Evaluation of a multiplex PCR assay for detection of respiratory viruses and *Mycoplasma pneumoniae* in oropharyngeal swab samples from outpatients

Ying Zhang\(^1\) | Lan Cao\(^1\) | Zhi Xu\(^2\) | Pingting Zhu\(^1\) | Bing Huang\(^1\) | Kuibiao Li\(^1\) | Yang Xu\(^1\) | Zhoubin Zhang\(^1\) | Yong Wu\(^2\) | Biao Di\(^1\)

\(^1\)Guangzhou Center for Disease Control and Prevention, Guangzhou, China  
\(^2\)Ningbo Health Gene Technologies Co., Ltd, Ningbo, China

**Correspondence**  
Biao Di, Guangzhou Center for Disease Control and Prevention, No.1, Qide Road, Baiyun District, Guangzhou, Guangdong 510440, China.  
Email: biaodi55@yahoo.com

**Funding information**  
National Science and Technology Major Project of the Ministry of Science and Technology of China, Grant/Award Number: 2017ZX10103011; Key Medicine Discipline Construction of Guangzhou Municipality, Grant/Award Number: 2017-2019-07; Science and Technology Program of Guangdong Health Department, Grant/Award Number: A2017489; Science and Technology Program of Guangzhou Health Department, Grant/Award Number: 20181A011052

**Abstract**  
**Background:** Respiratory viruses, such as influenza viruses, initially infect the upper airways but can manifest as severe lower respiratory tract infections in high-risk patients with significant morbidity and mortality. For syndromic diagnosis, several multiplex nucleic acid amplification tests have been developed for clinics, of which SureX 13 Respiratory Pathogen Multiplex Kit (ResP) can simultaneously detect 13 pathogens directly from airway secretion specimens. The organisms identified are influenza virus A, influenza virus A pdmH1N1 (2009), influenza virus A H3N2, influenza virus B, adenovirus, boca virus, rhinovirus, parainfluenza virus, coronavirus, respiratory syncytial virus, human metapneumovirus, *Mycoplasma pneumoniae*, and Chlamydia.

**Methods:** This study provides performance evaluation data of this assay by comparing with pathogen-specific PCRs from oropharyngeal swab samples.

**Results:** Ten pathogens were detected in this assay, of which rhinovirus, adenovirus, and influenza virus A pdmH1N1 (2009) were the most common. The overall agreement between the ResP and the comparator tests was 93.8%. The ResP demonstrated 86.5% agreement for positive results and 97.8% agreement for negative results.

**Conclusion:** The ResP assay demonstrated a highly concordant performance comparing with pathogen-specific PCRs for detection of respiratory pathogens in oropharyngeal swabs from outpatients and could aid in the diagnosis of respiratory infections in a variety of clinical scenarios.

**KEYWORDS**  
acute respiratory infections, influenza virus A, multiplex PCR, oropharyngeal swab, respiratory virus

**1 | INTRODUCTION**  
Acute respiratory infections (ARIs) are common and contribute significantly to morbidity and mortality. They are the leading causes of outpatient visits and hospitalizations in all age groups, especially for children under 5 years of age.\(^1\) Most ARIs in children and outpatients are caused by nine common respiratory viruses, including respiratory syncytial virus (RSV), influenza virus A, influenza virus B, rhinovirus, adenovirus, parainfluenza virus, coronavirus, human metapneumovirus, and boca virus\(^2\)\(^3\). Additionally, atypical pathogens, such as *Mycoplasma pneumoniae*, are
also major causes of ARLs in children. The symptoms caused by these pathogens are largely similar, thus definitive diagnosis requires effective laboratory testing. By using multiplex assay targeting these pathogens, early diagnosis can be made in a timely manner. Consequential antimicrobial or antiviral therapy may thus be administered promptly and appropriately. Most importantly, the early diagnosis of influenza viruses, which are contagious, is beneficial for early isolation of patients, thus reducing the spread of influenza viruses.

The routine clinical laboratory testing for respiratory viruses is largely conducted by direct fluorescent-antibody assays and rapid antigen tests in China. Given the poor sensitivity and complicated manual operation, these methods have been gradually replaced by nucleic acid amplification tests (NAATs), which are more sensitive and more specific. However, majority of the NAAT kits are based on real-time polymerase chain reaction (PCR), which can only detect one or two pathogens of ARLs within a single tube, thus are not syndromic testing. The clinical and economic impacts of syndromic testing for respiratory pathogens have been evaluated in several studies. Overall, the implementation of syndromic testing can decrease the time of diagnosis, decreased healthcare resource utilization, decrease inpatient length of stay and time in isolation, and improve antiviral use for influenza virus-positive patients.

SureX 13 Respiratory Pathogen Multiplex Kit (ResP) is a syndromic multiplex molecular test for simultaneous detection of 13 pathogens in a single tube. The aim of this study was to evaluate the application of the ResP for detection of respiratory pathogens in outpatients with flu-like manifestations.

2 | MATERIALS AND METHODS

2.1 | Samples

The inclusion criteria for this study were as follows: (a) patients admitted to hospitals between Feb. 2017 and Aug. 2018; (b) oropharyngeal swabs were collected from hospitals and Centers for Disease Control in Guangzhou; (c) patients had the following flu-like manifestations: (a) fever (>38°C); (b) cough or sore throat. After sampling, specimens were kept in 4°C and transferred to the laboratory for testing within one week.

2.2 | Nucleic acid extraction

The specimen was shaken vigorously for 5 minutes in phosphate-buffered saline solution, centrifuged at 9.6 g for 20 minutes, and the supernatant was aspirated. About 50 µL of RNA was extracted from 140 µL supernatant using the QIAamp Viral RNA extraction kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions and was stored at −80°C.

2.3 | Detection of influenza viruses

Influenza virus nucleic acid detection was performed by Influenza A/B Influenza Virus Nucleic Acid Detection Kit (Cat. No. DA-BN147, Daan Gene). Positive samples were further tested for influenza virus A pdmH1N1 (2009) and seasonal influenza virus H3N2 using a separate kit (Cat. No. JC10209, Daan Gene). Both tests were carried out on ABI Quant Studio 7 System (Thermo Fisher Scientific) according to the instructions. A typical S amplification curve and Cq value ≤35.0 were determined positive.

2.4 | Detection of other respiratory pathogens

For influenza virus-negative samples, more PCR tests were performed to detect the following pathogens: adenovirus (ADV), bovavirus (BOV), human rhinovirus (HRV), parainfluenza virus (PIV), human metapneumovirus (HMPV), Mycoplasma pneumoniae (MP), and respiratory syncytial virus (RSV), using corresponding NAAT kits from Daan Gene. All tests were carried out on ABI Quant Studio 7 System (Thermo Fisher Scientific) according to the instructions. A typical S amplification curve and Cq value ≤38.0 were determined positive.

2.5 | Multiplex detection of respiratory pathogens

The nucleic acid was subjected to multiplex amplification for all specimens using SureX 13 Respiratory Pathogen Multiplex Detection Kit (Cat. No. 1060144, Ningbo Health Gene Technology) on ABI GeneAmp PCR System 9700 (Thermo Fisher Scientific). The 13 respiratory pathogens were as following: influenza A virus, influenza A virus H1N1 (2009), seasonal H3N2 influenza virus, influenza B virus, adenovirus, bova virus, rhinovirus, parainfluenza virus, chlamydia, human metapneumovirus, Mycoplasma pneumoniae, coronavirus, and respiratory syncytial virus. The PCR product was subjected to capillary electrophoresis using GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter) according to the instructions. Each pathogen, if detectable, produced a distinctive fragment size after PCR amplification. The results of fragment analysis were used to determine the outcomes of testing. In brief, if the peak height of a targeted fragment size is lower than the lower peak of the signal standard, the targeted pathogen is determined negative; if the peak height of a targeted fragment size is higher than the higher peak of the signal standard, the targeted pathogen is determined positive; if the peak height of a targeted fragment size is between the higher and the lower peaks of the signal standard, the targeted pathogen is determined uncertain and the test should be repeated.

2.6 | Statistical analysis

The results were analyzed using EXCEL2007. The Cohen’s kappa statistics were calculated to measure the agreement between pathogen-specific PCRs and multiplex PCR results (≤0 = poor, 0.0-0.2 = slight, 0.21-0.4 = fair, 0.41-0.6 = moderate, 0.61-0.8 = substantial, and 0.81-1 = almost perfect). P value was calculated by CHITEST, and P < .05 indicates statistical significance.
A total of 420 oropharyngeal swabs were enrolled from 10 hospitals and 10 CDCs in Guangzhou from 2017 to 2018. Samples were collected from a wide range of ages, with the average age of 27.2 (Table 1). About 55% specimens were from male.

A pathogen-positive result was determined when the pathogen-specific fragment(s) was positive, as shown in Figure 1. A negative result was determined when none of the 13 pathogen-specific fragment was positive, while the controls (huDNA, huRNA, and IC) were positive (Figure 2). In this study, the ResP detected positive results in 141 samples, accounting for 33.6%, while the comparator tests detected positive results in 127 samples, with positive rate 30.2%. Among the detected pathogens, rhinovirus was the most common, followed by adenovirus and influenza virus A pdmH1N1 (2009) (Table 2). Of the 420 specimens, the ResP yielded consistent positive results in 121 specimens (86.5%, 121/141), and consistent negative results in 273 specimens (97.8%, 273/279) comparing with pathogen-specific PCRs, leading to an overall agreement of 93.8%.

No specimen was detected positive with coronavirus or Chlamydia. In six of the ten detected pathogens, the Cohen’s kappa

### Table 1: Samples enrolled in this study

| Item         | n    | Average age (y) |
|--------------|------|-----------------|
| Gender       |      |                 |
| Male         | 231  | 26.5            |
| Female       | 189  | 28.2            |
| Age group    |      |                 |
| <5           | 111  | 2.1             |
| 5 ~ 18       | 92   | 8.8             |
| 18 ~ 60      | 150  | 36.4            |
| >60          | 67   | 73.8            |
| Total        | 420  | 27.2            |

### Figure 1

An example of influenza virus A pdmH1N1 (2009)-positive result from the multiple PCR assay ResP. A, X-axis represents the sizes of amplification products, and Y-axis represents the signal strength. InfA, influenza virus A; 09H1, influenza virus A pdmH1N1 (2009); huRNA, human RNA; huDNA, human DNA; IC, internal control; B, Sanger sequencing result of partial sequence of influenza virus A pdmH1N1 (2009)
values were over 0.8 with P value <.01 (Table 2). The lowest kappa (0.70) was observed on human metapneumovirus.

4 | DISCUSSION

Multiplex PCR-based NAATs have been increasingly used for syndromic diagnosis, due to their high throughput, high sensitivity, high specificity, cost-effectiveness, and great clinical significance. The ResP assay is based on multiplex PCR amplification and capillary electrophoretic separation of PCR amplicons by length. This technique has been used for pathogen detection and subtype classification of pediatric acute lymphoblastic leukemia. By comparing the results with a standard size marker of targeted pathogens, pathogens in samples can be separated and identified as expected. The subtypes of most viruses were not designed to be further distinguished by this assay, except for influenza virus A. The influenza virus A pdmH1N1 (2009) and H3N2 are the two subtypes which are most popular in China recently. Therefore, a patient whose specimen is positive for influenza virus A but negative for influenza virus A pdmH1N1 (2009) or H3N2 is probably infected by an uncommon influenza virus A, such as H7N9, H5N1, H5N6 avian influenza virus and has to be immediately quarantined once it is confirmed. It should be noted that

| Pathogen                    | ResP | Pathogen-specific PCRs | Kappa | P   |
|-----------------------------|------|------------------------|-------|-----|
| Rhinovirus                  | 34   | 28                     | 0.82  | < .01|
| Adenovirus                  | 30   | 28                     | 0.81  | < .01|
| Influenza virus A pdmH1N1 (2009) | 30   | 30                     | 1.00  | < .01|
| Respiratory syncytial virus | 16   | 14                     | 0.93  | < .01|
| Influenza virus B           | 12   | 11                     | 0.96  | < .01|
| Human metapneumovirus       | 10   | 7                      | 0.7   | < .01|
| Parainfluenza virus         | 7    | 6                      | N/A   | N/A |
| Mycoplasma pneumoniae       | 7    | 6                      | N/A   | N/A |
| Boca virus                  | 4    | 4                      | N/A   | N/A |
| Influenza virus A H3N2      | 1    | 1                      | N/A   | N/A |
| Total                       | 151  | 135                    | 0.86  | < .01|

Abbreviation: N/A, not available.
hospitals, not CDCs, are the first to reach such patients, so this assay helps hospitals identifying such high-risk patients and make appropriate quarantine measurement in a timely manner to control further spread of avian influenza A virus.

This assay has previously been clinically applied to detection of respiratory pathogens in hospitalized children suffered with community-acquired pneumonia (CAP)\textsuperscript{14} or lower respiratory tract infections.\textsuperscript{20} The assay was evaluated by comparing with Sanger sequencing, showing great performance with 100% positive prediction value (PPV) and 99.85% negative prediction value (NPV).\textsuperscript{20} To our knowledge, this is the first study evaluating the performance of the ResP in oropharyngeal swab specimens from outpatients with ARIs.

Our study showed almost perfect kappa statistics for the ResP on rhinovirus, adenovirus, influenza virus A pdmH1N1(2009), respiratory syncytial virus, and influenza virus B, suggesting that the performance of ResP on these viruses was as effective as pathogen-specific PCRs. On human metapneumovirus, the kappa statistics were lower than 0.8, presumably due to the small number of positive cases. Overall, this assay demonstrated 86.5% PPV and 97.8% NPV. This work suggested that the performance of ResP was sufficient enough be used for respiratory pathogen identification in outpatients with flu-like manifestations.

The major limitation of this study is the small number of human metapneumovirus, parainfluenza virus, Mycoplasma pneumoniae, bocavirus, influenza virus A H3N2, coronavirus, and Chlamydia. Further investigation is needed to evaluate the performance of ResP on these pathogens.

In conclusion, the performance of ResP showed a high-degree agreement with pathogen-specific PCRs in oropharyngeal swabs from outpatients. The implementation of ResP may facilitate the diagnosis of respiratory infections in a variety of clinical scenarios.

**FUNDING INFORMATION**

The study was supported by the grants from the National Science and Technology Major Project of the Ministry of Science and Technology of China (2017ZX10103011), the Project for Key Medicine Discipline Construction of Guangzhou Municipality (2017-2019-07), Science and Technology Program of Guangdong Health Department (A2017489), Science and Technology Program of Guangzhou Health Department (2018A011052).

**ORCID**

Ying Zhang \(\text{https://orcid.org/0000-0003-2666-6575}\)

**REFERENCES**

1. Liu L, Oza S, Hogan D, et al. Global, regional, and national causes of under-5 mortality in 2000–15: an updated systematic analysis with implications for the Sustainable Development Goals. *Lancet*. 2016;388:3027-3035.

2. Jain S, Williams DJ, Arnold SR, et al. Community-acquired pneumonia requiring hospitalization among U.S. children. *N Engl J Med*. 2015;372:835-845.

3. Self WH, Williams DJ, Zhu Y, et al. Respiratory viral detection in children and adults: comparing asymptomatic controls and patients with community-acquired pneumonia. *J Infect Dis*. 2016;213:584-591.

4. Rapp U, Schuetz AN, Jenkins SG, et al. Impact of early detection of respiratory viruses by multiplex PCR assay on clinical outcomes in adult patients. Caliendo AM, ed. *J Clin Microbiol*. 2016;54:2096-2103.

5. Ramanan P, Bryson AL, Binnicker MJ, Pritt BS, Patel R. Syndromic panel-based testing in clinical microbiology. *Clin Microbiol Rev*. 2018;31:e00024-e117.

6. Subramony A, Zachariah P, Krones A, et al. Impact of multiplex polymerase chain reaction testing for respiratory pathogens on healthcare resource utilization for pediatric inpatients. *J Pediatr*. 2016;173:196-201.e2.

7. Rogers BB, Shankar P, Jerris RC, et al. Impact of a rapid respiratory panel test on patient outcomes. *Arch Pathol Lab Med*. 2015;139:636-641.

8. Brendish NJ, Malachira AK, Armstrong L, et al. Routine molecular point-of-care testing for respiratory viruses in adults presenting to hospital with acute respiratory illness (ResPOC): a pragmatic, open-label, randomised controlled trial. *Lancet Respir Med*. 2017;5:401-411.

9. Landis JR, Koch GG. The measurement of observer agreement for categorised data. *Biometrics*. 1977;33:159-174.

10. Wang S, Yang F, Li D, et al. Clinical application of a multiplex genetic pathogen detection system remaps the aetiology of diarrhoeal infections in Shanghai. *Gut Pathog*. 2018;10:37.

11. Vallières E, Renaud C. Clinical and economical impact of multiplex respiratory virus assays. *Diagn Microbiol Infect Dis*. 2013;76:255-261.

12. Uyeki TM, Bernstein HH, Bradley JS, et al. Clinical Practice Guidelines by the Infectious Diseases Society of America: 2018 Update on Diagnosis, Treatment, Chemoprophylaxis, and Institutional Outbreak Management of Seasonal Influenza. *Clin Infect Dis*. 2019;68:e1-e47.

13. Zhang H, Cheng H, Wang Q, et al. An advanced fragment analysis-based individualized subtype classification of pediatric acute lymphoblastic leukemia. *Sci Rep*. 2015;5:12435.

14. Wang L, Zhao M, Shi Z, et al. A GeXP-based assay for simultaneous detection of multiple viruses in hospitalized children with community-acquired pneumonia. *PLoS ONE*. 2016;11:e0162411.

15. Xie Z, Luo S, Xie L, et al. Simultaneous typing of nine avian respiratory pathogens using a novel GeXP analyzer-based multiplex PCR assay. *J Virol Methods*. 2014;207:188-195.

16. Wu Z-Q, Zhang Y, Zhao N, et al. Comparative epidemiology of human fatal infections with novel, high (H5N6 and H5N1) and low (H7N9 and H9N2) pathogenicity avian influenza A viruses. *Int J Environ Res Public Health*. 2017;14(3):263.

17. Pan M, Gao R, Lv Q, et al. Human infection with a novel, highly pathogenic avian influenza A (H5N6) virus: virological and clinical findings. *J Infect*. 2016;72:52-59.

18. Qin Y, Horby PW, Tsang TK, et al. Differences in the epidemiology of human cases of Avian Influenza A(H7N9) and A(H5N1) viruses infection. *Clin Infect Dis*. 2015;61:563-571.

19. Zhang H, Chen X, Lyu F, et al. Detection of viral and atypical pathogens in children with lower respiratory tract infection by multiple PCR technique. *J Wenzhou Med Univ*. 2017;47:791-800.

20. Li X, Chen B, Zhang S, et al. Rapid detection of respiratory pathogens for community-acquired pneumonia by capillary electrophoresis-based multiplex PCR. *SLAS Technol*. 2019;24:105-116.

**How to cite this article**: Zhang Y, Cao L, Xu Z, et al. Evaluation of a multiplex PCR assay for detection of respiratory viruses and Mycoplasma pneumoniae in oropharyngeal swab samples from outpatients. *J Clin Lab Anal*. 2020;34:e23032. \(\text{https://doi.org/10.1002/jcla.23032}\)