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Evaluation of three rapid assays for detecting *Mycoplasma pneumoniae* in nasopharyngeal specimens

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Abstract

During the 2023 autumn–winter period in China, *Mycoplasma pneumoniae* (MP) infections have increased. To address this, rapid and accurate MP DNA detection methods are crucial. Three nucleic acid detection assays (Ustar, Coyote Flash10, Coyote Flash 20) that are widely used in China are currently being evaluated for their effectiveness in detecting MP DNA in nasopharyngeal specimens. Reference standard materials for MP and a total of 35 NPS collected from Peking Union Medical College Hospital were tested using the Ustar, Coyote Flash10 and Coyote Flash 20 assays to assess analytical sensitivity, analytical specificity, diagnostic performance and workflow. The assays showed differing limits of detection (LOD) based on the absolute quantification of reference standards, with LODs of 500 copies/mL for the Ustar assays and 200 copies/mL for both Coyote Flash10 and Coyote Flash 20 assays. Additionally, all three assays displayed excellently analytical specificity in detecting MP DNA.The clinical correlation analysis demonstrated that the Ustar assay exhibited a sensitivity of 90.00%, specificity of 100%, positive predictive value (PPV) of 100%, and negative predictive value (NPV) of 62.50%. In contrast, both the Coyote Flash10 and Coyote Flash 20 assays displayed perfect diagnostic accuracy with 100% sensitivities, specificities, PPVs, and NPVs. Despite variations in detection principles, sample volume, and pre-preparation among the three assays, they all had a turnaround time of less than 30 min with low-throughput processing. Overall, all three rapid nucleic acid detection assays displayed excellent clinical performance in detecting MP DNA, offering a solid foundation for the quick clinical diagnosis of MP infection.

Keywords *Mycoplasma pneumoniae*, Rapid nucleic acid detection assays, Analytical sensitivity, Analytical specificity, Diagnostic performance, workflow

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Introduction

Mycoplasma pneumoniae (MP) is a bacterial pathogen capable of infecting humans, primarily causing upper respiratory tract infections and pneumonia. It is also recognized as the primary causative agent of pediatric community-acquired pneumonia (CAP) (Kumar 2018). Extrapulmonary involvement has been associated with MP, however, the exact mechanisms remain unclear (Abdulhadi and Kiel 2023). Similar to SARS-CoV-2, MP predominantly affects the respiratory tract but can also impact systemic organs (Yang et al. 2022). CAP attributable to MP may increase frequently during epidemics that occur every four to seven years due to waning herd immunity and introduction of new subtypes (Gadsby et al. 2012).

The ongoing COVID-19 pandemic has necessitated the implementation of various hygiene protocols, such as social distancing, mask-wearing, frequent handwashing, and limited social interactions. Consequently, there has been a substantial decline in the number of visits to hospital emergency rooms and private practices for pediatric infectious diseases acquired within the community even beyond the lockdown periods. Nevertheless, it is important to note that the primary catalysts for these infections are early childhood viral infections, largely inevitable during the initial years of life. In the event of a pandemic, an insufficient immune response due to personal non-pharmaceutical interventions (NPI), could lead to an immune debt and negatively affect the health of the population (Cohen et al. 2021). There is some evidence that respiratory syncytial virus (RSV) and possibly influenza epidemics will be more intense in the future based on mathematical models (Baker et al. 2020).

According to an international surveillance study on MP involving 24 countries across four continents, the introduction of NPI measures against COVID-19 delayed the reemergence of epidemics of MP (Meyer Sauteur and Beeton 2023). During the autumn and winter of 2023 in China, multiple pathogens caused illness, primarily MP (Gong et al. 2024). Despite the mild and self-limiting nature of Mycoplasma pneumoniae pneumonia (MPP), some children will develop severe pulmonary complications (e.g., obliterative bronchitis, bronchiectasis, and necrotizing pneumonia) after timely treatment with antibiotics, and the incidence of refractory patients is increasing yearly (Kumar 2018; Esposito et al. 2021). Therefore, early and accurate diagnosis is essential for a positive prognosis for patients with MPP.

A variety of methods can be used to diagnose MPP, including culture, serological tests, and molecular methods. Culture is unreliable and time-consuming, while serological tests require a paired serum sample that shows the infection (Daxboeck et al. 2003), The molecular methods have been found to be effective in detecting MP in respiratory tract specimens quickly, sensitively, and specifically(Dumke et al. 2007). A real-time PCR test is the most commonly used method for clinically detecting MP (Dumke et al. 2017); however, it requires complicated techniques, trained professionals and 4–6 h to get the detection results, so it cannot be used in the emergency department. Methods such as isothermal detection and denaturation bubble-mediated strand exchange amplification for MP have been reported (Petrone et al. 2015; Shi et al. 2019). While in China, rapid molecular diagnosis methods for MP have just been applied to clinical practice. In this study, we compared the analytical performance of the three rapid nucleic acid detection assays for MP in nasopharyngeal specimens (NPS) to explore their clinical application.

Materials and methods

The reference standard candidate and samples

Reference standard material was provided by Division II of In Vitro Diagnostics for Infectious Diseases, Institute for In Vitro Diagnostics Control, National Institutes for Food and Drug Control (Lot No.:370090-202001). It was made from inactivated Mycoplasma pneumoniae cultures, and was determined by the digital PCR method based on different platforms. The initial concentration of bacterial DNA was 2.2×10^6 copies/mL, quantified by digital PCR, and was diluted in viral transport media for the calculation of limit of detection (LOD). 35 NPS were collected from patients admitted to the emergency department of Peking Union Medical College Hospital (PUMCH) from October 1st, 2023 until April 30th, 2024. These samples were detected for MP DNA using real-time PCR assay (Sansure Biotech, Changsha, China), and the remaining specimens were stored at -20℃ for this study. The Ethics Committee of PUMCH (I-23PJ732) reviewed and approved the study. This study did not require informed consent for participation in accordance with national legislation and institutional requirements.

Ustar assay

1 mL extraction reagent was transferred into an automatic detection tube, and then 500 μL of sample, negative control or positive control was added to the detection tube of the Nucleic Acid Detection Kit for Mycoplasma Pneumoniae (MP). Isothermal amplification real-time fluorescence assay was conducted using Ustar EasyNAT® nucleic acid amplification and detection analyzer (Ustar Biotechnologies Co., Ltd,Hangzhou,China). The results could be concluded positive when the Ct values of MP DNA or Inner Control(IC) were≤40; otherwise, if the Ct values of MP DNA or IC were N/A, a negative result was considered. When the result was "Invalid" or "No results", retesting was required. The whole detection time was approximately 30 min.

Coyote Flash 10 and Flash 20 assays

For Coyote Flash10 assay, 300 µL NPS sample was added into the sample chamber of FlashDetect™ LyocartE RSV & MP & ADV assay (Real-time PCR), then the chamber was placed into the COYOTE® Flash10 fully-automated nucleic acid detection and analysis system (Coyote Bioscience Co., Ltd, Beijing, China) for the detection of MP DNA. When the Ct value of MP was ≤ 42, the result was judged as positive; otherwise, if the Ct value of MP was > 42, the result was judged as negative.

For Coyote Flash20 assay, PCR mixture containing 32 µL reagent I, 3µL reagent II and 2µL enhancer was prepared for usage. A 15 µL aliquot of the NPS specimen was transferred into a specimen lysis tube containing 15 µL of lysis buffer and vortexed for 15 s, then 15 µL mixture was added to the PCR reaction mixture. Real- time PCR for MP DNA detection was performed using a FlashDetect™ LyocartE RSV & MP detection Kit (Real-time PCR) on COYOTE® Flash20 Real-time quantitative PCR instrument(Coyote Bioscience Co., Ltd., Beijing, China). The results could be concluded as positive when the Ct values of MP were ≤ 27; otherwise, when the Ct values of MP were > 27, a negative result was considered.

Clinical performance evaluation

The diagnostic performance evaluation of three assays was compared with our routine MP-DNA testing protocol using Sansure real-time PCR assay (Sansure Biotech Inc., Changsha, China). Thirty MP-positive and five MP-negative samples confirmed by Sansure assays were detected by three rapid nucleic acid detection assays simultaneously, and then the sensitivity, specificity, positive percent agreement (PPA), negative percent agreement (NPA), and Cohen's kappa (k) were calculated.

Statistical analysis

Comparisons between two groups with or without interfering substances were made using the Wilcoxon test, and a p-value less than 0.05 (two-sided) was considered statistically significant. The analyses were performed using Prism 7.0 (GraphPad, La Jolla, CA, USA) software. The PPA, NPA, kappa, and two-sided (upper/lower) 95% confidence interval (CI) were calculated using Microsoft Office Excel 365 MSO software (Microsoft, Redmond, WA). To assess the overall level of agreement, Cohen's kappa (k) value was calculated. The interpretation of the kappa value is as follows: a value less than 0.2 indicates poor agreement, between 0.2 and 0.4 represents fair agreement, between 0.4 and 0.6 signifies moderate agreement, between 0.6 and 0.8 denotes strong agreement, and a value between 0.8 and 1.0 indicates very strong agreement (McHugh 2012).The McNemar's chi-square test was conducted using SPSS 20.0 (Statistical Package for the Social Sciences, IBM Corporation).

Table 1 Comparison of the LODs of three rapid nucleic acid detection assays

Assay	Positivity rate(%) at indicated dilution(copies/mL)		
	1000	500	200
Ustar	100(5/5)	100(15/15)	80(12/15)
Coyote Flash10	100(5/5)	100(15/15)	100(15/15)
Coyote Flash20	100(5/5)	100(15/15)	100(15/15)

Results

Analytical sensitivity

The MP standards with a concentration of 2.2×10^6 copies/mL were diluted to 1000 copies/mL, 500 copies/mL and 200copies/mL to verify the LOD of each assay according to their instructions. As shown in Table 1, the LOD was 500 copies/mL for the Ustar assay, and 200 copies/mL for both the Coyote Flash 10 and Coyote Flash 20 assays respectively.

Analytical specificity and specificity

To compare the specificity of three rapid nucleic acid detection assays, cross- reaction and capacity of resisting disturbance were assessed using three assays. Three pathogens (*Streptococcus pneumoniae, human Boca virus and coxsackievirus*) that may cause the same clinical symptoms as MP were tested, and all three assays reported negative results. These pathogens are included in National Reference Panel A for the Specificity Evaluation of Respiratory Pathogen Nucleic Acid Detection Kits, which was supplied by Division II of In Vitro Diagnostics for Infectious Diseases, Institute for In Vitro Diagnostics Control, National Institutes for Food and Drug Control. Besides, hemoglobin (100 g/L), triglycerides (25.47 mmol/L), and Azithromycin (3 µg/mL) were added into the NPS from MP-positive samples to explore whether these interfering substances would have an effect on the Ct value of MP detection. It was shown that the Ct values of MP DNA were not influenced by the interfering substances (Fig. 1).

Clinical performance evaluation

For diagnostic performance evaluation, 30 MP-positive NPS and 5 MP-negative NPS confirmed by the Sansure assay were involved. The Ustar assay exhibited detection of 32 out of 35 samples, whereas the Coyote Flash10 and Coyote Flash20 assays achieved all detection for 35 samples. The analysis revealed that the Ustar assay possessed a sensitivity of 90.00% (± 95% CI, 0.74–0.97), specificity of 100% (± 95% CI, 0.57–1.00), and positive predictive value (PPV) of 100% (± 95% CI, 99.00% to 100%). Additionally, the three samples yielding false-negative results in the Ustar assay were identified to have MP DNA Ct values of 39.69, 39.17, and 39.88 when tested with the Sansure

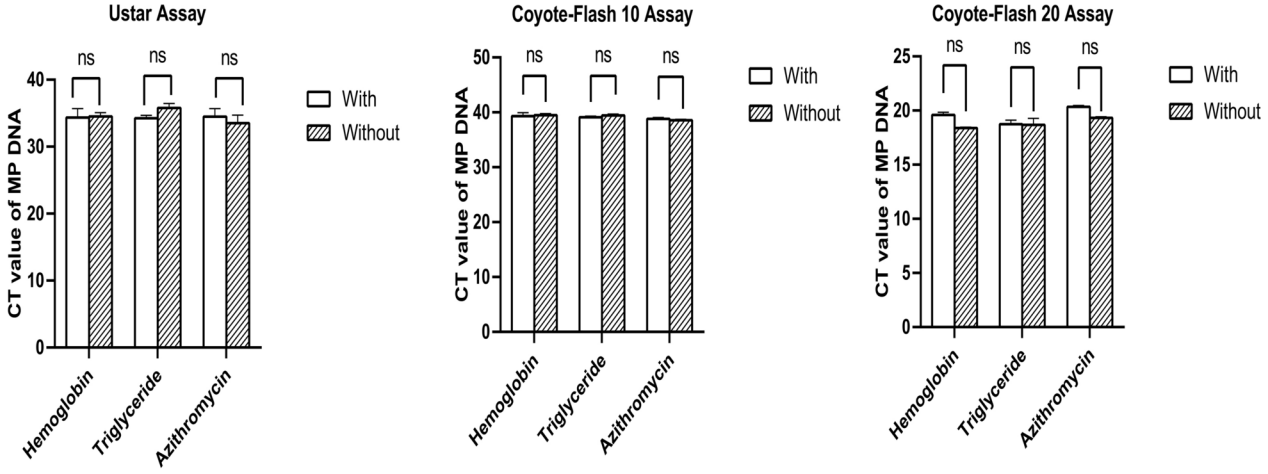

Fig. 1 The impact of NPS on Ct values of MP DNA detected by three rapid nucleic acid detection assays with and without the interfering substance

Table 2 Clinical performance comparison of three rapid nucleic acid detection assays for 35 NPS

Assay		Sansure Assay		Kappa value (±95% CI)	McNemar's test (P)	Sensitivity(%) (±95% CI)	Specificity(%) (±95% CI)	PPV (%) (±95% CI)	NPV (%) (±95% CI)
		Positive	Negative						
Ustar	Positive	27	0	0.72(0.43–1.00)	0.25	90(0.74–0.97)	100(0.57–1.00)	100(0.99–1.00)	62.50(0.29–0.96)
	Negative	3	5						
Coyote Flash10	Positive	30	0	1.00(1.00–1.00)	1.00	100(0.89–1.00)	100(0.57–1.00)	100(0.99–1.00)	100(0.46–1.00)
	Negative	0	5						
Coyote Flash20	Positive	30	0	1.00(1.00–1.00)	1.00	100(0.89–1.00)	100(0.57–1.00)	100(0.99–1.00)	100(0.46–1.00)
	Negative	0	5						

assay, suggesting that the viral loads in these samples were near the detection threshold of the Ustar assay, yielding a negative predictive value (NPV) of 62.50% (±95% CI,0.29–0.96). Notably, the Coyote assays demonstrated perfect performance in terms of sensitivity, specificity, and PPV, all achieving values of 100%. Regarding the remaining 5 MP-negative samples, all of them (5/5; 100%) were correctly reported as negative by both assays, exhibiting a NPV of 100% (±95% CI, 0.46–1.00) (Table 2).

Workflow

Workflow parameters along with basic assay characteristics are presented in Table 3. Both Ustar and Coyote Flash10 assays were performed on fully automated platforms utilizing magnetic bead-based nucleic acid extraction without preliminary preparation of the reaction mixture before PCR. Conversely, the Coyote Flash20 assay employed separate steps for nucleic acid extraction and amplification, with alkali lysis serving as the extraction method. Reagents of the Coyote Flash10 assay could be stored at room temperature, while the other two assays should be stored at low temperature. All three assays do not belong to high-throughput processes, but the TATs were no more than 30 min.

Discussion

MPP and CAP caused by other factors are difficult to distinguish at an early stage due to their similarities in clinical, radiological, and histopathological characteristics, making differentiation between them in the early stages of illness difficult (Chen et al. 2020; Kim et al. 2022). Therefore, it is challenging for laboratory diagnosis to select early, rapid, and accurate diagnostic methods. China's rapid nucleic acid detection technology became widely used following the COVID-19 epidemic in 2020. The Chinese Food and Drug Administration has approved a variety of nucleic acid POCT products for COVID-19, including EasyNAT (Ustar Biotechnologies, China), AGS8830 (Daan Gene Co., Ltd., China), iPonatic (Sansure Biotech Inc., China), and Flash 10 and Flash20 (Coyote Bioscience Co., Ltd., China). The testing time of these products ranges from 30 to 79 min, and their performance is similar to that of similar international products, but there has been little research on the comparison of these assays for MP DNA detection. In this study, we evaluated the analytical sensitivity, analytical specificity, clinical performance and workflow of three rapid molecular assays that were widely applied in Chinese clinical settings.

The Ustar EasyNAT®nucleic acid amplification and detection analyzer, the first instrument of its kind to be listed in China, received registration certification from the National Medical Products Administration in 2019. Capable of

Table 3 Basic detection characteristics and workflow parameters of three rapid nucleic acid detection assays

Characteristic	Ustar	Coyote Flash10	Coyote Flash20
Detection platform/system	Ustar EasyNAT®nucleic acid amplification and detection analyzer	COYOTE®Flash10 fully-automated nucleic acid detection and analysis system	COYOTE®Flash20 Real-time quantitative PCR instrument
Detection principles	Real-time fluorescence loop mediated isothermal amplification	Real-time PCR	Real-time PCR
Reagent storage temperature	2–8 ℃	2–28 ℃	−20 ± 5 ℃
Sample type	NPS	NPS	NPS
Nucleic acid extraction method	Magnetic bead	Magnetic bead	Alkali lysis
Sample volume required	500μL	300μL	15μL
High-throughput processing	No	No	No
Pre-preparation the reaction mixture before PCR	No	No	Yes
Number of samples that can be tested simultaneously	8	4	4
Overall TAT/run	25 min	30 min	25 min

simultaneously detecting two samples, this instrument utilizes cross-primer isothermal amplification technology with a detection time of 79 min. In contrast, the Coyote Flash20 COVID-19 nucleic acid rapid detection system features independent temperature control designs for each sample well, facilitating the sequential detection of samples and offering a more convenient solution in emergencies(Ye et al. 2022). The evaluation of SARS-CoV-2 RNA detection using Ustar and Coyote Flash20 assays has been previously reported by our team (Yi et al. 2021). While certain advancements have been achieved in MP DNA detection, such as the reduction in detection time and the enhancement of throughput compared to previous versions of these two assays, it is of utmost importance to conduct a rigorous evaluation of the analytical performance after improvements.

The LOD serves as a critical analytical performance parameter for rapid molecular assays. Assays with lower LODs have a higher likelihood of detecting cases with a low viral burden, thereby reducing the occurrence of false negatives (Yi et al. 2021). Our study revealed that the LODs of the Coyote Flash10 and Flash20 assays were comparable to those of routine real-time PCR assays, while the LOD of the Ustar assay was found to be higher than that of the Coyote assays. This discrepancy may be attributed to variations in the detection methods employed. The Ustar assay utilizes an isothermal amplification real-time fluorescence technique, which, as indicated by previous research, has shown assay sensitivity ranging from 50 to 100% when compared to real-time PCR (Amaral et al. 2021; Inaba et al. 2021; Cao et al. 2022; Iqbal et al. 2022). In our study, the sensitivity of the Ustar assay for MP DNA was 90.00%, which was quite comparable to the real-time PCR. However, it was worth noting that the Ustar assay exhibited a slight limitation in detecting samples with low pathogen loads, as it tended to miss these cases. In the two Coyote assays utilizing real-time PCR technology, it was observed that while the detection time was reduced, the LOD was not impacted. This suggested that the Coyote assays maintained comparable sensitivity to the conventional real-time PCR method despite the expedited detection time. This is indeed a significant advantage in clinical settings where rapid results are often needed.

Sensitivity and specificity are inversely related, as sensitivity increases, specificity tends to decrease, and vice versa (Naeger et al. 2013). While taking into account the specificity offered by these three different assays, it is evident that they represent a robust and reliable diagnostic tool with 100% specificity. Specificity is a critical factor in clinical diagnostics, as it ensures that the test accurately identifies the target pathogen without giving false-positive results, which is a critical factor in clinical diagnostics. This is particularly important in scenarios where an accurate diagnosis is vital to prevent unnecessary treatments or undue worry for patients.

One important factor for laboratories implementing these assays is the need to strike a balance between speed, simplicity, and reliability. For example, the Ustar and Coyote Flash10 assays may be more suitable for high-paced settings where quick results and user-friendly procedures are essential. However, the Coyote Flash 20 assay, which involved separate processes for nucleic acid extraction and amplification, may be less convenient during a pandemic. Additionally, the ability to store reagents at room temperature for Coyote Flash 10 significantly reduces the dependence on specialized storage facilities, making it a cost-effective option for laboratories with limited resources. In contrast, the requirement for low-temperature storage in the other two assays may limit their accessibility in certain settings. Furthermore, the Coyote Flash10 and Flash20 assays featured multiple targets, allowing for a thorough analysis of samples and making them suitable for various applications. It was important to consider that the lack of high-throughput processing in all three assays limited their capacity to handle a greater number of samples in a condensed timeframe. Despite their quick turnaround times, the assays varied in terms of workflow intricacy, stability of reagents, and storage prerequisites, making each assay suitable for different scenarios.

This study is limited by its focus on verifying the LODs of three assays rather than establishing the LODs. The assays were able to detect the MP standard at a dilution of 200 copies/mL for the Coyote assays, which suggested that the LODs may actually be lower than 200 copies/mL. Furthermore, the small sample size used to analyze diagnostic performance indicated that a significantly larger dataset was needed to validate the diagnostic performance of the assays. In conclusion, the three rapid detection methods evaluated in this study offered excellent performance in detecting MP, and the choice of method should be based on the specific needs and conditions of each laboratory. The combined use of multiple methods and periodic updates of testing protocols is essential for accurate and efficient MP detection.

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Author Contribution
C.Y and J.Y conceived the presented study. C.Y, Z.Y and L.K carried out experiments, data curation, formal anaylsis and validation. J. Y supervised the project. C.Y wrote the initial script. J.Y and J.D reviewed, discussed and contributed to the final version of the manuscript. J.Y obtained funds for the project.

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Data availability
All data generated or analysed will be available on request

Declarations

Ethical approval and consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethics committee at PUMCH.

Competing interests
The authors declare that they have no competing interests.

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