



# Accurate, rapid and low-cost diagnosis of *Mycoplasma pneumoniae* via fast narrow-thermal-cycling denaturation bubble-mediated strand exchange amplification

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## Abstract

*Mycoplasma pneumoniae* is a strong infectious pathogen that may cause severe respiratory infections. Since this pathogen may possess a latent period after infection, which sometimes leads to misdiagnosis by traditional diagnosis methods, the establishment of a rapid and sensitive diagnostic method is crucial for transmission prevention and timely treatment. Herein, a novel detection method was established for *M. pneumoniae* detection. The method, which improves upon a denaturation bubble-mediated strand exchange amplification (SEA) that we developed in 2016, is called accelerated SEA (ASEA). The established ASEA achieved detection of 1% *M. pneumoniae* genomic DNA in a DNA mixture from multiple pathogens, and the limit of detection (LOD) of ASEA was as low as  $1.0 \times 10^{-17}$  M (approximately  $6.0 \times 10^3$  copies/mL). Considering that the threshold of an asymptomatic carriage is normally recommended as  $1.0 \times 10^4$  copies/mL, this method was able to satisfy the requirement for practical diagnosis of *M. pneumoniae*. Moreover, the detection process was finished within 20.4 min, significantly shorter than real-time PCR and SEA. Furthermore, ASEA exhibited excellent performance in clinical specimen analysis, with sensitivity and specificity of 96.2% and 100%, respectively, compared with the “gold standard” real-time PCR. More importantly, similar to real-time PCR, ASEA requires only one pair of primers and ordinary commercial polymerase, and can be carried out using a conventional fluorescence real-time PCR instrument, which makes this method low-cost and easy to accomplish. Therefore, ASEA has the potential for wide use in the rapid detection of *M. pneumoniae* or other pathogens in large numbers of specimens.

**Keywords** ASEA · *Mycoplasma pneumoniae* · Rapid pathogen detection · Sputum specimens · Clinical diagnosis

## Abbreviations

2019-nCoV 2019 Novel coronavirus  
ASEA Accelerated strand exchange amplification  
CAP Community-acquired pneumonia  
LOD Limit of detection

NTC No template control  
SEA Strand exchange amplification  
T<sub>1</sub> First temperature  
T<sub>2</sub> Second temperature  
T<sub>m</sub> Melting temperature  
T<sub>t</sub> Threshold time

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## Introduction

Pneumonia, a common respiratory infectious disease with widespread global prevalence, is one of the major disease states resulting in mortality in children younger than 5 years [1, 2]. This disease is triggered by a variety of pathogens, including *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Streptococcus pneumoniae* and some types of coronavirus [3–6], among which *M. pneumoniae* is the cause of approximately 10–30% of community-acquired pneumonia (CAP), especially in children and adolescents [7]. Besides the typical

symptoms of pneumonia such as headache, sore throat, fever and cough, the infection of *M. pneumoniae* can also lead to multiple complications, including nervous system disorders, diarrhea, hemolytic anemia and myocarditis [8, 9]. However, these clinical symptoms are similar to those of respiratory diseases induced by other bacterial or viral infections, which thus might lead to misdiagnosis and delayed treatment [10]. Moreover, the incubation period of *M. pneumoniae* is roughly 2–4 weeks [11], and therefore most patients are unaware of the invasion of this pathogen for 14 days after infection, which greatly increases the risk of pathogen transmission in crowded places, such as schools. This is also one of the reasons why *M. pneumoniae* has caused frequent CAP outbreaks in school-aged children aged 5 to 15 years [8]. For these reasons, rapid and sensitive detection methods capable of accurately diagnosing *M. pneumoniae* early in the incubation period are critical in preventing the outbreak of *M. pneumoniae*-caused CAP.

Traditional diagnostic methods for *M. pneumoniae* include isolation culture and serological test, which are still generally used in medical institutions [12]. However, isolation culture is time-consuming, space-occupying and laborious, and usually takes several days to even more than 1 week to obtain detectable strains [13], since the antibodies can barely be detected in serum during the incubation period [14]. Besides, poor specificity has been reported for serological testing [15]. Therefore, these two traditional methods cannot meet the requirement for accurate, rapid and early diagnosis. Compared with the traditional methods, nucleic acid amplification technologies are normally free of the pathogen culture process and able to detect pathogens during the incubation period, exhibiting excellent specificity and sensitivity [16, 17]. Thus, these technologies are more desirable for early diagnosis, among which real-time polymerase chain reaction (real-time PCR) is the most widely used in clinical diagnosis [18, 19]. However, the amplification progress of conventional real-time PCR usually takes 1–1.5 h [20], which makes it difficult for the technology to meet the demands of situation requiring quick testing of a large number of samples, such as 2019-nCoV detection in airports or seaports. Although some extreme PCR methods greatly shorten the detection time to less than 100 s, even to 46 s according to some reports, these efficient approaches are dependent on special DNA polymerase and sophisticated instruments, and generally require more than a tenfold series of primers and polymerase, all of which make the process costly and restrict the widespread application of this technology [21, 22]. In our previous work, we developed an isothermal denaturation bubble-mediated strand exchange amplification (SEA) method, which is started by the invasion of one pair of primers into the denaturation bubbles randomly formed in a DNA duplex triggered by DNA respiration [23]. This isothermal reaction relies on *Bst* 2.0 WarmStart DNA, an easily available commercial enzyme with polymerase and strand displacement activity which allows

the primers to be extended at a constant reaction temperature [10, 24–26]. Although SEA takes less time than conventional real-time PCR to detect pathogens, including *M. pneumoniae*, the time required for this method is only approximately 25% shorter than real-time PCR, and its sensitivity is obviously lower, though this isothermal amplification method requires no sophisticated temperature control equipment [27]. We recently discovered that the amplification efficiency of SEA could be significantly improved by introducing a narrow range of rapid thermal cycling [28], which does not require special equipment and can be performed by conventional fluorescence real-time PCR instruments. Also, similar to SEA, this accelerated SEA (ASEA) is initiated by *Bst* 2.0 WarmStart DNA polymerase and one pair of primers, rather than expensive special polymerase. Therefore, this method possesses the potential to fulfill the requirements for fast and low-cost detection of epidemic pathogens from a large number of specimens.

Due to the advantages described above, herein, the ASEA method was established for *M. pneumoniae* detection for the first time. Specifically, we first optimized reaction conditions, followed by feasibility, specificity, sensitivity and anti-jamming capacity assessment. Finally, ASEA was employed for *M. pneumoniae* nucleic acid testing of clinical specimens to verify the accuracy of this novel method by comparison with real-time PCR, the current “gold standard.” The objective was to provide a rapid, low-cost and sensitive detection method with conventional instruments and easily available commercial polymerase for early diagnosis of *M. pneumoniae* and other epidemic pathogens.

## Materials and methods

### Materials and reagents

Fifty sputum specimens from patients with respiratory tract infection symptoms were provided by the Affiliated Hospital of Qingdao University. All of the sputum specimens were immediately stored at  $-20\text{ }^{\circ}\text{C}$  after collection for subsequent use. *M. pneumoniae* (ATCC 15531) and nine other reference pathogen strains that may also infect humans and cause pneumonia with similar symptoms, including *Streptococcus pneumoniae* (ATCC BAA-255), methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300), *Escherichia coli* (ATCC 43895), *Pseudomonas aeruginosa* (ATCC 47085), *Mycobacterium tuberculosis* (ATCC 27294), *Legionella pneumophila* (ATCC 33823), *Stenotrophomonas maltophilia* (ATCC 17666), *Haemophilus influenzae* (ATCC 49247) and *Acinetobacter baumannii* (ATCC 19606), were provided by Navid Biotechnology Co., Ltd. (Qingdao China). Genomic DNA of sputum specimens was extracted using a High Pure Viral Nucleic Acid extraction kit purchased from Roche Applied Science (Mannheim, Germany)

according to the manufacturer's instructions. dNTP was purchased from Sangon Biotech (Shanghai, China). EvaGreen was purchased from Bridgen (Beijing, China). *Bst* 2.0 WarmStart DNA polymerase and ISO buffer were purchased from New England Biolabs Inc. (Beijing, China). The 20-bp DNA ladder was purchased from Takara Co., Ltd. (Dalian, China).

ASEA primers specific to *M. pneumoniae* 16S rDNA were designed and optimized by NCBI primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>), NUPACK software (<http://www.nupack.org/>) and the DINAMelt web server (<http://unafold.rna.albany.edu/?q=DINAMelt>), and were synthesized by Sangon Biotech (Shanghai, China) and purified by HPLC. All other reagents and buffers used in this study were of analytical grade.

### ASEA reaction

The ASEA reaction was performed in 20  $\mu\text{L}$  amplification mixture containing 2  $\mu\text{L}$  of the relevant templates,  $3.0 \times 10^{-6}$  M primers F and R, 2  $\mu\text{L}$  ISO buffer, 0.5  $\mu\text{L}$  EvaGreen, 0.2  $\mu\text{L}$  *Bst* 2.0 WarmStart DNA polymerase and 1.6  $\mu\text{L}$  dNTPs. The amplification mixture was subjected to rapid thermal cycles between a first temperature ( $T_1$ ) and a second temperature ( $T_2$ ) using the CFX Connect™ Real-Time PCR System (Bio-Rad, CA, USA). In particular, each thermal cycle consisted of incubating the amplification mixture at  $T_1$  for 1 s, then immediately reducing the reaction temperature to  $T_2$  at a rate of 3  $^\circ\text{C}/\text{s}$ , and incubating the amplification mixture at  $T_2$  for another 1 s, before immediately increasing the temperature back to  $T_1$  at a rate of 5  $^\circ\text{C}/\text{s}$ . The fluorescence signal emitted from the amplification mixture was scanned every thermal cycle and plotted over time to monitor the amplification in real time. Reaction temperatures including  $T_1$  and  $T_2$  were optimized prior to using this method for sputum specimen detection. Specifically, two series of temperatures from 76  $^\circ\text{C}$  to 72  $^\circ\text{C}$  and from 61  $^\circ\text{C}$  to 57  $^\circ\text{C}$  were selected as  $T_1$  and  $T_2$ , respectively.  $T_1$  and  $T_2$  at which the reaction showed the lowest threshold time ( $T_t$ ) and highest specificity were employed for sputum sample detection.

### Specificity, sensitivity and anti-jamming capability of ASEA

Genomic DNA extracts of the *M. pneumoniae* strain and the nine pathogens mentioned above were used as template for ASEA to assess the feasibility of this method for detecting *M. pneumoniae* and distinguishing this pathogen from others. The sensitivity of ASEA was evaluated by employing tenfold serial dilution of *M. pneumoniae* genomic DNA ranging from  $1.0 \times 10^{-11}$  M to  $1.0 \times 10^{-18}$  M as the templates. Additionally, a series of multicomponent blends containing 0%, 1%, 5%, 10%, 50% and 100% of *M. pneumoniae* genomic DNA were

prepared by mixing genomic DNA extracts of the abovementioned pathogens. These mixtures, which possessed the same final DNA weight of 200 ng, were subsequently utilized for anti-jamming assay of ASEA, and the amplification products were subsequently loaded on 4% agarose gel and subjected to electrophoresis for 0.5 h at 110 V. Each assay was conducted in triplicate.

### Detection of *M. pneumoniae* in sputum samples

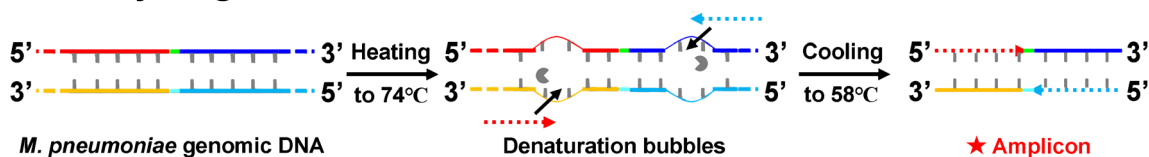
The feasibility of ASEA in actual samples was validated by detecting *M. pneumoniae* in the genomic DNA extracts of sputum specimens collected from patients with the symptoms of respiratory infection. Prior to the detection, all of the above sputum specimens were tested by gold-standard real-time PCR. The sensitivity and specificity of ASEA were assessed by comparing the number of negative and positive detections, respectively, with the real-time PCR results.

## Results and discussion

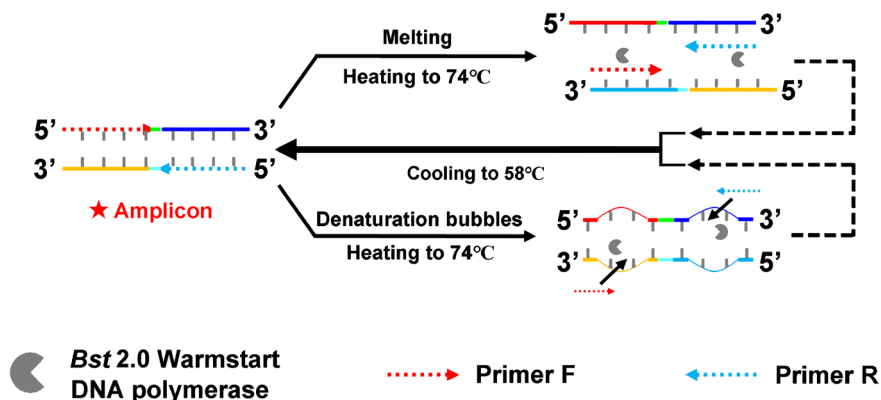
### Design of ASEA for *M. pneumoniae* detection

The mechanism of ASEA is shown in Fig. 1. The initial cycling of ASEA is similar to SEA, which is also initiated by the invasion of the primers into the denaturation bubbles and extended with the help of *Bst* 2.0 WarmStart DNA polymerase. As the amplification reaction progresses, the short amplicons, which can melt to ssDNA at  $T_1$  of the thermal cycling and bind with primers at  $T_2$ , largely accumulate. Therefore, the later cycling is triggered by both melting and annealing of amplicons, as well as the invasion of primers into denaturation bubbles. Based on the primer design strategy we established previously [29], a pair of primers specific to *M. pneumoniae* 16S rDNA was designed with amplicon length of 36 bp (Table 1), while *M. pneumoniae* genomic DNA at a concentration of  $1.0 \times 10^{-11}$  M was applied as template. Subsequently, in order to achieve the melting of the short amplicons and binding of primers with targets during the thermal cycling, a series of temperatures was set as  $T_1$  (76  $^\circ\text{C}$ , 75  $^\circ\text{C}$ , 74  $^\circ\text{C}$ , 73  $^\circ\text{C}$  and 72  $^\circ\text{C}$ ) and  $T_2$  (61  $^\circ\text{C}$ , 60  $^\circ\text{C}$ , 59  $^\circ\text{C}$ , 58  $^\circ\text{C}$  and 57  $^\circ\text{C}$ ) for reaction temperature optimization based on the melting temperature ( $T_m$ ) value of amplicon and primers, respectively. According to our previous work on SEA, a reaction temperature slightly lower than the average value of the primers'  $T_m$  value is the most beneficial for the combination of primers to target; therefore, 60  $^\circ\text{C}$  was first fixed as  $T_2$  for  $T_1$  optimization. As shown in Fig. 2a, the reaction with  $T_1$  of 74  $^\circ\text{C}$  exhibited the lowest  $T_t$  value, suggesting this temperature was best for this amplification reaction. It was observed that even a small number of amplification products were detected in the reaction with 76  $^\circ\text{C}$  as  $T_1$ , which may be attributed to the fact that the

## Initial cycling



## Later cycling



**Fig. 1** Schematic illustration of *M. pneumoniae* detection by ASEA. The initial cycling was mediated mainly by the invasion of primers into denaturation bubbles formed between the duplex of *M. pneumoniae*

genomic DNA, whereas the later cycling was triggered by both melting of amplicons and invasion of primers into denaturation bubbles

polymerase was inactivated during the rapid thermal cycling ranging from 76 °C to 58 °C. We then fixed 74 °C as  $T_2$  for  $T_1$  optimization, the result of which is shown in Fig. 2b. To our surprise, the reaction with  $T_2$  of 58 °C instead of 60 °C exhibited the lowest  $T_1$  value. Finally, to confirm that 74 °C was still the optimal  $T_1$  when 58 °C was employed as  $T_2$ , we fixed 58 °C as  $T_2$  to optimize  $T_1$  again. As expected, the reaction with 74 °C as  $T_1$  still showed the lowest  $T_1$  value (Fig. 2c). In summary, thermal cycling in the range of 74 °C to 58 °C was most beneficial for this ASEA reaction; thus, 74 °C and 58 °C were employed as  $T_1$  and  $T_2$ , respectively, for subsequent research in this work. It was found that  $T_1$  and  $T_2$  were both slightly lower than the amplicon's  $T_m$  value and average  $T_m$  value of primers, respectively. On one hand, a temperature lower than the primers'  $T_m$  value ensured the strong binding of primers to the target sequence [30, 31], which was crucial for the

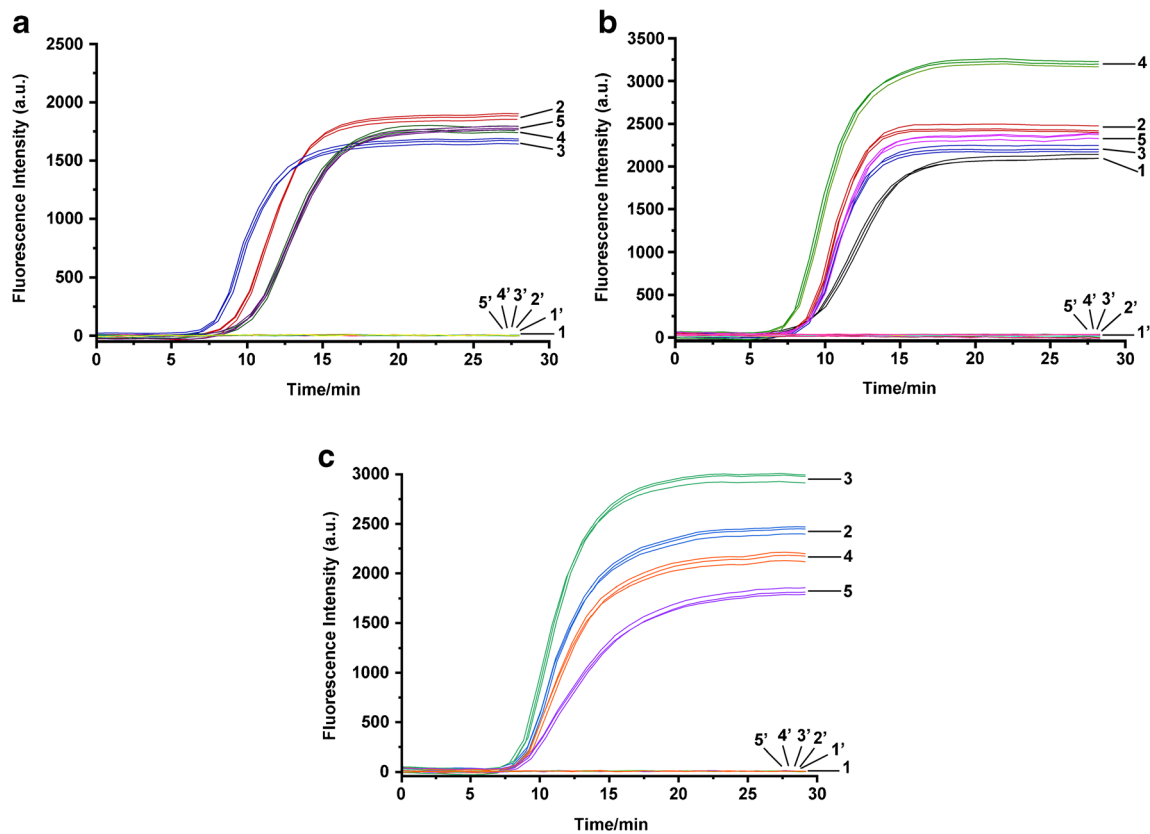
subsequent amplification. On the other hand, the polymerase applied in this work displayed the best reaction performance at 60–72 °C, and would be inactivated within 20 min at 80 °C; thus a reaction temperature far beyond this range is not conducive to amplification reaction. Besides, an excessive temperature difference between  $T_1$  and  $T_2$  would also increase the time consumption of the temperature change process, thus extending the total reaction time. Therefore, a temperature higher than the amplicon's  $T_m$  value in this research was not favorable for amplification, though denatured bubbles appeared in the DNA duplex more frequently at the target portion at a temperature higher than the amplicon's  $T_m$  value, which would increase the probability of primers invading denatured bubbles [26]. Combining the above factors, thermal cycling between 58 °C and 74 °C was most beneficial for the ASEA reaction utilized in this work.

**Table 1** Sequences of primers used in this work

Name	Sequence (5'–3')	$T_m$ value (°C)
<i>M. pneumoniae</i> 16S rDNA ( <sup>a</sup> NR_041751.1, <sup>b</sup> 697–732)	GGCGAAAACCTTAGGCCATTA CTGACTCTTAGGCTTG	75.9
Primer F	GGCGAAAACCTTAGGCCAT	60.8
Primer R	CAAGCCTAAGCGTCAGTA	60.9

<sup>a</sup> GenBank accession number

<sup>b</sup> The position of amplicon sequence in 16S rDNA



**Fig. 2**  $T_1$  and  $T_2$  optimization of the ASEA method for detecting *M. pneumoniae*. (a)  $T_1$  optimization at the  $T_2$  value of 61 °C. ASEA reactions were carried out at  $T_1$  values of (1) 76 °C, (2) 75 °C, (3) 74 °C, (4) 73 °C and (5) 72 °C. 1'–5' represent the corresponding NTC. (b)  $T_2$  optimization at the  $T_1$  value of 74 °C. ASEA reactions were carried

out at  $T_2$  values of (1) 61 °C, (2) 60 °C, (3) 59 °C, (4) 58 °C and (5) 57 °C. 1'–5' represent the corresponding NTC. (c)  $T_1$  optimization at the  $T_2$  value of 58 °C. ASEA reactions were carried out at  $T_1$  values of (1) 76 °C, (2) 75 °C, (3) 74 °C, (4) 73 °C and (5) 72 °C. 1'–5' represent the corresponding NTC

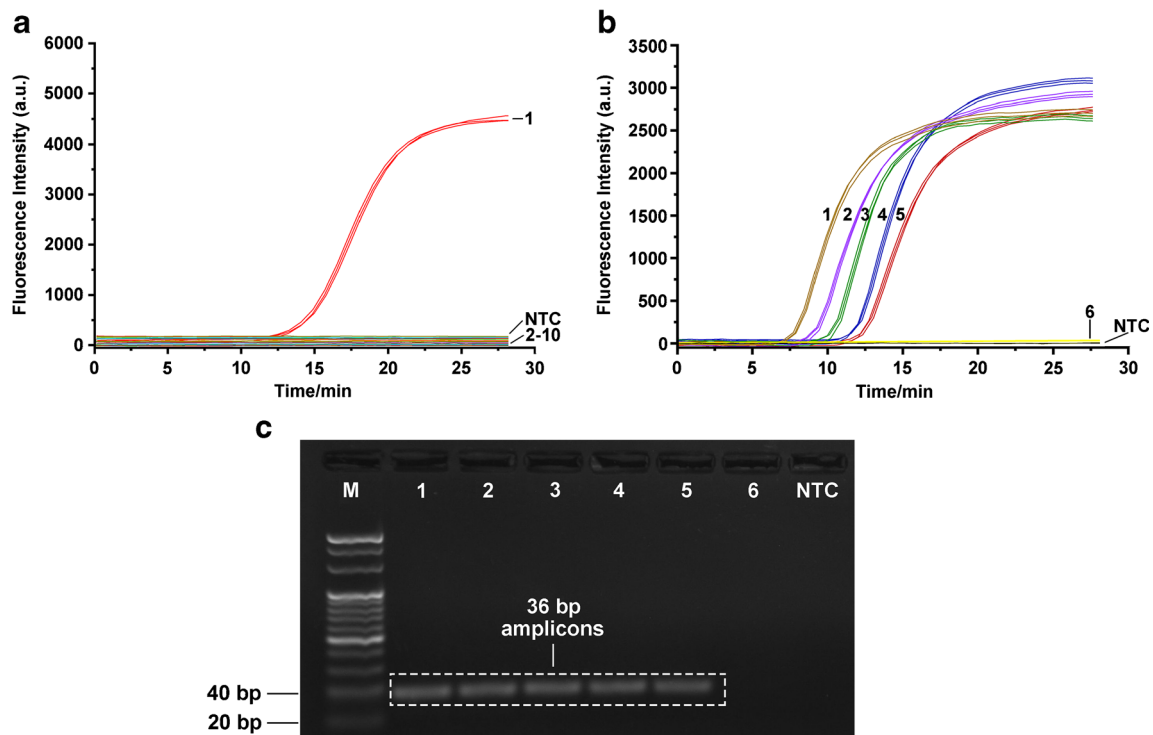
The italic portion is the same with primer F. The sequence complementary to primer R is shown in bold.

### Evaluation of ASEA for *M. pneumoniae* detection

The feasibility and specificity of ASEA were evaluated by performing amplification using genomic DNA of *M. pneumoniae* and nine other species of common pathogens mentioned above as templates, all at concentrations of  $1.0 \times 10^{-13}$  M (approximately 1.1 ng). As shown in Fig. 3a, significant fluorescence signal accumulation was observed only in the reactions with genomic DNA of *M. pneumoniae* as template, while hardly any fluorescence signal accumulation was detected in the reactions with other templates, which exhibited no obvious differences with NTC. This result demonstrated that ASEA was able not only to accomplish the amplification of the target sequence of *M. pneumoniae*, but also to avoid non-specific amplification and to distinguish *M. pneumoniae* from other common pathogens, suggesting the feasibility and good specificity of this method for *M. pneumoniae* detection.

With respect to practical detection application, *M. pneumoniae* is normally coexistent with multiple pathogens in the samples collected from patients. In order to assess the

capability of detecting *M. pneumoniae* from the specimens comprising multiple pathogens, DNA mixtures with ratios of 50%, 10%, 5% and 1% *M. pneumoniae* genomic DNA prepared by blending genomic DNA extracts of the above pathogens were utilized as templates to carry out ASEA reactions with *M. pneumoniae* 16S rDNA-specific primers. *M. pneumoniae* genomic DNA extracts and a mixture of the other nine pathogen genomic DNA extracts were also employed as positive and negative control. The total amount of DNA in all the above mixtures was 200 ng to eliminate the influence of DNA content on amplification efficiency. As expected, the result showed that the fluorescence signals were obviously enhanced in genomic DNA extracts of *M. pneumoniae* in the mixtures containing 50%, 10%, 5% and 1% *M. pneumoniae* genomic DNA, with  $T_t$  values of  $7.34 \pm 0.09$ ,  $8.52 \pm 0.15$ ,  $9.78 \pm 0.24$ ,  $11.35 \pm 0.08$ , and  $12.04 \pm 0.11$  min, respectively. Low fluorescence signals were detected in the mixtures without *M. pneumoniae* genomic DNA and NTC (Fig. 3b), demonstrating that *M. pneumoniae* genomic DNA even as low as 1% could be accurately detected. Additionally, agarose gel electrophoresis was carried out, the result of which was consistent with that shown by fluorescence curves. The bands of 36-bp amplicons framed by the white

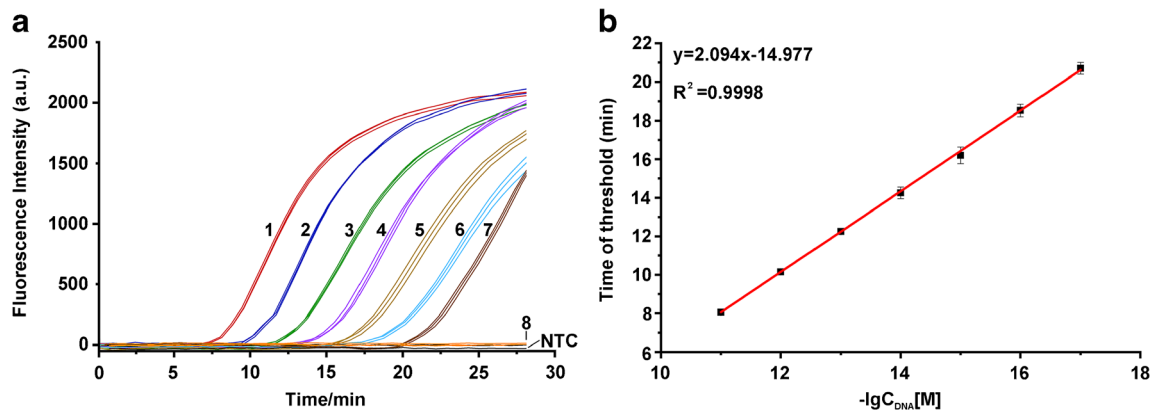


**Fig. 3** (a) Feasibility and specificity of ASEA for *M. pneumoniae* detection with genomic DNA of *M. pneumoniae* and nine other common pathogens as template. 1 represents genomic DNA of *M. pneumoniae*, and 2–10 represent genomic DNA of *S. pneumoniae*, MRSA, *E. coli*, *P. aeruginosa*, *M. tuberculosis*, *L. pneumophila*, *S. maltophilia*, *H. influenzae* and *A. baumannii*, respectively. (b) Anti-jamming capability of ASEA for *M. pneumoniae* detection and (c)

agarose gel electrophoresis image of corresponding ASEA products. 1 represents genomic DNA extract of *M. pneumoniae*, and 2–6 represent a mixture of the genomic DNA of the above pathogens with 50%, 10%, 5%, 1% and 0% genomic DNA of *M. pneumoniae*, respectively. The white dashed line frames the bands of 36-bp amplicons. M and NTC represent a 20-bp DNA ladder and no target control, respectively

dashed line were observed in the lane of the mixture containing *M. pneumoniae* genomic DNA, while hardly anything was detected in the lane with the mixture of genomic DNA from the other nine pathogens as well as NTC (Fig. 3c). The above results suggest that ASEA possesses high specificity and strong anti-jamming capability in DNA mixtures of multiple species.

Moreover, since the  $T_t$  value exhibited significant correlations with the target DNA content in the mixtures, this method also showed great potential for the quantitative analysis of *M. pneumoniae*. Therefore, ASEA is feasible for the detection and analysis of *M. pneumoniae* in actual specimens collected from patients.



**Fig. 4** Sensitivity of ASEA for *M. pneumoniae* detection. (a) The fluorescence curves of ASEA reactions with the template of *M. pneumoniae* genomic DNA at concentrations of (1)  $1.0 \times 10^{-11}$  M, (2)  $1.0 \times 10^{-12}$  M, (3)  $1.0 \times 10^{-13}$  M, (4)  $1.0 \times 10^{-14}$  M, (5)  $1.0 \times 10^{-15}$  M, (6)  $1.0 \times 10^{-16}$  M, (7)  $1.0 \times 10^{-17}$  M and (8)  $1.0 \times 10^{-18}$  M. (b) Linear

relationship between the threshold time and the negative logarithmic values of the concentration of *M. pneumoniae* genomic DNA ( $-\lg C_{DNA}$ ). Error bars represent the standard deviations of three independent measurements

**Table 2** Accuracy of ASEA for *M. pneumoniae* detection in clinical sputum specimens

ASEA	Real-time PCR		Total
	Positive <sup>a</sup> (26)	Negative <sup>a</sup> (24)	
Positive	25	0	25
Negative	1	24	25
Total	26	24	50

<sup>a</sup> The number in the brackets represents the number of positive or negative results diagnosed by real-time PCR

### Sensitivity of ASEA for *M. pneumoniae* detection

According to a previous report, asymptomatic carriers of *M. pneumoniae* are common, especially among children [32], and the pathogens that they carry are still infectious and may cause widespread transmission, despite the absence of symptoms in these healthy carriers. Since the amount of *M. pneumoniae* in an asymptomatic carrier is normally at a lower level than that in patients, the establishment of sensitive diagnostic approaches that can distinguish asymptomatic carriers from uninfected individuals is critical for *M. pneumoniae* transmission prophylaxis. To verify the sensitivity of the ASEA method, a tenfold serial dilution of *M. pneumoniae* genomic DNA ranging from  $1.0 \times 10^{-11}$  M to  $1.0 \times 10^{-18}$  M was employed as the templates for ASEA reaction, and the results are shown in Fig. 4a. The fluorescence signal increased significantly in the reactions with the DNA template ranging from  $1.0 \times 10^{-11}$  M to  $1.0 \times 10^{-17}$  M, with  $T_t$  values of  $8.14 \pm 0.08$ ,  $10.34 \pm 0.07$ ,  $12.13 \pm 0.10$ ,  $14.11 \pm 0.18$ ,  $16.02 \pm 0.36$ ,  $17.82 \pm 0.25$  and  $20.39 \pm 0.16$  min, respectively. In contrast, similar to the NTC, no obvious signal changes were observed in the reaction with the template concentration of  $1.0 \times 10^{-18}$  M, suggesting that ASEA could detect a concentration as low as  $1.0 \times 10^{-17}$  M of *M. pneumoniae* genomic DNA ( $1.2 \times 10^2$  copies in our 20- $\mu$ l reaction system) within 20.4 min. Moreover, the  $T_t$  value exhibited a significant linear relationship with the negative logarithm (lg) of genomic DNA amount from  $1.0 \times 10^{-11}$  M to  $1.0 \times 10^{-17}$  M, with a correlation equation of  $T_t = -14.977 + 2.094 \times (-\lg C_{\text{DNA}})$  and a corresponding correlation coefficient ( $R^2$ ) of 0.9998, demonstrating the excellent stability of this method for *M. pneumoniae* detection in this concentration range (Fig. 4b). Compared with the SEA method we established previously, the detection limit and corresponding processing time were  $1.0 \times 10^{-14}$  M and approximately 60 min (only 14.1 min was required by ASEA to detect *M. pneumoniae* at this concentration), respectively. Therefore, this new method displayed a lower limit of detection (LOD) in obviously shorter time. In addition, while real-time PCR and loop-mediated isothermal amplification (LAMP) were reported as having lower LOD, reaching  $2.2 \times 10^3$  copies/mL and  $1.1 \times 10^3$  copies/mL

(equivalent to  $3.7 \times 10^{-18}$  M and  $1.8 \times 10^{-18}$  M), respectively [33, 34], ASEA exhibited an equal advantage with regard to detection time in comparison with 1 h–1.5 h for conventional real-time PCR, while LAMP had a high rate of false-positive amplification according to some reports [35]. More importantly, ASEA can be operated using common real-time PCR devices and is free of complicated primer design processes and expensive special polymerase or instruments that are required for LAMP, digital PCR or extremely rapid PCR [21, 36–38]. Furthermore, since  $1.0 \times 10^4$  copies/mL (equivalent to  $1.7 \times 10^{-17}$  M) genomic DNA is generally recommended as the threshold for distinguishing clinical infection of *M. pneumoniae* from asymptomatic carriage [39, 40], this rapid and sensitive method, whose LOD was lower than this threshold, possesses the potential for diagnosis of *M. pneumoniae* infection from an asymptomatic carriage in practical application. To sum up, the abovementioned advantages render ASEA a promising approach for rapid, accurate and low-cost diagnosis of *M. pneumoniae* infection.

### Application of ASEA for diagnosis of *M. pneumoniae* infection in clinical specimens

A total of 50 specimens from patients with symptoms of respiratory tract infection, which had been assayed via the gold-standard real-time PCR by the hospital that provided these specimens in advance, were collected for verification of the potential application of ASEA in clinical diagnosis. As shown in Table 2, 26 of the 50 specimens were detected as positive by real-time PCR, while 25 of these positive specimens were also successfully diagnosed by ASEA, indicating that ASEA yielded a positive detection rate of 96.15% taking PCR as a reference, which was higher than SEA and LAMP, whose positive detection rates were 90.5% and 95.23%, respectively, according to our previous work [10]. The false-negative result of ASEA demonstrated that the sensitivity of ASEA was slightly lower than real-time PCR. However, from another perspective, the possibility of real-time PCR giving false detection results cannot be excluded. In contrast to the positive specimens, ASEA exhibited diagnostic results identical to those with real-time PCR for all remaining 24 specimens, which were diagnosed with the negative results by this gold standard, thus indicating that no false-positive diagnostic results appeared in the ASEA assay. That is, ASEA achieved specificity of 100.0%, higher than the 98.91% of LAMP. Although ASEA failed to exhibit 100.0% sensitivity based on the premise that real-time PCR would not give false results, this method still showed 100% specificity and had a favorable performance for *M. pneumoniae* detection in clinical specimens. Moreover, the slight differences between diagnostic results of these two methods are not statistically significant according to the result of chi-square tests ( $p > 0.05$ ). In addition to excellent specificity and sensitivity, the running time of

ASEA was only approximately 30 min, significantly shorter than real-time PCR, SEA and LAMP, whose processing took around 80 min, 60 min and 70 min, respectively. Furthermore, each testing of ASEA was estimated to cost approximately \$0.24, which was close to SEA, including \$0.05 for the reaction tube and pipette tip, and \$0.19 for reagents, lower than PCR and LAMP, since *Taq* polymerase is normally more expensive than *Bst* 2.0 WarmStart DNA polymerase, while LAMP requires multiple pairs of primers. Hence, this rapid and low-cost method still presents great advantages in terms of efficiency, making ASEA more suitable for the situations that require fast detection of a large number of specimens, such as epidemic outbreaks or inspection and quarantine of airports and seaports.

## Conclusion

In this present work, the ASEA method was successfully established for *M. pneumoniae* detection by introducing a narrow range of a rapid thermal cycling process in the SEA method that we developed previously. The range of the thermal cycling should be between the temperature slightly lower than the amplicon's  $T_m$  value and that slightly lower than the average  $T_m$  value of primers to ensure high polymerase possessing activity and strong binding of primers to the target sequence. This method was able not only to accurately distinguish genomic DNA extracts of *M. pneumoniae* from other common pathogens, but also to detect 1% *M. pneumoniae* genomic DNA in a multi-DNA mixture. Moreover, the diagnosis time was shortened dramatically by this method, which detected  $1.0 \times 10^{-17}$  M of *M. pneumoniae* genomic DNA in 20.4 min via conventional amplification instruments and ordinary commercial polymerase. Since the LOD of ASEA was lower than the asymptomatic carriage threshold and its diagnostic time was much shorter than commonly used real-time PCR, we believe this rapid, low-cost, sensitive and highly specific method possesses great application potential for the rapid diagnosis of *M. pneumoniae* and other pathogens, as well as the development of relevant kits.

**Authors' contributions** Chen Yang and Jie Deng performed the experiments; Chen Yang, Yang Li and Jie Deng analyzed the data; Yang Li, Cuiping Ma and Chao Shi designed the study; Yang Li, Chen Yang and Mengzhe Li wrote the manuscript; and all authors contributed to the writing of the paper, had primary responsibility for the final content, and read and approved the final manuscript.

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**Availability of data and material** The authors declare that all data and materials support our published claims and comply with field standards.

## Compliance with Ethical Standards

**Conflict of interest** There are no conflicts of interest to declare.

**Ethics approval** The authorized Human Health and Ethics Committee of the Affiliated Hospital of Qingdao University approved this study, and all methods were carried out in accordance with the relevant guidelines and regulations.

**Consent to participate** All donors of clinical sputum specimens provided informed consent.

**Consent for publication** All the authors and participants consent to publication of this manuscript in *Analytical and Bioanalytical Chemistry*.

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