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Article

CHAMP: A Centrifugal Microfluidics-Based CRISPR/Cas12b-Combined Real-Time LAMP One-Pot Method for *Mycoplasma pneumoniae* Infection Diagnosis

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Cite This: *ACS Omega* 2024, 9, 38989−38997

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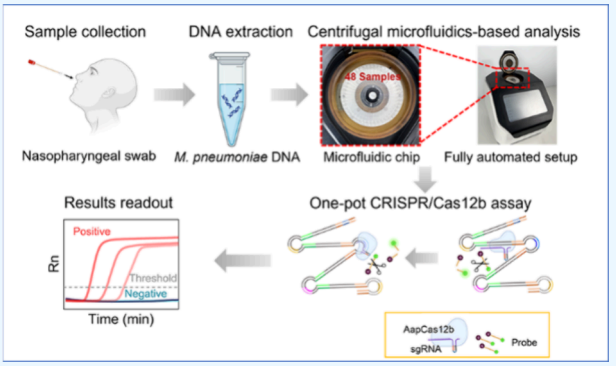
ABSTRACT: The *Mycoplasma pneumoniae* outbreak poses health risks to community residents. However, it still has limitations for current clinical diagnostic methods (qPCR nucleic acid assay or IgM immunoassay), including specialized handling, expensive equipment, prolonged turnaround time, and false positives and negatives, highlighting the need to improve clinical diagnostic methods. Herein, we present a novel centrifugal microfluidics-based method for rapidly diagnosing *M. pneumoniae* infections (CHAMP system). This user-friendly method combines CRISPR/Cas12b and real-time loop-mediated isothermal amplification (LAMP) in a one-pot reaction, offering high sensitivity, specificity, and simplicity for methodology. By adding fully automated nucleic acid magnetic bead-extracted samples to a prepackaged centrifugal microfluidics chip, 48 samples can be automated tested simultaneously within 15 to 60 min at 60 °C. 427 clinical nasopharyngeal swab specimens were used for validation, demonstrating good positive and negative predictive values and good diagnostic sensitivity, specificity, and significant time savings. This method is particularly suitable for detecting low nucleic acid copies of *M. pneumoniae* samples.



INTRODUCTION

Mycoplasma pneumoniae is the most common bacterial pathogen in community-acquired pneumonia (CAP) in patients. Usually, it causes lower respiratory tract infections with a prolonged incubation period, high morbidity, and recurrent infections, often leading to severe illness and extrapulmonary complications.[1−4] *M. pneumoniae* is primarily transmitted through respiratory routes with an incubation period of several weeks. The peak of infections typically occurs from January to March and can account for up to 70% of CAP cases in closed settings. However, due to its asymptomatic and nonspecific characteristics and the lack of sensitive and specific diagnostic tests, distinguishing *M. pneumoniae* infections from other respiratory diseases poses challenges. This complexity in diagnosis often leads to underestimating the prevalence and actual impact of *M. pneumoniae* infection on public health.[5−7] The commonly employed conventional quantitative Polymerase Chain Reaction (qPCR)-based nucleic acid assay used in clinical settings is a method with a lower likelihood of contamination and provides quantitative and reliable data. However, its limitations include specialized handling, expensive equipment, and prolonged turnaround time.[8] Additionally, the IgM antibody assay, which is often used in clinical as an alternative, can produce false positives due to nonspecific antibody binding and false negatives caused by delayed antibody production, which makes it unsuitable for accurate diagnosis, especially in vulnerable populations such as elderly individuals and children with weakened immune systems.[8−10]

Loop-mediated isothermal amplification (LAMP) technology is a cost-effective and sensitive method to be widely used in research areas, which could rapidly replicate small amounts of nucleic acid molecules in a shorter time when compared with qPCR. However, false-positive results can affect diagnosis interpretation. In recent years, CRISPR-Cas has enabled specific detection of targeted nucleic acid sequences due to its programmability and enzymatic activity.[11] Combining nucleic acid isothermal amplification technology with CRISPR-Cas enhances sensitivity and specificity and has a high potential to enable advanced point-of-care (PoC) diagnostic platforms.[12−14] Previous studies utilized the CRISPR/RPA combination approach to reduce false positives in *M. pneumoniae* detection effectively.[15−22] However, their

Received: June 12, 2024
Revised: July 21, 2024
Accepted: August 9, 2024
Published: September 6, 2024

amplification and CRISPR steps still need to be performed in two steps and require liquid handling, which can lead to sample cross-contamination and hinder automation. This limitation is particularly significant when dealing with a large number of samples. AapCas12b is a type V CRISPR-associated heat-stable enzyme derived from *Alicyclobacillus acidiphilus*, which exhibits endonuclease activity within the temperature range of 37−65 °C, aligning with the optimal amplification temperature range of LAMP (60−65 °C) thus suitable for a simplified workflow where amplification and CRISPR-based detection can be accomplished in a single step.[15,23−25] Therefore, the CRISPR/Cas12b-LAMP-based method has the potential to achieve high sensitivity, specificity, rapid detection, and simplification in the detection of *M. pneumoniae* infections.

Herein, we developed a novel centrifugal microfluidics-based method for the diagnosis of *M. pneumonia*, namely, the CHAMP system (a **C**entrifugal microfluidics-based **H**igh-throughput one-pot method combining CRISPR/**A**apCas12b and real-time LA**M**P for *Mycoplasma* ***P****neumoniae* infection diagnosis). This method combines CRISPR/Cas12b and real-time LAMP in a one-pot reaction, which is user-friendly, involving the addition of fully automated nucleic acid magnetic bead-extracted samples into a homemade centrifugal microfluidics chip with prepackaged reagent. Operating at 60 °C enables rapid and simultaneous testing of 48 samples within 15 to 60 min. This method has been validated with 427 clinical nasopharyngeal swab specimens, showing outstanding clinical predictive accuracy for both negative and positive samples as well as high diagnostic sensitivity and specificity and significant time savings when compared with qPCR and LAMP methods, making it an ideal automated rapid diagnostic method that is particularly suitable for detecting *M. pneumoniae* samples with low nucleic acid copies. The inclusion of a large number of patient samples enhances the statistical power, reliability, and generalizability of this method.

EXPERIMENTAL SECTION

Reagents and Instruments. The nucleic acid extraction kit (model: MCR01) and *M. pneumoniae* real-time PCR kit (25 persons/kit) were purchased from Shanghai ZJ Biotech Co., Ltd. (China). Bst 2.0 DNA polymerase was purchased from Novoprotein (Shanghai, China). AapCas12b was purchased from Tolo Biotech Co., Ltd. (Shanghai, China). LAMP primers, probes, and sgRNA sequences were synthesized by General Biol Corp. (Anhui, China). *M. pneumoniae* nucleic acid standards were extracted from the pure cultures of the *M. pneumoniae* M129 strain, and the concentration was diluted to 10^6 copies/μL. Clinical nasopharyngeal swab collection tubes (model: X105) and cell preservation solution used for specimen collection and storage, consisting of tris hydrochloride, guanidine thiocyanate, sodium hydroxide, ethylenediaminetetraacetic acid disodium salt dehydrate, RNase inhibitor, ProClin 300, and phenol red, were purchased from Sansure Biotech Inc. (China). dTTP, dATP, dCTP, and dGTP reagents were purchased from BBI Life Sciences Corporation (Shanghai, China). The freeze-dried lyophilized beads used for the CRISPR-LAMP reaction were custom-made at Zhuhai Biori Biotechnology Co., Ltd., China, following our laboratory's developed procedures (Supporting Information Table S12). All other reagents were purchased from Sigma (USA). The nucleic acid extraction and purification were performed using an automated nucleic acid extraction system (model EX3600, Shanghai ZJ Biotech Co., Ltd.). Conventional qPCR and LAMP detection was conducted on a qPCR instrument (model: SLAN-96S, Shanghai Hongshi Medical Technology Co., Ltd., China). The CHAMP method was performed on an in-development centrifugal isothermal amplifier instrument (model: CH−CI48, Suzhou Changhe Biotech Inc., China) with detailed pictures and parameters of the key structures in Supporting Information Figure S3 and Table S11.

Design and Fabrication of the CHAMP Microfluidic Chip. SolidWorks was used to create 3D drawings of the homemade microfluidic chip. The microfluidic chip was fabricated with a computer numerical control machining center (Dingya, model: 850, Shanghai). The microfluidic channels were coated with a hydrophilic buffer Vistex 111−50 (FSI Coating Technologies, USA), and the reaction reagents were pre-embedded in different chambers in liquid or lyophilized bead forms. The freeze-drying procedure can be found in Supporting Information Table S12. After preloading, the microfluidic chip with patterned channels upside was sealed using a one-sided adhesive PET film and stored at −20 °C until use.

Evaluation of the Analytical Performance of the CHAMP Assay. The whole-genome nucleic acid extraction from the *M. pneumoniae* strain pure culture was used to assess the analytical performance of the CHAMP system. The original concentration of the DNA template was measured using UV−vis spectrophotometry (Thermo Fisher, USA). It was then diluted to various concentrations (ranging from 1×10^5 to 3×10^{-2} copies/μL) to compare the sensitivities of the CHAMP system with qPCR and LAMP. The specificity was confirmed by testing the whole-genome nucleic acid extract from pure cultures of 15 common respiratory pathogens, including *M. pneumoniae*, Influenza B virus, RSV B, *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, GAS, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, RSV A, ADV3, ADV7, *Chlamydia pneumoniae*, H1N1, and H3N2. Each experiment was repeated in triplicate.

Design and Population Characteristics of the Study. 440 clinical nasopharyngeal swab specimens for retrospective analysis of *M. pneumoniae* infection were consecutively obtained between August 2023 and January 2024. The Laboratory Department of Yangpu District Central Hospital, Shanghai, China, provided these specimens. Specimens were collected from individuals with suspected respiratory infections and healthy donors. The samples consisted of individuals diagnosed as *M. pneumoniae* positive based on clinical symptoms and qPCR (n = 180), those diagnosed with other respiratory diseases (n = 194), and healthy donors (n = 53). This study underwent review and approval by the ethics committee review boards of Yangpu Hospital, Shanghai, China (Ethics Committee approval no. LL-2024-SCI-001). All experiments were conducted in the same hospital.

Clinical Specimens' Collection, Manipulation, and DNA Extraction. Nasopharyngeal swabs were collected from participants with suspected respiratory infections and healthy donors on the day of the visit. The collected swabs were rinsed into clinical nasopharyngeal swab collection tubes containing 2 mL of a cell preservation solution. The specimens were stored at 4 °C for a maximum of 2 h. Following routine clinical procedures, 1 mL of the specimens was utilized for DNA extraction and qPCR-based diagnosis of the *M. pneumoniae* target gene. The remaining specimens were transferred to −80 °C for long-term storage without DNA extraction. After

https://doi.org/10.1021/acsomega.4c05489
ACS Omega 2024, 9, 38989−38997

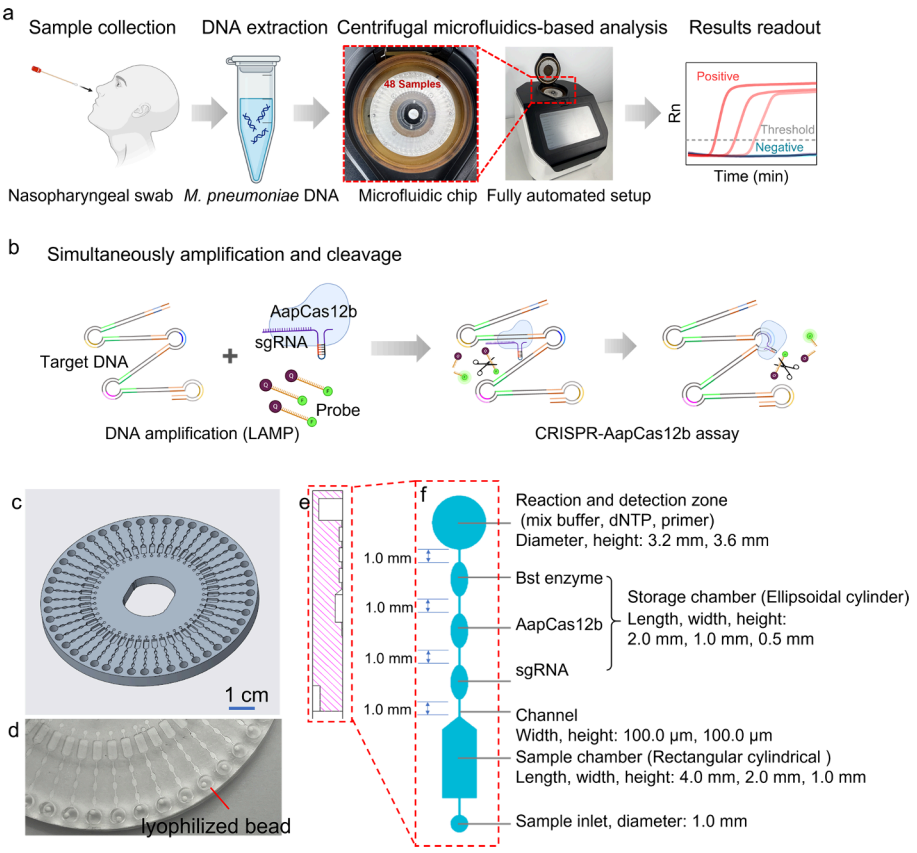


Figure 1. Overview of the CHAMP system (the CRISPR/Cas12b-LAMP analysis system). (a) Schematic of the operation workflow. (b) The detection strategy of CHAMP assay. (c) The 3D image depicts the structure of the microfluidic chip. (d) The image displays a section of the microfluidic chip with reloaded lyophilized beads. The cover film has not been sealed to provide a clearer view of the channels and the lyophilized beads. (e) The schematic picture of the microfluidic chip in the longitudinal section. (f) The schematic illustrates each component of the microfluidic channel.

anonymization and renumbering, centralized DNA extraction was conducted using an automated DNA extraction instrument mentioned in the Reagent and instrument part. The resulting DNA samples were then used for detection using the LAMP and CHAMP methods. More detailed information can be found in the Supporting Information methods.

Comparing the Diagnostic Performance of CHAMP with Conventional qPCR and LAMP. To assess the performance of the CHAMP system, a blinded test was performed using 180 *M. pneumoniae* positive samples and 247 *M. pneumoniae* negative samples, including 194 samples diagnosed with other respiratory diseases and 53 samples from healthy donors. The negative and positive results of LAMP and CHAMP were statistically analyzed and compared with those confirmed positive and negative by clinical symptoms and qPCR. The positive and negative predictive values, sensitivity, and specificity of the developed CHAMP method for detecting *M. pneumoniae* in nasopharyngeal samples were calculated by following the statistical analysis method.

Statistical Analysis. Blinded testing of clinical samples was conducted to ascertain the values. Continuous variables were summarized using the median and interquartile ranges (IQR), while categorical variables were expressed as counts and percentages. The diagnostic performance of the CHAMP system was assessed by comparing its sensitivity and specificity to clinical qPCR diagnosis and LAMP analysis using online MedCalc Software Ltd. Diagnostic test evaluation calculator (MedCalc Software Ltd., n.d.).[26] Proportions and their corresponding 95% confidence intervals (95% CIs) were calculated and reported. One-way ANOVA, one sample t, Wilcoxon test, and *t* test were performed with a significance level (α) of 0.05. Original data analysis and visualization were conducted using Prism GraphPad 10 software. GraphPad Prism (version 10).

RESULTS AND DISCUSSION

Establishment of the CHAMP System. The flowchart schematic view of the CHAMP system for *M. pneumoniae* infection diagnosis is depicted in Figure 1a. First, nasopharyngeal swab samples are collected from participants and immediately rinsed into clinical nasopharyngeal swab collection tubes containing cell preservation solution. DNA extraction took approximately 15 min, and 25 μL of DNA was injected into a Poly(methyl methacrylate) (PMMA) centrifugal microfluidic chip. The gel-mixed reagent liquefied at 40 °C, enabling manipulation through centrifugal force. The microfluidic chip was rotated at 2000 rpm for 30 s to allow the DNA samples and reaction buffer to flow smoothly into the reaction and detection zone, enabling effective interaction between the sample, buffer, and lyophilized bead. The reaction was conducted at 60 °C for 60 min. Real-time detection curves were generated to determine the positive and negative results of qualitative detection. The detailed reagent composition and reaction conditions are presented in Supporting Information Tables S1−S10. The real-time normalized reporter signals

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ACS Omega 2024, 9, 38989−38997

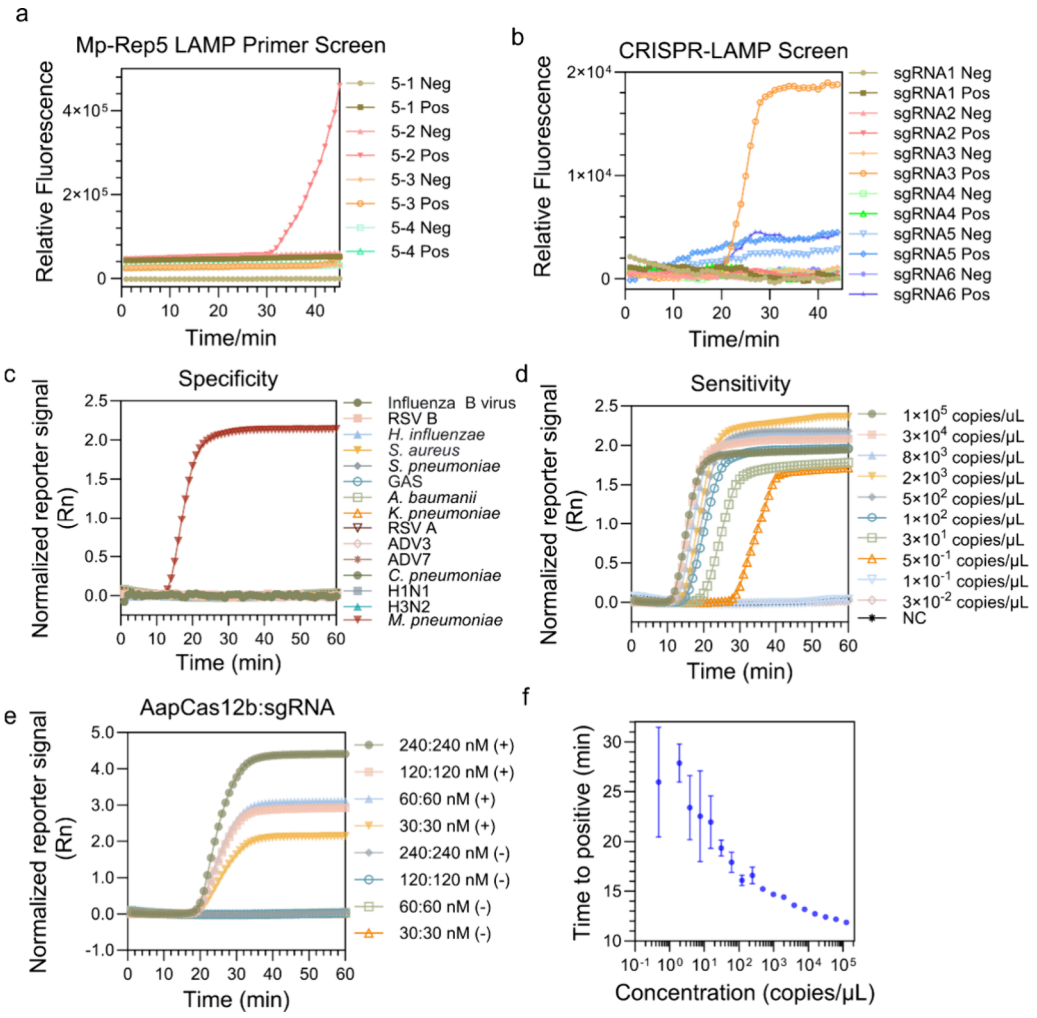

Figure 2. The analytical performance of the CHAMP system for detecting *M. pneumoniae* nucleic acid. (a) Results of the LAMP primer screening for Mp-Rep5. (b) The screening of sgRNA in the CHAMP system. (c) Specificity evaluation of the CHAMP method compared to 14 other common respiratory pathogens species. (d) Sensitivity evaluation of the CHAMP method through dilution of DNA extractions from pure cultures of the *M. pneumoniae* strain. (e) The optimization of the ratio of AapCas12b enzyme to sgRNA using positive and negative standard nucleic acid samples. (f) The illustration depicts how the time to positive trends vary with an increase in the concentration of *M. pneumoniae* nucleic acid copy number.

(Rn) were recorded to determine the detection results in qualitative testing (positive or negative).

Mechanism of the CHAMP System. The mechanism of the CHAMP system is depicted in Figure 1b. The LAMP amplification generated substantial DNA amplification products, which served as the target DNA for the CRISPR/Cas12b reaction. The reaction system initiates CRISPR-mediated signal amplification while inhibiting further amplification by destroying the amplifiable units. The LAMP amplicons function as the CRISPR activator, which cannot be reamplified upon recognition and cleavage by CRISPR/Cas12b. The collateral cleavage activity of AapCas12b induces signal transduction by cleaving single-stranded fluorophore-quencher-labeled (ssFQ) molecules, resulting in fluorescence. Figure 1c illustrates the 3D structure of the homemade microfluidic chip with 48 separate patterned channels. The Bst enzyme, AapCas12b, and sgRNA were mixed with 0.5% low melting point agarose and preloaded into the chambers, while the mix buffer, dNTP, and primer were preloaded as lyophilized beads in the reaction and detection zones (Figure 1d). The microfluidic chip has a diameter of 83 mm and consists of two layers. The first layer was made of Poly(methyl methacrylate) (PMMA) with a thickness of 4.13 mm. The second layer was a 0.1 mm thick one-sided adhesive PET film cover to seal the microfluidic channels. There are five chambers in each microfluidic channel after the inlet (Figure 1e). The sample chamber is rectangular cylindrical with a length of 4 mm, width of 2 mm, height of 1 mm, and volume of 8 μL. The sgRNA storage chamber, AapCas12b storage chamber, and Bst enzyme storage chamber are ellipsoidal cylinders with a length of 2 mm, width of 1 mm, height of 500 μm, and volume of approximately 3 μL. The reaction and detection zone is a cylinder with a diameter of 3.2 mm, height of 3.6 mm, and volume of approximately 29 μL. Figure S3 shows the fluorescence detection module positioned beneath the microfluidic chip's plate holder for fluorescence signal detection and analysis. The LED light source excites the sample, and the emitted fluorescence light is collected and measured for the intensity. A fluorescence beam hole on the plate holder enables both the entry of excitation light and the collection of emitted fluorescence light by the detection module.

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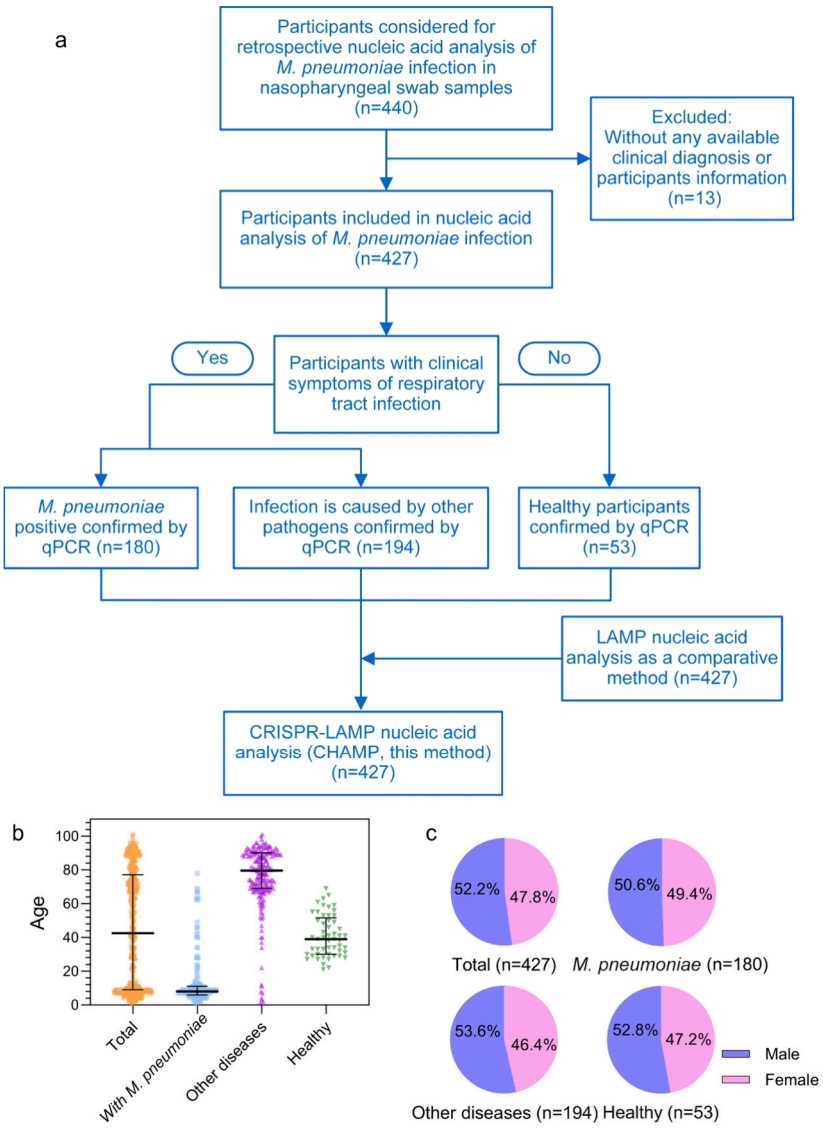

Figure 3. Study participants and illustration of included and excluded. (a) Participants included individuals with respiratory infectious diseases (including *M. pneumoniae* and other respiratory diseases) and healthy donors. DNA extractions from clinical nasopharyngeal swab specimens were used to evaluate the CHAMP method's performance. The evaluation has three groups: the *M. pneumoniae* positive group, the infection caused by other pathogens group, and the healthy participants group. (b) Age distribution among different groups. The error bars represent the median with the interquartile range. (c) The sex distribution of study groups.

Primers and sgRNA Design. The LAMP primers were designed using the online PrimerExplorer software (http://primerexplorer.jp/e/index.html). A conserved region within eight RepMp5 repetitive sequences of the *M. pneumoniae* strain was selected through sequence alignment for designing LAMP primers. It is part of the ORF6 gene and is involved in the adhesion of the bacterium to its host cell. Detailed RepMp5 repetitive element sequences used for short common sequence screening are shown in Supporting Information Table S1. Four designed LAMP degenerate primers were verified by using standard samples, and primer 5−2 was chosen as the final LAMP primer for the experiment (Figure 2a). The sequences of the LAMP primer are shown in Supporting Information Table S2. The LAMP primers' complementary sequence of the F2−B2 amplification region was utilized to design the single guide RNA (sgRNA) sequence for AapCas12b. Detailed information about sgRNA sequences is shown in Supporting Information Table S3. The sgRNA can be designed anywhere within the F2−B2 regions of the LAMP amplicon without constraints. Six sgRNA sequences were developed, and their performance was evaluated using the CHAMP assay. As shown in Figure 2b, sgRNA3 exhibited robust functionality and proved highly effective and was chosen for this study.

Evaluation of the Analytical Performance of the CHAMP System. To assess its performance, the CHAMP system was evaluated for its specificity, sensitivity, and time to positive detection. Figure 2c illustrates the specificity of the CHAMP system. The whole-genome nucleic acid extractions of pure cultures from 15 common respiratory pathogens were tested. The results indicated that the *M. pneumoniae* sample produced positive results. In comparison, the other 14 pathogens, including Influenza B virus, RSV B, *H. influenzae*, *S. aureus*, *S. pneumoniae*, GAS, *A. baumannii*, *K. pneumoniae*, RSV A, ADV3, ADV7, *C. pneumoniae*, H1N1, and H3N2, showed negative results. These findings indicate good performance in specific assays of the developed CHAMP

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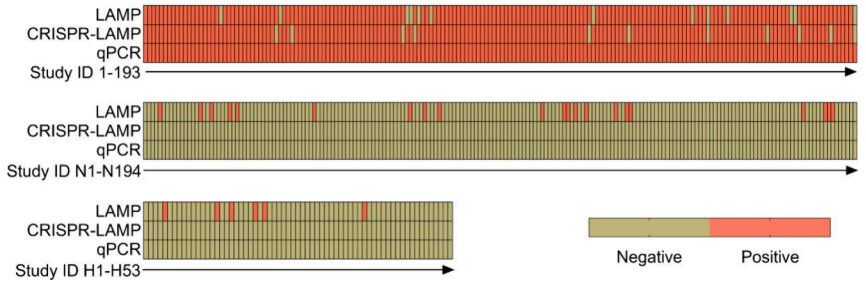

Figure 4. The comparison of detection results among groups. The CHAMP method is shown as CRISPR-LAMP in this figure. The orange color represents a positive result, while the brown color represents a negative result. The clinical qPCR results were used as the reference. The study IDs 1−193 comprised participants with confirmed *M. pneumoniae* infection. The study IDs N1−N194 represented participants with other confirmed respiratory diseases, while the study IDs H1−H53 represented participants confirmed as healthy.

method. The sensitivity of this method was also evaluated using nucleic acid extracts of *M. pneumoniae* pure cultures (Figure 2d). The ratio of the AapCas12b enzyme to sgRNA was optimized by using positive and negative standard nucleic acid samples (Figure 2e). Balancing signal intensity and reagent cost, a ratio of 60 nM:60 nM was chosen as the optimal ratio for subsequent experiments. The gradient dilutions of DNA extract samples were performed starting from an initial concentration of 2×10^6 copies/μL, using a DNA-free buffer as a control to determine the sensitivity. The detection limit of the assay was found to be 5×10^{-1} copies/μL. The detection time to positive decreased from approximately 30 min to around 12 min as the concentration of *M. pneumoniae* pure culture's nucleic acid extracts increased from 5×10^{-1} copies/μL to 1×10^5 copies/μL (Figure 2f).

Evaluation of the Diagnostic Performance of the CHAMP System for Rapid M. pneumoniae Detection. The reported sensitivity represents the ideal sensitivity under the optimal conditions. In clinical samples, there may be interfering substances that could affect the detection. Therefore, the clinical diagnostic performance of the CHAMP system was validated via a blinded test. The test included many clinical samples and was compared with conventional LAMP without CRISPR, using clinical qPCR results as a reference for the clinical diagnosis. We first studied the characteristics of the study population, including the collection of specimens, criteria for inclusion and exclusion, and composition of the sample groups. Our specimen collection method closely resembled the real hospital setting during the collection period, and we had a relatively large sample size. Only a few samples with incomplete information were excluded. Therefore, the test results of these samples reflect the diagnostic performance of the method and provide valuable insights into the recent *M. pneumoniae* infection epidemic to some extent. Samples were included based on the criteria shown in Figure 3a, excluding 13 samples that did not meet the criteria. Statistical analysis results showed significant age distribution differences among the *M. pneumoniae* positive group, other respiratory disease group, and healthy donors (Figure 3b, Supporting Information Figure S1) ($p < 0.01$). *M. pneumoniae* infections were mainly observed in the age group of 0−17 years, with a median age of 7 years (quartiles of 5−10 years old). In contrast, the other respiratory disease group consisted mainly of patients over 60, with a median age of 80 (quartiles 60−91). The overall sex ratio of the included samples and within each group was approximately 1:1 (Figure 3c).

To perform the evaluation, the automated rapid nucleic acid extraction using a magnetic-bead-based method on the preservation solution of the nasopharyngeal swab samples was conducted, which took approximately 15 min. The extracted nucleic acids were used for validation with the CHAMP and LAMP methods. The qualitative results of qPCR, LAMP, and CHAMP of each specimen are shown in Figure 4. The results of clinical qPCR are taken as the control. It is observed from the qualitative results of the three methods that most of the test results of samples are consistent, but a few inconsistent results are also encountered. Therefore, the diagnostic accuracy and specificity differences between LAMP and CHAMP methods were further analyzed and compared based on the validation results (Figure 5). The CHAMP exhibited a sensitivity of 94.2% (95% Confidence Interval 89.9−97.1%) for diagnosis, slightly higher than the sensitivity of LAMP at 93.0% (95% Confidence Interval 88.8−

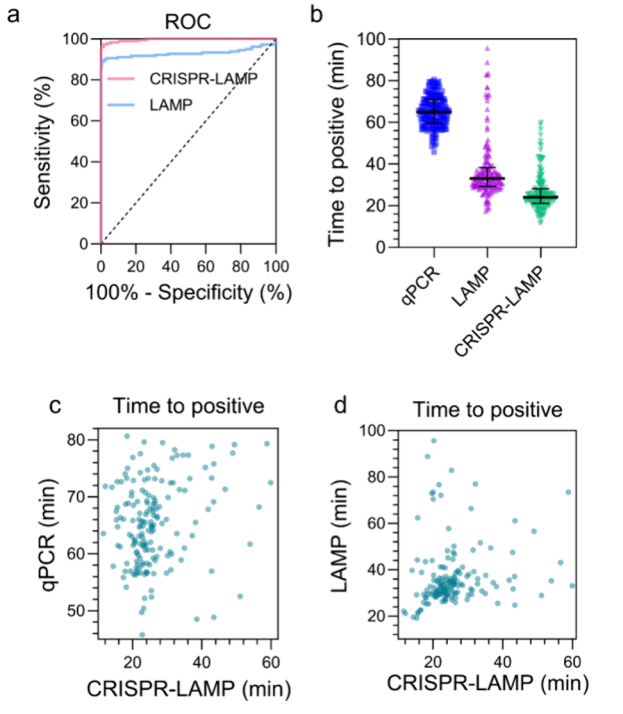

Figure 5. The clinical performance of the CHAMP system (shown as CRISPR-LAMP). (a) The ROC analysis comparing CHAMP and LAMP. (b) The comparison of the detection time-to-positive among qPCR, LAMP, and CHAMP methods. (c) The correlation of detection time to positive between qPCR and CHAMP methods. (d) The correlation of detection time to positive between LAMP and CHAMP methods.

https://doi.org/10.1021/acsomega.4c05489
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Table 1. Positive and negative predictive values, sensitivity, and specificity of this CHAMP method for diagnosing *M. pneumonia* in nasopharyngeal Samples

	LAMP		CRISPR/Cas12b-LAMP (CHAMP, this method)		
Nasopharyngeal Swabs	Negative	Positive	Negative	Positive	Total
Positive confirmed by clinical symptoms and qPCR	13	167	11	169	180
Negative confirmed by clinical symptoms and qPCR	220	27	247	0	247
Total	233	194	258	169	427
Statistics	**Value (%)**	**95% Confidence Interval (%)**	**Value (%)**	**95% Confidence Interval (%)**	**Best Performance**
Sensitivity	93.0	88.8−96.4	94.2	89.9−97.1	CHAMP
Specificity	90.0	86.0−93.4	100	98.5−100	CHAMP
Positive predictive value	87.0	82.3−90.5	100	98.0−100	CHAMP
Negative predictive value	95.0	91.8−97.0	95.7	92.7−97.6	CHAMP
Accuracy	91.0	88.5−93.8	97.5	95.6−98.7	CHAMP

96.4%), but the difference was not significant. The specificity of CHAMP was 100% (95% Confidence Interval 98.5−100%), significantly higher than the specificity of LAMP at 90% (95% Confidence Interval 86.0−93.4%) ($p < 0.01$) (Table 1). The ROC analysis comparing CHAMP and LAMP (Figure 5a) demonstrated a significant improvement in the ROC of CHAMP, indicating its superior performance ($p < 0.01$).

Additionally, a comparison of detection time to positive of CHAMP, qPCR, and LAMP revealed that CHAMP had a significantly shorter detection time (median 23.5 min, quartiles 21.4−28.5) compared to conventional LAMP (median 32.2 min, quartiles 29.1−37.9) and qPCR (median 66.0 min, quartiles 59.9−69.9) ($p < 0.01$) (Figure 5b). As shown in Figure 5c,d, despite the good sensitivity and specificity of CHAMP in diagnostic performance, the detection time trends of the three methods varied when analyzing each positive sample individually. This inconsistency may stem from differences in the reaction principles of the methods; the details are further discussed in the Discussion section. The detection time-to-positive results for each DNA extraction sample related to study ID in different methods and the qPCR Ct value are shown in Supporting Information Figure S2.

CONCLUSIONS

In conclusion, our method offers new insights into CRISPR/Cas12b and LAMP for one-step *M. pneumonia* diagnosis, offering potential benefits in terms of time, labor savings, and resource utilization. While the CRISPR-LAMP technology allows for specific primer design targeting mutations, our current method utilizes universal LAMP primers, offering specificity in detecting *M. pneumoniae* without differentiating antibiotic resistance genes due to the routine determination of whether a mycoplasma infection is present, which holds greater significance in the current clinical diagnosis. Since macrolide-resistant *M. pneumoniae* remains high in some countries, such as China and South Korea,[27] we plan to introduce primers for important mutation sites in future detection methods. Previous research has also reported that *M. pneumoniae* genotypes may not significantly impact determining specific clinical outcomes.[28] Further studies of the genotypes could be explored in future experiments. Based on current results, we believe the significant advantages of this method make it a powerful tool for future efficient diagnostic testing and genetic research.

The detection time trends of the three methods are inconsistent when each positive sample is considered individually. Certain limitations could affect the study's interpretation. Different reaction principles among the methods contribute to variations in the required readout time. The nucleic acid samples from clinically frozen nasal swab specimens in a preservation solution potentially cause nucleic acid degradation and negatively impact the evaluation of the CHAMP method's detection performance. In future research, we plan to test more fresh and dried nasal swab samples from multiple centers. This will help confirm our assumptions and further validate the method's usability.

In addition, following the Sex and Gender Equity in Research (SAGER) guidelines and reviewing the available references, we found a lack of reports on sex differences or similarities in studies related to Mycoplasma pneumonia disease. Our statistical analysis revealed no significant differences in the percentage of each group between males and females. Therefore, we assumed that sex does not play a significant role in this disease study, as indicated by our limited analysis results.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c05489.

Nucleic acid extraction, sample preparation, reactions, optimization, buffer, freeze-drying procedure, and other detection methods and tables (PDF)

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https://doi.org/10.1021/acsomega.4c05489
ACS Omega 2024, 9, 38989−38997

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Shanghai Municipal Science and Technology Major Project (ZD2021CY001); the National Natural Science Foundation of China (82302580); the Science and Technology Commission of Shanghai Municipality (23YF1434600); the National Natural Science Foundation of China (82302635). Biorender generated the partial elements in Figure 1.

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https://doi.org/10.1021/acsomega.4c05489
ACS Omega 2024, 9, 38989−38997