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ORIGINAL ARTICLE

Laboratory Evaluation of the Xpert Xpress Flu/RSV Test in Hubei, China

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SUMMARY

Background: **Early confirmation of infections with influenza virus and/or respiratory syncytial virus (RSV) is beneficial for prompt treatment and outbreak management. This study aimed to assess the Cepheid Xpert Xpress Flu/RSV assay in Central China, using Sanger sequencing as the reference method.**
Methods: **Nasopharyngeal swab (NP) samples from pediatric and adult patients with influenza-like illnesses were collected by the Hubei Province Disease Control and Prevention Center. The Xpert Xpress Flu/RSV assay was performed according to the manufacturer's guidelines, and the cycle threshold (Ct) values of each positive sample were recorded. Sanger sequencing was then performed on the NP samples, and discordant results were verified with a self-built fluorescent PCR method and virus tissue culture.**
Results: **The Xpert assay demonstrated a sensitivity of 97.06%, a specificity of 95.81%, an accuracy of 95.93%, a PPV of 71.74%, and a negative predictive value (NPV) of 99.66% for influenza A (Flu A) detection. Influenza B (Flu B) detection had a sensitivity of 100%, a specificity of 88.52%, an accuracy of 91.86%, a PPV of 78.12%, and an NPV of 100%. RSV detection had a sensitivity of 100%, a specificity of 95.67%, an accuracy of 98.80%, a PPV of 60.00%, and an NPV of 100%. False positive samples had significantly higher mean Ct values compared to true positive samples for detecting Flu A, Flu B, or RSV ($p < 0.001$).**
Conclusions: **The Xpert Xpress Flu/RSV test is a rapid, accurate, and reliable molecular diagnostic method for Flu A, Flu B, and RSV. Positive Xpert results with a low Ct value are more likely to indicate a clinical infection with Flu/RSV to allow timely diagnosis and treatment.**
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influenza A, influenza B, respiratory syncytial virus, Xpert Xpress Flu/RSV, Sanger sequencing

INTRODUCTION

Respiratory viruses such as the influenza virus (*Orthomyxoviridae spp.*) and the respiratory syncytial virus (RSV; a single-stranded RNA virus) are a major global contributor to hospital admissions, increased mortality, and the rising cost of healthcare. These viruses primarily affect young children, pregnant women, the elderly, and patients with comorbidities [1,2]. Influenza A (Flu A) and influenza B (Flu B) viruses are recurrent respiratory diseases and annual seasonal epidemics. The an-

nual number of seasonal influenza-associated respiratory deaths is estimated to be between 291,243 and 645,832 worldwide [3,4]. RSV is a common cause of hospitalizations and emergency room visits for lower respiratory tract infections [5,6]. Acute lower respiratory infections in adults and the elderly are primarily caused by influenza viruses in China, whereas RSV is the most prevalent viral pathogen in young children under the age of two years [7,8].

Early confirmation of respiratory viral infections is beneficial in clinical settings as it allows for prompt treatment and outbreak management [9]. Rapid and accurate pathogen diagnosis allows for targeted antiviral treatment as soon as possible after the onset of symptoms, while reducing unnecessary antibiotic prescriptions [10, 11]. However, the clinical diagnosis of Flu A, Flu B, and RSV can be challenging, because many of the symptoms are nonspecific and overlap with other respiratory pathogens such as adenovirus and coronavirus [1, 2]. Many clinicians use immunoassays for the rapid detection of influenza virus and RSV infection because of their simplicity and quick turnaround time [12]. These tests have low negative predictive values and perform poorly in terms of clinical and analytical sensitivity, making nucleic acid-based testing a promising alternate choice as it is more sensitive and specific [13,14].

In the GeneXpert system (Cepheid, France), nucleic acid extraction, PCR amplification, and real-time detection are integrated into an automated platform supplied with a single-use disposable cartridge [15]. Compared to traditional molecular assays, GeneXpert does not require precision pipetting and can be used by anyone without prior training, significantly reducing the possibility of errors and the time of operation [16,17]. An improved version of the former Xpert Flu/RSV XC, called Xpert Xpress Flu/RSV, needs less time to complete (63 minutes versus 32 minutes) and can detect RSV genome RNA, human and avian Flu A strains, and Flu B simultaneously from respiratory specimens, with a higher level of sensitivity and specificity than the original system [18]. In 2016, the use of Xpert Xpress Flu/RSV for in vitro diagnosis was approved by the FDA and European Medicines Agency, followed by the National Medical Products Administration (NMPA) in China in 2019 [19].

In China, the prevalence of Flu/RSV infection varies by season, duration, and region [8]. No study evaluating the use of this assay in Central China has been performed [19-21]. In this study, we collected samples from a Central China province, Hubei, to evaluate the Xpert Xpress Flu/RSV assay compared to a Sanger sequencing reference method. Our findings indicate an excellent overall performance of the Xpert assay in Chinese clinical settings. This new evidence supports using point-of-care testing to improve patient throughput and guide clinical decision-making, particularly in the event of pandemics or respiratory infectious outbreaks.

MATERIALS AND METHODS

Study design

The study was designed to assess the clinical performance of the Xpert Xpress Flu/RSV assay to detect Flu A, Flu B, and RSV in upper respiratory samples compared to Sanger sequencing. The Medical Ethical Committee approved the study protocol, and all participants and/or their families provided written informed consent (Ethics approval 2017-006-02).

Participants and sample storage

During the respiratory virus season, the Hubei Province Disease Control and Prevention Center provided nasopharyngeal swab (NP) samples from pediatric and adult patients with signs or symptoms of a respiratory infection (such as cough, rhinorrhea, etc.). NP specimens were collected in UTM viral transport media (Copan Diagnostics) through one nostril, stored at 2 - 8°C, and were then sent to the clinical microbiology laboratory within 24 hours for the Xpert assay. Following the assay, the remaining UTM was split into two aliquots and stored at -80°C for Sanger sequencing within 24 hours of thawing.

Data sources and measurement of Xpert Xpress Flu/RSV test

Xpert Xpress Flu/RSV assay was performed according to the manufacturer's instructions [22]. Briefly, after five times of inversion and mixture, 300 µL of NP specimen in UTM were transferred to the test cartridge for subsequent loading into the Gene Xpert instrument. The test processing time was approximately 30 minutes. The cycle threshold (Ct) values generated by the assay for each sample with a positive result were recorded for analysis, and the Ct values from fluorescence channel 2 were used for Flu A. For an initial indeterminate result, a single retest was performed with a fresh aliquot specimen and a new cartridge. If an additional indeterminate result was obtained during the repeat testing, it was excluded from the final analysis. One negative and one positive external control sample containing all of the RNA targets for Flu A, Flu B, and RSV were used in quality control assays.

Data sources and measurement of Sanger sequencing

Specimens tested by the Xpert assay were also analyzed by Sanger sequencing by Kingmed Diagnostics (Guangzhou, China). The internal validation results showed that the detection specificity, accuracy, and precision of this method were 100%. The sensitivity therefore met the requirements of national reference standards, reaching 2.1×10^2 TCID50/L for influenza virus and 100 TCID50/mL for RSV. Briefly, viral RNA extracted by Qiagen MinElute Virus Spin Kit was reverse transcribed using Superscript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA). The cDNA template was further amplified by using AmpliTaq Gold

Table 1. Oligonucleotide sequences of the PCR primers for reference Sanger sequencing.

Primer name	Primer sequence	Amplified fragment size (bp)
Flu-A_MP_Seq_Fwd1	CCCTCAAAGCCGAGATC	280
Flu-A_MP_Seg_Rev1	GCCCCATGGAACGTTAT	
Flu-A_PB2_Seq_Fwd1	CCAGCATTAAGCATCAATGAAC	164
Flu-A_PB2_Seq_Rev1	TAATTGATGGCCATCCGAAT	
Flu-B_MP_Seq_Fwd1	TCGCTGTTTGGAGACAC	244
Flu-B_MP_Seq_Rev1	GGGGCTCTGTGATGAATC	
Flu-BNS_Seq_Fwd1	AACATTCCTCAAACACCCC	376
Flu-B_NS_Seq_Rev1	ACCATGTCAGCTATTATGGAG	
RSV-A_Seq_Fwd1	GCATAACTACACTCCATAGTCC	363
RSV-A_Seq_Rev1	CATGATATCCCGCATCTCTG	
RSV-B_Seq_Fwd1	TATTCCCTAAACAACAGTGCTC	339
RSV-B_Seq_Rev1	GTGTCTTCCCTTCCTAACC	

Table 2. Demographic characteristics of the study population.

Characteristic	Number (percentage)
Gender	
Male	147 (42.7%)
Female	197 (57.3%)
Age group (year)	
≤ 1	82 (23.8%)
2 - 18	31 (9.0%)
19 - 60	196 (57.0%)
≥ 60	35 (10.2%)

Table 3. Xpert Xpress Flu/RSV performance evaluation referencing Sanger sequencing.

		TP	FP	TN	FN	Kappa	Sensitivity	Specificity	Accuracy	PPV	NPV
							TP/ (TP + FN)	TN/ (TN + FP)	(TP+TN)/ 344	TP/ (TP + FP)	TN/ (TN + FN)
Xpert	Influenza A	33	13	297	1	0.8026	97.06%	95.81%	95.93%	71.74%	99.66%
	Influenza B	100	28	216	0	0.8177	100%	88.52%	91.86%	78.12%	100%
	RSV	21	14	309	0	0.7293	100%	95.67%	98.80%	60.00%	100%

TP - true positive, FP - false positive, TN - true negative, FN - false negative, PPV - positive predictive value, NPV - negative predictive value.

360 Master Mix in ABI Veriti DX with primers targeting Flu-A matrix protein (280 ± 30 bp, Flu-A_PB2 Flu-A alkaline polymerase (164 ± 30 bp), Flu-B matrix protein (Flu-B_MP, 244 ± 30 bp), Flu-B non-structural protein (Flu-B_NS, 376 ± 30 bp), RSV-A nucleocapsid A (363 ± 30 bp), and RSV-B nucleocapsid B (339 ± 30 bp) (Table 1). Sequencing reactions were cleaned using the Big Dye Xterminator v3.1 (Life Technologies,

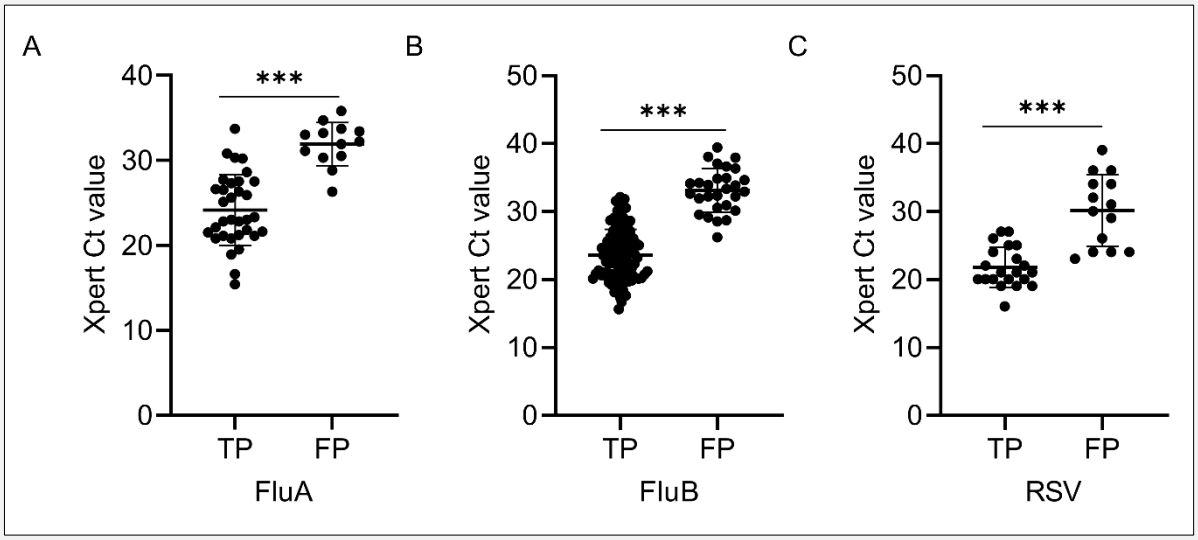

Figure 1. Comparison of the Ct values of Xpert Xpress Flu/RSV assay between true positive (TP) and false positive (FP) samples for the detection of FluA (A), FluB (B), and RSV (C) (* p < 0.001).**

Carlsbad, CA, USA) according to the kit's instructions before loading on an Applied Biosystems 3500 xl dx. Sequencing traces were analyzed with the Applied Biosystems SeqScanner 2, and the pathogen identification of the qualifying reads was verified through alignment with the reference viral genome. If each amplicon contained at least one acceptable trace in either direction, the specimen was deemed positive. Samples with discordant Xpert and sequencing results were verified at Zhujiang Hospital of Southern Medical University daily, using a self-built fluorescent PCR method and virus tissue culture [23].

Quantitative variables and statistical methods

The percentage of positive or negative results compatible with the sequencing approach across all samples was used to evaluate the efficacy of the Xpert assay for each virus. The Xpert assay result was classified as true positive (TP) or true negative (TN) depending on whether the sequencing matched. For every potential specimen, two-by-two tables were used to calculate the test features of sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) for each pathogen. The concordance between Xpert and sequencing was assessed by using the Kappa test (SPSS 25, SPSS Inc., Chicago, IL, USA). The exact (Clopper-person) method was used to calculate 95% confidence intervals (95% CI). Analysis of variance was used to compare the mean Ct values. Statistical analysis was performed using GraphPad 7 (La Jolla, CA), and a p-value of < 0.05 was considered statistically significant.

RESULTS

Study subjects

In this study, 401 NP samples were collected, out of which 50 samples without guardian signature, 1 duplicate sample, and 6 samples without medical treatment information were excluded, leaving 344 samples finally included. The final valid sample included in the statistical analysis was 344 patients, with 147 males (42.7%) and 197 females (57.3%). Among them, 82 patients (23.8%) were younger than one year old, 31 (9.0%) were 2 - 18 years old, 196 (57.0%) were 19 - 60 years old, and 35 (10.2%) were over the age of 60 (Table 2).

Performance of Xpert assay

Using Sanger sequencing as the reference method, there were 33 TP, 13 FP, 297 TN, and 1 FN samples for Flu A detection, yielding a sensitivity of 97.06%, a specificity of 95.81%, an accuracy of 95.93%, a PPV of 71.74% and an NPV of 99.66% for the Xpert assay (Table 3). The real-time PCR of 13 FP samples revealed 7 negative and 6 positive results. The virus culture of these 7 negative samples yielded 2 negative and 5 positive results. For Flu B detection, there were 100 TP, 28 FP, 216 TN, and 0 FN samples, with a sensitivity of 100%, a specificity of 88.52%, an accuracy of 91.86%, a PPV of 78.12%, and an NPV of 100%. The real-time PCR of 28 FP samples yielded 4 negative and 24 positive results. The available virus culture results for the 28 FP samples were 8 negatives and 4 positives. For RSV detection, there were 21 TP, 14 FP, 309 TN, and 0 FN samples, with a sensitivity of 100%, a specificity of

95.67%, an accuracy of 98.80%, a PPV of 60.00% and an NPV of 100%. The real-time PCR of 14 FP samples yielded 2 negative results. The 12 positive cross-reaction assays demonstrated that the Xpert does not produce false positive results due to coinfection with other respiratory pathogens. The above results also demonstrated that Xpert and Sanger sequencing have good clinical equivalence, with Kappa values of 0.8026, 0.8177, and 0.7293 for detecting FluA, FluB, and RSV, respectively.

Xpert Ct value analysis

In qualitative real-time PCR assays, Ct values represent the number of cycles required to achieve a positive result and are inversely related to the concentration of the target in a given sample. The Xpert assay produced Ct values for all samples, and a positive result indicated an accumulated, fluorescent signal that could be used as a semi-quantitative indicator of the amount of virus present in the sample. In the present study, the mean Ct value for TP samples was 24.15 (n = 33), 23.60 (n = 100), and 21.76 (n = 21) for the detection of Flu A, Flu B, and RSV, respectively. The mean Ct value for FP samples was 31.92 (n = 13), 33.09 (n = 28), and 30.14 (n = 14) for the detection of FluA, FluB, and RSV, respectively, which were significantly higher than those with TP results ($p < 0.001$) (Figure 1). There is a known linear correlation between Xpert Ct values and logarithms of viral loads, thus some of the FP samples in our study cannot be detected by sequencing due to their too low viral loads, not because of the bias of the Xpert methodology itself [19].

DISCUSSION

This study used 344 patient samples to evaluate the performance of the Xpert Xpress Flu/RSV assay in China compared to Sanger sequencing. The Xpert assay demonstrated high sensitivity, specificity, and accuracy in detecting FluA, FluB, and RSV, with a high observed concordance rate. Moreover, our results demonstrated a strong correlation between the TP results in the samples and the lower Ct values reported by Xpert, enabling a semi-quantitative assessment of the viral amounts in the examined samples. The assay is simple to use and provides clinicians with quick results, allowing them to make more confident clinical decisions and improve clinical outcomes.

The Xpert Xpress Flu/RSV, Filmarray respiratory panel, and Cobas® Liat® System are the most common point-of-care nucleic acid amplification tests for respiratory virus detection on the market. In terms of diagnostic performance, the pooled sensitivity and specificity of Cobas-modified RT-PCR were 0.9683 and 0.9913 for detecting influenza A, and 0.9878 and 1.00 for influenza B, respectively [22]. The pooled sensitivity of Filmarray for detecting influenza A, influenza B, and RSV was 0.911, 0.822, and 0.911, respectively [24]. The pooled sensitivity of Xpert Xpress Flu/RSV for detecting influenza A, influenza B, and RSV was 0.97, 0.98, and 0.96, and the specificities were 0.97, 1.00, and 1.00, respectively [18]. Additionally, the Xpert Xpress Flu/RSV test has significantly shorter turnaround times than other molecular devices [15,25]. The Xpert Xpress assay requires only one manual pipetting step to add samples into the cartridge [26]. It is clear, that Xpert Xpress can be used for point-of-care respiratory pathogen testing, particularly during infectious outbreaks or pandemics.

In China, three studies were reported to evaluate the clinical performance of Xpert Xpress Flu + RSV Assay. When compared to the reference multiplex influenza real-time RT-PCR LDT, Chen et al. discovered that the Xpert assay had 100% and 96.3% sensitivity for Flu A and Flu B, respectively, with 100% specificity for both viruses on 134 NP specimens from Hong Kong [20]. In another study, To et al. found that compared to patients' infection status, the clinical sensitivity of the Xpert assay was 98%, 100%, and 92.0% for Flu A, Flu B, and RSV, while the clinical specificity was 98.8%, 99.5%, and 98.9%, respectively [21]. The third report, which examined 658 specimens from Beijing, used a laboratory-developed real-time PCR test as the reference method. The Xpert assay had a sensitivity of 100% for Flu A, 100% for Flu B, and 90.5% for RSV, with an accuracy of 98.9%, 99.4%, and 99.4%, and specificities of 98.6%, 99.2%, and 99.7%, respectively [19].

For the first time, the present study collected samples from Central China for the Xpert Xpress Flu/RSV Assay, with Sanger sequencing serving as the reference method. Our findings showed a sensitivity of 97.06%, 100%, and 100% for Flu A, Flu B, and RSV, respectively, which is consistent with previous studies [18]. However, the specificity found in the current study was 95.81%, 88.52%, and 95.67%, which was slightly lower than that reported in similar studies [18]. We speculate that this is because the reference method used in this study is Sanger sequencing, which has a higher specificity than real-time PCR and clinical diagnosis. Both real-time PCR and virus culture in our study supported this and also revealed that some FP samples had negative results, indicating their true negative value. On the other hand, the fact that several other FP samples were verified positive via real-time PCR and virus isolation suggests that the sequencing had a detection specificity, accuracy, and precision of 100%, but not sensitivity. Viral load, nucleic acid extraction, and amplification procedures can all contribute to negative sequencing results. The use of PCR Ct values as a proxy for genomic load is influenced by the assay and factors in the sample that affect amplification efficiency [27]. Ct values, which are an integer scale representing a log result measurement of a log biological process, should be interpreted with caution [28]. Ct values can provide semi-quantitative information for clinically relevant outcomes [29]. Several studies found positive associations between low Ct values and disease severity, ICU admission, and

length of hospital stay for influenza virus and RSV infection [29-31]. For Xpert Xpress Flu/RSV, Zou et al. discovered samples with false positive results for Flu A, Flu B, and RSV had significantly higher Ct values than those with true positive results, which is consistent with our results [19]. These findings suggest that positive Xpert results with a low Ct value are more likely to indicate a clinical infection with Flu/RSV. Therefore, caution is advised when interpreting positive Xpert results with a high Ct value to indicate infection.

Limitations of the study

Our study is limited by lack of evaluation of test characteristics in carriers who are asymptomatic or who have prolonged virus shedding after a recent acute illness [32]. We cannot comment on test results and their relation to disease severity because we did not evaluate clinical diagnosis and treatment efficacy. Future implementation studies of the Xpert Xpress Flu/RSV test with larger sample sizes and carriers are recommended.

CONCLUSION

Our study found the Xpert Xpress Flu/RSV test to have a high clinical sensitivity, specificity, and accuracy for detection of Flu A, Flu B, and RSV in nasopharyngeal swab samples. It is a rapid, accurate, and reliable molecular diagnostic method that can be used in a variety of Chinese clinical settings. Positive Xpert results with a low Ct value were more likely to indicate a clinical infection with Flu/RSV, allowing for timely diagnosis and treatment.

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Data Availability Statement:

The data used in developing the results presented in this manuscript are available from the corresponding author upon request.

Declaration of Interest:

The authors declare that they have no conflicts of interest.

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