and HMPV was noted. Meanwhile, the peak incidence of infection differed for some viruses. HBoV infection peaked between August and October, CoV peaked in August, and HMPV was uncommon between October and December. In the present study, InfA and InfB infections were uncommon, while 09H1 and H3N2 infections had a high incidence and peaked in January and July, respectively. These findings highlight the need for the development of preventive and control strategies for viral infections in the region.

Co-infection, defined as the detection of more than one pathogen in the same specimen, is a phenomenon that is underestimated when using viral culture and/or quantitative RT-PCR assays. The multiplex RT-PCR assay has a high capability for detecting co-infection and has substantially improved our understanding of the actual incidence of co-infection. The clinical significance of the association between co-infection and substantially worse disease severity remains unclear. Some studies link co-infection with more severe clinical outcomes, while some show no overall difference between single infection and co-infections [23, 24]. Nonetheless, rapid and accurate detection of a wide spectrum of RTPs is important and can help identify the actual rate of co-infections and their correlation with clinical symptoms and severity of disease. The co-infection rate for LRTI viruses ranges from 6.1 to 62 % [8, 25, 26]. Co-infection with two viruses is common, and co-infection with five viruses, although rare, has also been reported. In the present study, co-infection was seen in 965 cases (29.9%). HRV was the most common RTP among the co-infections, and this result is consistent with that of Arbefeville and Ferrieri's study [27]. As shown in Table 3, co-infection was more frequent in infants and toddlers than in preschoolers. Most studies have shown that RSV and HRV are the most common pathogens in hospitalized children with co-infection [28]. This study also found that HRV+RSV was the most common co-infection, followed by HRV+ADV and HRV+PIV co-infections. The monthly distributions of both

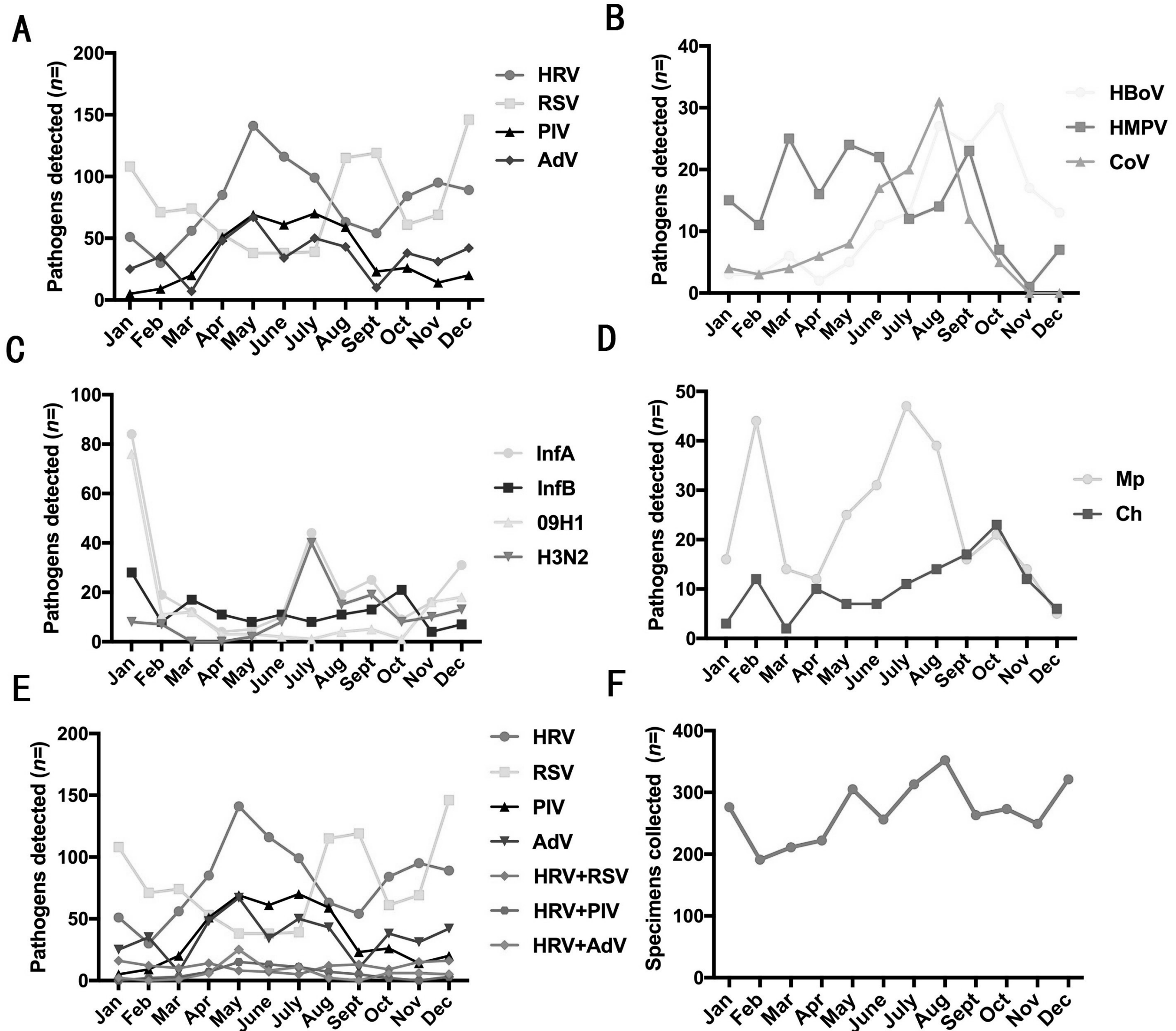



**Fig. 2.** Monthly distribution of RTPs detected in paediatric inpatients with acute respiratory infections in Wenzhou.

HRV+RSV and HRV+PIV co-infections were similar to those for RSV and PIV rather than HRV. Previous studies have shown that the incidence of co-infection is not correlated with the incidence of single-virus infection, but with the occurrence of a multi-virus cycle in a year [29]. Co-infection may be related to age, sex, immune status, climate, environment and virus characteristics [30].

In total, approximately 88.9 % of the cases tested presented with viral and/or atypical bacterial infections, and this rate is comparable to that reported in a study by Raymond *et al.* [3], which utilized two NAATs for 221 specimens from 3-year-old paediatric inpatients with LRTIs. Using a 14-virus NAAT, Arbefeville and Ferrieri reported a 73.5 % positivity rate in 344 specimens from hospitalized children aged <1 year [27]. Similarly, Wang *et al.* described a 70 % positivity rate in hospitalized children aged <3 years with community-acquired pneumonia [4]. The higher positivity rate observed in this study can be attributed to the symptoms and detection range of RTPs. Arbefeville and Ferrieri's study was performed on specimens from mixed clinics, with the detection range exclusive of HBoV and atypical bacteria.

Mp and Cp are known to cause mild, moderate, or severe LRTIs in children, with significant annual and regional differences worldwide [31, 32]. The results of the present study show that Mp was one of the main causes of LRTIs in hospitalized children, with a positivity rate of 4.94 %. Notably, Ch was only detected in infants. Because the primer pair designed for Ch was able to amplify both Ct and Cp, Sanger sequencing

was further performed for these 16 Ch-positive specimens, and BLAST on NCBI showed that the detected pathogen was Ct, and not Cp. The Ct infection in these infants was possibly due to exposure to the bacteria during birth [33].

Given that most respiratory pathogens produce similar symptoms, rapid and accurate laboratory diagnostic tests are necessary to identify the causative agents. From a public health perspective, information on the seasonality of pathogens is crucial to determine the optimal timing of interventions, particularly for a climatically and economically diverse country such as China. NAAT has increasingly been explored for the identification of RTPs in infectious respiratory diseases [34]. Multiplex PCR technologies can detect multiple pathogens simultaneously, not only saving on detection time and cost, but also optimizing sample utility. There are many multiplex molecular diagnostic kits available in developed countries. These include xTAG RVP by Luminex (Toronto, Canada) [35] and FilmArray's respiratory pathogen diagnostic kit (BioFire Diagnostics, Inc., Salt Lake City, UT, USA) [36]. In China, however, commercialized multiplex PCR detection kits are not readily available, and the national CDC and sentinel hospitals in China use different molecular diagnostic kits.

In the present study, we evaluated the capability of a commercialized multiplex RT-PCR assay to detect RTPs and applied it to 3232 NPSs from paediatric inpatients with LRTIs. We showed that this assay had high analytical sensitivity and specificity for simultaneous detection of multiple RTPs. The introduction of this assay into our clinical laboratory has enhanced our ability to detect RTPs that we would not have been able to determine using traditional culture methods or quantitative RT-PCR assays, thus enabling us to identify the 'hidden' burden of LRTIs in our patient population.

This study has some limitations. Our ability to detect respiratory viruses and atypical bacteria was limited by the sensitivity of testing specimens from the nasopharynx. Invasive approaches to obtain direct samples from the lower respiratory tract were not performed on account of feasibility and ethical considerations. Detecting a pathogen in nasopharyngeal specimens with the multiplex RT-PCR assay may not denote causation in LRTIs. However, with the exception of HRV, the detection of viruses and atypical bacteria in nasopharyngeal specimens can be considered to have identified the 'probable cause' pathogens in children presenting with community-acquired pneumonia [8, 37]. In addition, the sensitivity of current tests is likely incomplete. Li *et al.* evaluated this kit using 314 samples from patients with community-acquired pneumonia. The sensitivity, specificity, positive predictive value and negative predictive value were 97.31%, 100%, 100 and 99.85 %, respectively [38]. Our testing methods were limited by the small quantity of subtype information (with the exception of influenza A virus) obtained from the multiplex RT-PCR assay; thus, we were not able to assess the epidemiological information for PIV-3 (an important infectious agent causing bronchiolitis and pneumonia in infants, second only to RSV [39]) and HRV-C (the most recently identified species associated with severe lower respiratory disease in infants and young children, and elderly and immunocompromised patients).

In conclusion, our study showed that viruses and atypical bacteria were quite common in hospitalized children with RTIs in Wenzhou. The SureX 13 respiratory pathogen multiplex kit has the ability to detect a wider range of RTPs in paediatric inpatients with LRTIs.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. GBD 2015 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* 2016;388:1545–1602.
2. Baron EJ, Miller JM, Weinstein MP, Richter SS, Gilligan PH *et al*. A guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: 2013 recommendations by the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM)(a). *Clin Infect Dis* 2013;57:e22–e121.
3. Raymond F, Carbonneau J, Boucher N, Robitaille L, Boisvert S *et al*. Comparison of automated microarray detection with real-time PCR assays for detection of respiratory viruses in specimens obtained from children. *J Clin Microbiol* 2009;47:743–750.
4. Wang L, Zhao M, Shi Z, Feng Z, Guo W *et al*. A GeXP-Based assay for simultaneous detection of multiple viruses in hospitalized children with community acquired pneumonia. *PLoS One* 2016;11:e0162411.
5. Wang W, Ren P, Sheng J, Mardy S, Yan H *et al*. Simultaneous detection of respiratory viruses in children with acute respiratory infection using two different multiplex reverse transcription-PCR assays. *J Virol Methods* 2009;162:40–45.
6. Pabbaraju K, Tokaryk KL, Wong S, Fox JD. Comparison of the Luminex xTAG respiratory viral panel with in-house nucleic acid amplification tests for diagnosis of respiratory virus infections. *J Clin Microbiol* 2008;46:3056–3062.
7. Zhang H, Cheng H, Wang Q, Zeng X, Chen Y *et al*. An advanced fragment analysis-based individualized subtype classification of pediatric acute lymphoblastic leukemia. *Sci Rep* 2015;5:12435.
8. Jain S, Williams DJ, Arnold SR, Ampofo K, Bramley AM *et al*. Community-acquired pneumonia requiring hospitalization among U.S. children. *N Engl J Med* 2015;372:835–845.
9. Brown RJ, Nguipdop-Djomo P, Zhao H, Stanford E, Spiller OB *et al*. *Mycoplasma pneumoniae* epidemiology in England and Wales: a national perspective. *Front Microbiol* 2016;7:157.
10. Feng L, Li Z, Zhao S, Nair H, Lai S *et al*. Viral etiologies of hospitalized acute lower respiratory infection patients in China, 2009–2013. *PLoS One* 2014;9:e99419.
11. Oumei H, Xuefeng W, Jianping L, Kunling S, Rong M *et al*. Etiology of community-acquired pneumonia in 1500 hospitalized children. *J Med Virol* 2018;90:421–428.
12. Chen J, Hu P, Zhou T, Zheng T, Zhou L *et al*. Epidemiology and clinical characteristics of acute respiratory tract infections among hospitalized infants and young children in Chengdu, West China, 2009–2014. *BMC Pediatr* 2018;18:216.
13. Haynes AK, Prill MM, Iwane MK, Gerber SI. Respiratory syncytial virus–United states, July 2012–June 2014. *MMWR Morb Mortal Wkly Rep* 2014;63:1133–1136.

14. Haynes AK, Manangan AP, Iwane MK, Sturm-Ramirez K, Homaira N *et al*. Respiratory syncytial virus circulation in seven countries with global disease detection regional centers. *J Infect Dis* 2013;208:S246–S254.
15. Meissner HC. Viral bronchiolitis in children. *N Engl J Med* 2016;374:62–72.
16. Khor CS, Sam IC, Hooi PS, Quek KF, Chan YF. Epidemiology and seasonality of respiratory viral infections in hospitalized children in Kuala Lumpur, Malaysia: a retrospective study of 27 years. *BMC Pediatr* 2012;12:32.
17. Guerrier G, Goyet S, Chheng ET, Rammaert B, Borand L *et al*. Acute viral lower respiratory tract infections in Cambodian children: clinical and epidemiologic characteristics. *Pediatr Infect Dis J* 2013;32:e8–13.
18. Do LA, Bryant JE, Tran AT, Nguyen BH, Tran TT *et al*. Respiratory syncytial virus and other viral infections among children under two years old in southern Vietnam 2009–2010: clinical characteristics and disease severity. *PLoS One* 2016;11:e0160606.
19. Chen YW, Huang YC, Ho TH, Huang CG, Tsao KC *et al*. Viral etiology of bronchiolitis among pediatric inpatients in Northern Taiwan with emphasis on newly identified respiratory viruses. *J Microbiol Immunol Infect* 2014;47:116–121.
20. Li J, Tao Y, Tang M, Du B, Xia Y *et al*. Rapid detection of respiratory organisms with the FilmArray respiratory panel in a large children's hospital in China. *BMC Infect Dis* 2018;18:510.
21. Louie JK, Roy-Burman A, Guardia-Labar L, Boston EJ, Kiang D *et al*. Rhinovirus associated with severe lower respiratory tract infections in children. *Pediatr Infect Dis J* 2009;28:337–339.
22. Drysdale SB, Alcazar M, Wilson T, Smith M, Zuckerman M *et al*. Functional and genetic predisposition to rhinovirus lower respiratory tract infections in prematurely born infants. *Eur J Pediatr* 2016;175:1943–1949.
23. Asner SA, Science ME, Tran D, Smieja M, Merglen A *et al*. Clinical disease severity of respiratory viral co-infection versus single viral infection: a systematic review and meta-analysis. *PLoS One* 2014;9:e99392.
24. Scotta MC, Chakr VC, de Moura A, Becker RG, de Souza AP *et al*. Respiratory viral coinfection and disease severity in children: a systematic review and meta-analysis. *J Clin Virol* 2016;80:45–56.
25. Martin ET, Fairchok MP, Stednick ZJ, Kuypers J, Englund JA. Epidemiology of multiple respiratory viruses in childcare attendees. *J Infect Dis* 2013;207:982–989.
26. Chorazy ML, Lebeck MG, McCarthy TA, Richter SS, Torner JC *et al*. Polymicrobial acute respiratory infections in a hospital-based pediatric population. *Pediatr Infect Dis J* 2013;32:460–466.
27. Arbefeville S, Ferrieri P. Epidemiologic analysis of respiratory viral infections mainly in hospitalized children and adults in a Midwest University medical center after the implementation of a 14-virus multiplex nucleic acid amplification test. *Am J Clin Pathol* 2016;147:43–49.
28. Mansbach JM, McAdam AJ, Clark S, Hain PD, Flood RG *et al*. Prospective multicenter study of the viral etiology of bronchiolitis in the emergency department. *Acad Emerg Med* 2008;15:111–118.
29. Cilla G, Oñate E, Perez-Yarza EG, Montes M, Vicente D *et al*. Viruses in community-acquired pneumonia in children aged less than 3 years old: high rate of viral coinfection. *J Med Virol* 2008;80:1843–1849.
30. Cui B, Zhang D, Pan H, Zhang F, Farrar J *et al*. Viral aetiology of acute respiratory infections among children and associated meteorological factors in southern China. *BMC Infect Dis* 2015;15:124.
31. Waites KB. New concepts of *Mycoplasma pneumoniae* infections in children. *Pediatr Pulmonol* 2003;36:267–278.
32. Kumar S, Hammerschlag MR. Acute respiratory infection due to *Chlamydia pneumoniae*: current status of diagnostic methods. *Clin Infect Dis* 2007;44:568–576.
33. Schachter J, Grossman M, Sweet RL, Holt J, Jordan C *et al*. Prospective study of perinatal transmission of Chlamydia trachomatis. *JAMA* 1986;255:3374–3377.
34. Li J, Mao NY, Zhang C, Yang MJ, Wang M *et al*. The development of a GeXP-based multiplex reverse transcription-PCR assay for simultaneous detection of sixteen human respiratory virus types/subtypes. *BMC Infect Dis* 2012;12:189.
35. Esposito S, Principi N. The role of the NxTAG® respiratory pathogen panel assay and other multiplex platforms in clinical practice. *Expert Rev Mol Diagn* 2017;17:9–17.
36. Babady NE. The FilmArray® respiratory panel: an automated, broadly multiplexed molecular test for the rapid and accurate detection of respiratory pathogens. *Expert Rev Mol Diagn* 2013;13:779–788.
37. Bhuiyan MU, Snelling TL, West R, Lang J, Rahman T *et al*. The contribution of viruses and bacteria to community-acquired pneumonia in vaccinated children: a case-control study. *Thorax* 2019;74:261–269.
38. Li X, Chen B, Zhang S, Li X, Chang J *et al*. Rapid detection of respiratory pathogens for community-acquired pneumonia by capillary electrophoresis-based multiplex PCR. *SLAS Technol* 2019;24:105–116.
39. Senchi K, Matsunaga S, Hasegawa H, Kimura H, Ryo A. Development of oligomannose-coated liposome-based nasal vaccine against human parainfluenza virus type 3. *Front Microbiol* 2013;4:346.